Guanylate cyclase C: a current hot target, from physiology to pathology

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Running title: GC-C and its ligands

Abstract

Guanylate cyclase C (GC-C) receptor is a transmembrane receptor, predominantly expressed in intestinal epithelial cells, that plays a main role in homeostasis and function of the digestive tract. The endogenous ligands for this receptor are the paracrine hormones uroguanylin and guanylin. The heat-stable enterotoxin, produced by enterotoxigenic bacteria, is also a natural ligand of this receptor. Upon ligand binding, GC-C receptors increase the second messenger cyclic guanosine monophosphate (cGMP) levels, regulating a variety of key cell-type specific processes such as chloride and bicarbonate secretion (which increases luminal fluid and intestinal motility), epithelial cell growth, regulation of intestinal barrier integrity and visceral sensitivity . It has been suggested that GC-C acts as an intestinal tumor suppressor with the potential to prevent the initiation and progression of colorectal cancer. In fact, loss of ligand expression is a universal step in sporadic colorectal carcinogenesis. On the other hand, the role of GC-C is not limited to the digestive tract but it has been extended to several other systems such as the cardiovascular system, kidney, and the central nervous system, where it has been involved in a gut-hypothalamus endocrine axis regulating appetite. Thus, available data points toward a relationship between these ligands and their receptor and pathological processes like gastrointestinal and renal disorders, colorectal cancer,, obesity and metabolic syndrome among others. Herein, we review the physiology of the GC-C receptor and its ligands, focusing on newly developed drugs like linaclotide, and their suggested role to reverse/prevent these diseases.

Keywords: guanylate cyclase C, heat-stable enterotoxin, diarrhea, irritable bowel syndrome, constipation, colorectal cancer, obesity, inflammatory bowel disease

INTRODUCTION

Guanylate or guanylyl cyclase C (GC-C) is a receptor for heat-stable enterotoxin (STa), produced by bacteria such as enterotoxigenic *Escherichia coli* (ETEC), which is responsible for the traveler's diarrhea [1, 2]. Diarrhea is produced by the activation of the receptor, which results in electrolyte (and water) secretion.

When delving into the pathogenic mechanisms underlying diarrhea, two peptidic endogenous agonists for GC-C, named guanylin and uroguanylin, were described and, subsequently, a physiological role of this receptor in maintaining intestinal homeostasis was suggested. In fact, mutations in this gene have been described leading to either diarrhea or ileus (failure of normal intestinal motility in the absence of mechanical obstruction). Furthermore, additional important roles in the intestine have been demonstrated for the receptor, including maintenance of a healthy intestinal barrier, anti-inflammatory effects, control of epithelial proliferation and tumorigenesis, and decrease of visceral pain sensation.

In addition to its intestinal location and function, other functions regarding other organs or systems have been suggested. Thus, guanylin and uroguanylin may regulate hydro-electrolytic balance, satiety, penile function or even attention.

Here, we will review different aspects of the GC-C receptor, including its structure and that of its ligands, its molecular mechanism of action, its location and physiological roles. Importantly, we will review the potential of GC-C as a target to prevent/reverse diseases associated to impaired signaling.

THE GUANYLATE CYCLASE C RECEPTOR AND ITS MOLECULAR MECHANISM OF ACTION

Structure and regulation of the GC-C receptor

GC-C is one of the members of particulate (membrane-bound) guanylate cyclases (pGC) found in mammals. GC-C was classified as an intestinal peptide-binding receptor, different from the other members of the pGC family, which bind natriuretic peptides (GC-A, GC-B), are expressed in the retina to be essential for vision (GC-E and GC-F) or are involved in rodent olfaction (GC-D and GC-G).

Like other pGC, GC-C exhibits several domain structures (Fig. 1): an extracellular binding domain at the N terminus; a transmembrane domain ; a cytoplasmic juxtamembrane domain; a regulatory domain; a linker or hinge region; a catalytic domain; and a carboxyl-terminal tail. These domains, their function and regulation will be briefly described below. More information can be found in previous extensive reviews on the topic (3, 4, 5 and references therein).

Several ligands are known for GC-C, including bacterial (STa), endogenous (guanylin, uroguanylin) and synthetic agonists (linaclotide, plecanatide, dolcanatide). Binding occurs at the extracellular domain, having been described that aminoacids 387 to 393 are essential for STa binding [6]. Post-translational glycosylation of the binding domain produces different molecular species of the protein from a single transcript [7]. This tissue-dependent process, altered in many diseases [5], seems to be important for ligand binding and receptor trafficking to the cell membrane. In contrast, it does not play a role for cell surface distribution, as shown in *in vitro* experiments [6, 8]. Ligand-independent oligomerization of receptor monomers may occur spontaneously by formation of disulfide bonds due to the existence of several cysteine residues in the extracellular domain [9, 10]. Interestingly, truncated mutants of GC-C, which contain the extracellular domain but lack the transmembrane spanning domain, are capable of forming dimers and binding ligand [11].

A short transmembrane domain of 21-25 hydrophobic amino acids bisects the extracellular and intracellular portions of the pGCs [5]. No inactivating or activating mutations have been identified in this domain [12] and, as mentioned above, it is not required for dimerization and ligand binding [11].

Like in other pGC, the short juxtamembrane domain of GC-C contains a consensus sequence that might mediate alternate signaling mechanisms, maybe through coupling to heterotrimeric G proteins and their downstream effectors [3].

The kinase homology domain (KHD) is a relatively long (~250 residues) domain ~30% homologous with a wide range of protein kinases. However, pGC (other than retinal pGC), including GC-C, does not possess the catalytic Asp residues in subdomain VI essential for kinase activity. Although its precise function is still unclear, it seems to participate in signal transduction [13] [and may be involved in allosteric regulation of the protein (see below). Particulate GC receptors lacking KHDs exhibit maximal cyclase activities that are unresponsive to ligand, which suggest that the KHDs repress guanylyl cyclase activity in the absence of ligand [13-15]. Prolonged exposure of GC-A and GC-C to ligands causes a shift from high to low affinity binding. This shift, which does not occur when lacking the KHD [15, 16], contributes to its activation (see below).

The hinge or linker region is an amphipathic α-helical structure, highly conserved in all five human pGC [12], which participates in dimerization, an essential process for activation of the catalytic domain [17-19]. However, mutagenesis studies have suggested that the primary role of this domain is to suppress cyclase activity in the absence of ligand [20].

The primary structure of the catalytic domain is highly conserved in pGCs (including GC-C), and closely related to the catalytic domain of adenylyl cyclases [21]. Particulate GCs appear to exist as preformed oligomers in the basal state, state that is not altered by ligand-receptor interaction. Oligomerization of GC-C is required for two catalytic subunits to convert guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). Although GC-C forms inactive homotrimers in a ligand-independent fashion, it appears to undergo ligand-dependent disulfide-stabilized dimerization [22]. In contrast to older models

suggesting that heterodimeric and homodimeric pGCs contain one and two catalytic domains, respectively [23], it has been more recently postulated that the catalytic domain of the full-length pGC receptor is an asymmetric homodimer with distinct and reciprocally regulated catalytic and allosteric sites that bind to GTP and ATP, respectively [24]. Point mutations in the purine-binding site of the catalytic domain abolished GC activity but not allosteric activation [24]. In addition, the crystallographic studies of GC-A suggest that its ligand induces an intermolecular rotation within the dimer [25]. This rotation would be transmitted across the transmembrane regions, would provoke a similar rotation in each of the GC catalytic domains of the dimer, approximate the GC domains to each other, and activate the synthesis of cGMP by GC-A [25, 26], and probably also by the other hormone-responsive pGCs, including GC-C [5].

The carboxyl terminal tail in GC-C might be important to cGMP production and phosphorylation by protein kinase C (PKC). Thus, phorbol ester exposure increases the phosphate content and maximum activation of GC-C by STa [27]. Mutation of Ser-1029, a PKC consensus site (SYK), to Ala abolished the ability of phorbol 12-myristate 13-acetate (PMA) to activate GC-C, consistent with PKC-dependent phosphorylation of GC-C at Ser-1029 [28]. In addition, this domain might be involved in associating GC-C with the cytoskeleton. In fact, GC-C is placed in detergent resistant membrane specializations of the intestinal brush border that are stabilized by an intricate and well-developed cytoskeleton [29]. Also, the C-terminal tail may mediate GC-C ligand-dependent internalization by endocytosis [30].

Two populations of binding sites were defined for STa in membranes from intestinal cells: one with high affinity but low capacity (R_H) and another one (R_L) with low affinity but high capacity (5% and 95% of total receptors, respectively). The molecular mechanism underlying these different sites is not clear but it might be related to receptor oligomerization, glycosylation, or ligand heterogeneity [3, 7, 22, 31-34].

Whatever the case may be, R_L is the only site leading to catalytic activation and production of cGMP. GC-C R_L exhibits biphasic association kinetics, demonstrating that these sites undergo a ligand-induced shift from higher (R_{L1}) to lower (R_{L2}) affinity [16, 35, 36]. Using different intracellular deletion mutants of GC-C expressed in COS-7 cells, it was shown that cytoplasmic domains of GC-C are not required for expression of high- and low-affinity binding sites. Rather, the transmembrane and extracellular domains are sufficient for expression of R_H and R_L. In contrast, the juxtamembrane and kinase homology domains mediate the ligand-induced affinity shift of R_{L1} to R_{L2}, important for catalytic activation, in an ATP-independent fashion [15]

In the R_L state, adenine nucleotides (ATP) bind to the KHD. ATP increases ligand-stimulated guanylyl cyclase activity of GC-C [7, 34, 37], but the effect of ATP on basal activities was equivocal [34, 37]. ATP increased maximal velocities of STa-stimulated GC-C [37]. However, unlike GC-A, STa activation of GC-C has not been reported to depend on the presence of adenine nucleotide. The rank order of adenine nucleotide activators for GC-C is ATP-γ-S >ATP= AMPPNP >AMP [34, 37]. It was suggested that ATP-dependent stimulation does not result from a direct activation of the enzyme, but rather from the stabilization of an active state or an inhibition of the deactivation process [7, 22, 34]. Additional studies by the same group demonstrated that ATP increased activity of immunopurified GC-C, consistent with

direct binding of ATP to the receptor [34]. In the presence of Mn^{2+}, adenine nucleotides activate and inhibit GC-C by regulating two unique sites [38]. Additional kinetic studies suggested that ATP binding inhibits GC-C catalytic activity by occupying a GTP allosteric site [39]. The mutation of Lys-516 in the KHD, which is homologous to Lys-535 in GC-A, reduced ligand-dependent activation of GC-C, and a monoclonal antibody against residues 491 to 568 that binds the wild type receptor in the absence but not presence of ATP failed to bind the K516A mutant form of GC-C, consistent with ATP causing a conformational change in the KHD of GC-C [40].

Intracellular transduction pathways of GC-C activation

Ligand-induced GC-C activation leads to intracellular elevation of [cGMP]. Two evolutionarily distinct allosteric sites for binding cGMP are present in eukaryotic cells. One occurs with significant sequence homology in cGMP-dependent and cAMP-dependent protein kinases (PKGs and PKAs, respectively), and in the cyclic nucleotide-gated (CNG) cation channels, while the other one occurs in cGMP-regulated phosphodiesterases (PDEs). The main molecular mechanisms of action of GC-C are summarized in figure 2.

PKG represents the principal intracellular mediator of cGMP signals. Activation of PKG due to a rise in intracellular cGMP concentration, leads to phosphorylation (using P from ATP) of the target protein, which in turn mediates the translation of the extracellular stimulus into a specific biological function. PKGII is an 86 kDa membrane-bound homodimer whose gene is located on human chromosome 4 [41]. The "classical" substrate for PKGII is cystic fibrosis transmembrane conductance regulator (CFTR), present in intestinal mucosal cells [42]. Phosphorilation of CFTR induces an electrogenic chloride current [43], leading to water secretion in the intestine. A similar system has been described in the inner medulla of rat kidney, suggesting a role for GC-C/cGMP/PKG/chloride channel in kidney function and hydroelectrolytic homeostasis (see below).

In the small intestine, PKGII is anchored by an NH_2-terminal myristoylation site to the apical membrane of the enterocytes, with its highest expression levels in duodenum and jejunum [44]. CFTR channel opening subsequent to GC-C/cGMP-induced PKGII activation leads to an efflux of Cl^- and secondary efflux of water [45, 46], and thus PKGII regulates ionic and water secretions for the hydration of intestinal content [47, 48]. Accordingly, in mice lacking genes for GC-C, PKGII or CFTR, the effects of guanylins and STa on intestinal secretion were markedly reduced or even abolished [45, 49, 50].

Interestingly, CFTR may also be activated indirectly. Thus, cGMP also regulates electrogenic chloride secretion by inhibiting the type 3 isoform of PDE. This occurs mainly in the colonic crypts, where PKGII is not involved in GC-C effect. Then, the consequent increase in intracellular cAMP activates PKA, which in turn activates CFTR [50, 51].

Another substrate of PKGII, important for intestinal fluid homeostasis, is the apical sodium-hydrogen exchanger NHE3. In this case, phosphorylation by PKGII leads to the inhibition of NHE3, which reduces sodium absorption and increases H^+ intracellular accumulation. Since regulation of NHE3 and CFTR may

be closely orchestrated (cellular trafficking and surface expression show opposite regulation) both effects might contribute to secretory diarrhea [8].

GC-C may be a key mediator for acid-induced bicarbonate secretion in the upper GI tract through an unknown transporter [52]. In duodenal mucosa mounted in Ussing chambers, it has been demonstrated that the bicarbonate secretion secondary to acid perfusion was reduced in GC-C knock-out (KO) compared to wild-type (WT) mice. In WT animals, treatment with HCl induced the phosphorylation of extracellular signal–regulated kinase (ERK), whereas this effect was attenuated in KO mice. Since cGMP levels were similar in both groups, a different mechanism for producing this mediator in GC-C KO mice was suggested [53]. Also with Ussing chambers, it was found that guanylin and uroguanylin provoke bicarbonate secretion [54, 55], but different mechanisms of action for the different GC-C agonists have been postulated. Using GC-C KO mice it was shown that guanylin and uroguanylin increased duodenal luminal bicarbonate in a CFTR-dependent way. In contrast, STa induced the same effect but involving CFTR independent pathways [56]. It was suggested that STa effect on luminal bicarbonate levels may not be a consequence of the stimulation of secretion but secondary to the reduction in H^+ secretion, the well-known effect of the toxin, via inhibition of NHE3, as reviewed above [57].

PKGI mediates the effects of GC-C ligands on the cytoskeleton and contractile apparatus. The vasodilator-stimulated phosphoprotein (VASP) is an actin binding protein controlling cytoskeletal remodeling, cell shape, and adhesion contacts in intestinal epithelial cells [58]. GC-C agonists activate PKGI to phosphorylate Ser239 in the carboxyl terminal VASP domain [59], suppressing F-actin polymerization and membrane protrusion formation [58, 60, 61].

The cyclic nucleotide-gated cation (CNG) channel is another important effector of GC-C activation. CNG channels are a family of voltage-gated cation (Na^+ and Ca^{2+}) channels expressed in a variety of cells. Although all CNG channels are activated by both cGMP and cAMP, certain isotypes are more sensitive to one nucleotide or the other. GC-C signaling through CNG channels slows down intestinal cell cycle progression by inducing intracellular Ca^{2+} influx and cytosolic Ca^{2+} elevations [62] [. This leads to the translocation of calcium-sensing receptors to membrane compartments [63]. Calcium-sensing receptor is a key mediator of tumor inhibitory activities by luminal Ca^{2+}. Thus, GC-C signaling after ligand binding may act as a regulatory system promoting physiological actions by dietary Ca^{2+} in the gut, including cytostasis and the proliferation-to-differentiation transition along the crypt-villus axis [62, 63]. The role of GC-C receptors in colorectal cancer is reviewed below in more depth.

Finally, signaling by cGMP is ended by the action of phosphodiesterase type 5 (PDE_5), a cGMP-dependent phosphodiesterase, which catalyzes the conversion of cGMP into GMP [64].

LIGANDS OF GUANYLATE CYCLASE C RECEPTORS

Overview

Different ligands are able to activate GC-C, with different affinities and potencies related to their different molecular structure (Figure 3). There are three different categories of agonists: the endogenous ligands, the bacterial enterotoxin, and the synthetic analogues. Table I offers an overview of the main characteristics of each GC-C agonist for an easier comparison. All agonists are peptides with 14-19 amino acid residues. The presence of disulfide bonds is important for their biologic activity, since they impact on their molecular flexibility to adopt different conformations, leading to different binding potency and different resistance to degradation by proteases. In addition, the different molecules show also different pH sensitivity depending on the presence or not of particular pH-sensing residues at the N-terminus. All GC-C agonists are minimally absorbed from the gastrointestinal lumen and exert their actions locally on the intestinal mucosa [65]. After binding of any of these ligands, GC-C signaling increases luminal fluid and accelerates intestinal motility [66]. These actions may have physiologic, pathologic or therapeutic consequences. Thus, in the context of intestinal fluid homeostasis, the endogenous ligands (guanylin and uroguanylin), less potent and resistant to degradation than the bacterial and synthetic agonists carrying three disulfide bonds ("superagonists"), exert a physiological role maintaining fluid homeostasis, and preventing hypernatremia and intestinal obstruction, without dehydration. The effects of the bacterial toxin STa on the lumen are more aggressive, and lead to secretory diarrhea and dehydration, characteristic of traveler's diarrhea, cholera infections and others. Finally, three synthetic drugs have been developed. Linaclotide was the first drug of this class to be approved by the FDA and the European Medicines Agency, in 2012, for treatment of CIC and IBS-C [67, 68]. Plecanatide has recently followed [69] for these indications. Both plecanatide and dolcanatide are currently being evaluated for inflammatory bowel disease (IBD) [70, 71]. Oral administration of these drugs is safe, with mild to moderate diarrhea being their main adverse effect [68-70]. Further details are provided below and may be found in previous reviews of the topic [70, 72, 73].

Some studies addressing the search for GC-C inhibitors will also be discussed in this section.

Endogenous guanylin peptides

Guanylin (GUCA2A) and uroguanylin (GUCA2B) are the endogenous ligands for GC-C activation in humans and other mammals [74]. Other guanylin peptides (GPs), namely lymphoguanylin and renoguanylin, have been found in the American opossum (*Didelphis virginiana*) [75] and European eel (*Anguilla japonica*) [76], respectively. All these GPs are cysteine-rich peptides produced as prepro-hormones that are cleaved to release the mature active peptide [77] .The main physiological function attributed to GPs is related to postprandial hypernatremia prevention, by sodium absorption inhibition in the intestine and kidneys [78].

Mature GPs consist of 15 or 16 amino acid residues, stabilized by two disulfide bonds (Fig. 3). Lymphoguanylin (showing 40% and 80% identity with guanylin and uroguanylin, respectively) is the exception: in contrast to other peptides, it is stabilized by a unique disulfide bond (between Cys^{98} and Cys^{106}), due to the presence of a tyrosine in position 109 in the C-terminus instead of a cysteine [79]. In spite of this, lymphoguanylin is an active compound and has recently attracted attention as a potential

candidate for the development of drugs to treat gastrointestinal disorders [80], similarly to the development of drugs based on uroguanynlin and STa structures (see below).

Guanylin and uroguanylin act as paracrine or autocrine mediators in the digestive tract, Interestingly, the different amino acid sequences of guanylin and uroguanylin enable them to function in a pH-dependent manner. Thus, uroguanylin is 10 times more potent in the slightly acidic mucosal environments of the duodenum and proximal jejunum (pH 5.0-6.0), where it is preferentially expressed, whereas guanylin is primarily expressed in the ileum and colon, activating GC-C receptors under more basic conditions (pH 7.0-8.0) [81, 86].

In Ussing chamber assays, it has been demonstrated that guanylin is mainly secreted to the luminal side of the epithelium, as pro-guanylin [82]. This probably occurs also *in vivo*, in response to food/salt intake [5]. It is not clear if uroguanylin is also released predominantly to the apical site. However, it is clear that both peptides only stimulate intestinal chloride secretion when they reach the intestinal lumen, highlighting the existence of a luminocrine axis involving these peptides and the GC-C, present in the epithelial brush-border, and not the basolateral membrane [45].

Importantly, pro-guanylin and pro-uroguanylin are also basolaterally released, reach circulation and are processed to the active peptides in other organs to exert endocrine functions [83, 84] (see below). Uroguanylin is present in the serum at concentrations of 5–7 pM [85].

Human guanylin is also known as guanylate cyclase activator 2A and is encoded by the *GUCA2A* gene (1p35-p34). Its precursor protein, prepro-guanylin, contains 115 amino acid residues, is cleaved first to pro-guanylin (94 amino acid residues) and then to the mature protein, consisting of its 15 C-terminal residues (101 to 115), with two disulfide bonds [86, 87]. Two different isoforms, named A and B, have been described for guanylin. A isoform is structurally similar to STa and is more active than isoform B [88].

Similarly, human uroguanylin (guanylate cyclase activator B) is encoded by the *GUCA2B* gene, located on chromosome 1p33-p34, which has approximately 2.5kb, including three exons and two introns [89]. The mature peptide, of 16 amino acid residues, is the result of two cleavage processes, first from prepro-uroguanylin, and second, from pro-uroguanylin, with 112 and 86 amino acid residues, respectively [90]. Uroguanylin has also two disulfide bonds and is found in two isoforms, A (more similar to STa) and B.

Although both uroguanylin and guanylin dysfunctions have been correlated with several diseases, studies on single nucleotide polymorphisms (SNPs) and their respective consequences at protein level are scarce. Computational analyses have been performed to predict deleterious SNPs for both guanylin [91] and uroguanylin [92].

Bacterial enterotoxin

STa, the exogenous heat-stable enterotoxin produced by diarrheagenic-enterotoxigenic bacteria (*Escherichia coli, Klebsiella sp., Citrobacterfreundii, Vibrio cholerae, Vibrio mimicus y Yersinia enterocolitica*) is also a natural ligand of GC-C [72]. STa causes secretory diarrhea in exposed individuals by over-activating the signaling pathway of the intestinal receptor GC-C, the only confirmed molecular target for STa in humans [3].

These enterotoxins were for the first time a focus of interest in the late 1970s Cultured bacteria isolated from patients suffering from diarrhea continued to be enterotoxic after being subjected to heat treatments [93]. Two families of heat-stable enterotoxins were described, STa and STb. STa, present in the human intestine, was the only one to exert its action through a cyclic-nucleotide dependent mechanism, in contrast with STb [94] [.

Bacterial STs are translated as precursor peptides and undergo intracellular (not extracellular) proteolytic processing to active peptides from 17 to 53 amino acids [95]. Isoforms of ST share a conserved C-terminal region of 13 amino acids containing three disulfide bonds responsible for heat stability and biological activity. In contrast to the endogenous ligands guanylin and uroguanylin and the synthetic uroguanylin derivatives (see below), the bacterial toxin STa is a constitutively active right-handed spiral formation stabilized by three intrachain disulfide bridges. This makes STa 10 times more potent than uroguanylin and 100 times more potent than guanylin in binding to GC-C receptors [48]. STa and its synthetic derivative (linaclotide) are the only ligands of GC-C with three disulfide bonds, which make them capable of maximally activating the intracellular transduction machinery and thus they are considered as “superagonists” of this receptor [73]. In addition, the third disulfide bond makes these ligands more resistant to cleavage by lumen proteases than guanylin and uroguanylin and, as such, their effect is more prolonged and intense than that of the endogenous agonists [79].

It has been proposed that ST is an essential survival factor facilitating escape of bacteria from nutrient-poor to nutrient-rich environments. Thus, synthesis and secretion of ST is reduced in a milieu enriched in glucose while depletion of this sugar stimulates ST production and secretion [96]. The binding of STa to GC-C provokes cGMP production, leading to chloride, bicarbonate and water secretion to the intestinal lumen, as described above. In fact, KO mice for GC-C exhibit resistance to STa-induced intestinal secretion and diarrhea [49, 97]. Thus, ST and enterotoxigenic diarrhea are prime examples of molecular mimicry and convergent evolution. Here, bacteria have co-opted a normal mammalian physiologic function, to produce an evolutionary population survival scheme that guarantees the adequacy of nutrient resources and dissemination into new environments and hosts [72] [. Interestingly, an inverse correlation seems to exist between incidence of enterotoxigenic diarrhea and risk of developing colorectal cancer (see below). Thus it has been suggested that STa and the host GC-C represent an evolutionary-conserved symbiotic system conferring mutual beneficial effects to microbes and mammals [73].

Synthetic GC-C agonists

Finally, some synthetic agonists have been developed to target GC-C [70-72, 98]. GPs and guanylin-like peptides (STa) have been used as templates for the development of these drugs. Up to now, three drugs have been approved or are in the approval process at the US Food and Drug administration (FDA). The first one was linaclotide (Ironwood Pharmaceuticals Inc, Boston, MA, USA and Forest Laboratories Inc, New York, NY, USA), derived from *E. coli* STa and, as such, carrying also three disulfide bonds. Plecanatide and dolcanatide (Synergy Pharmaceuticals Inc, New York, NY, USA) are both analogs of uroguanylin, with two disulfide bonds, and have been developed to treat gastrointestinal disorders, despite uroguanylin renal function (see below). These drugs may be also used to supply the absence of functional GPs, caused by deleterious mutations in guanylin [91] and/or uroguanylin [92]. The use of lymphoguanylin as a template is expected to counteract the constrictions represented in practice by the existence of two or three disulfide bonds in guanylin and guanylin-like molecules, which may make possible the occurrence of errors in creating the link between the cysteines leading to the generation of inactive isoforms of the peptide [80, 99].

Linaclotide acetate (MD-1100; Constella™ and Linzess™) is an orally administered available first-in-class 14–amino acid peptide with three disulfide bonds, for the treatment of irritable bowel syndrome with constipation (IBS-C), and chronic idiopathic constipation (CIC). Up to now, the FDA has approved 290 μg of linaclotide daily for IBS-C and 145 μg daily for CIC [100]. Recent preclinical studies suggest that it might be possible to expand its therapeutic range to post-operative ileus and opioid-induced constipation. Linaclotide potently and pH-independently binds to GC-C receptors in human colon carcinoma T84 cells (K_i=1.23–1.64 nmol/L), resulting in a significant, concentration-dependent accumulation of intracellular cGMP (EC_{50}= 99 nmol/L). It also inhibits sodium absorption from lumen via NHE3. In both rats and humans [65, 101, 102], linaclotide orally administered was shown to accelerate colonic transit, enhance intestinal secretion and reduce visceral perception of pain (see below). The adverse effects were primarily diarrhea and other gastrointestinal symptoms. Diarrhea is dose-dependent and may lead to dehydration when high doses are used (non-published observations from our own laboratory, in rat models). Interestingly, in these studies its oral bioavailability was found to be very low (0.1%), and was undetectable in the systemic circulation at therapeutic doses. The amino acid substitutions that differentiate linaclotide from STa further enhance the pharmacokinetic stability and proteolytic resistance of linaclotide, allowing it to remain active across a longer portion of the small intestine [103, 104]. Linaclotide is acid-stable and resistant to aminopeptidase and chymotrypsin under non-reducing conditions, but is degraded rapidly in the duodenum by the carboxypeptidase. This results in loss of the C-terminal tyrosine, and formation of the active 13–amino acid metabolite, MM-419447. MM-419447 also stimulates the accumulation of cGMP in T84 cells and accelerates gastrointestinal transit in rats [65]. The binding affinity of MM-419447 for GC-C on T84 cells and rat small intestine brush-border membranes was comparable to that of linaclotide.

Similar to linaclotide, plecanatide (SP-304) is an orally available synthetic GC-C agonist, under development by Synergy Pharmaceuticals Inc. for the treatment of CIC and IBS-C. In this case, the drug mimics uroguanylin, the endogenous agonist of GC-C receptor. Like uroguanylin, plecanatide exerts pH-dependent actions, and shows most favorable efficacy in the acidic environment of the duodenum.

Plecanatide showed eight times the binding potency of uroguanylin in preclinical models [105]. The longer side chain of Glu (compared to that of Asp) allows an interaction of Glu^3 with Asn^9, at pH values of 5.0 (at this pH value those residues are negatively and positively charged, respectively) that may explain why plecanatide binds more potently to GC-C receptors and is more stable in the gut than uroguanylin [106, 107]. Like linaclotide, plecanatide luminally activates the GC-C receptor on gastrointestinal mucosal epithelial cells, leading to secretory and anti-nociceptive effects via cGMP [108]. Oral plecanatide therapy improved stool frequency, stool consistency, straining and overall relief of chronic constipation symptoms, with no measurable systemic absorption at any doses. Diarrhea was the most prevalent side effect, but its frequency was not significantly different from placebo, and appeared not to be dose-related in the plecanatide-treated subjects. Other gastrointestinal events were nausea, abdominal discomfort and pain, and vomiting [106, 109, 110]. In January 2017, plecanatide (Trulance™) received its first global approval in the US for the treatment of adult patients with CIC and it is undergoing phase III investigation in IBS-C [69].

Finally, dolcanatide (SP-333) has been proposed as a potential drug in IBD therapy in patients with ulcerative colitis (UC). It has anti-inflammatory effect in dextran sodium sulfate (DSS)-induced colitis in mice [71] [through the downregulation of locally released autacoids, and has recently moved into clinical trials [73, 111].

Molecules interfering with GC-C action

Although the signaling cascade mediating STa-induced diarrhea is well characterized, anti-secretory therapy targeting this pathway has scarcely been developed, and has focused mainly on the downstream pathways, not on the GC-C receptors. To the best of our knowledge, there is no specific antagonist of GC-C receptor available. The development of this kind of drugs might offer new therapeutic tools to treat diarrheas associated to infection and new pharmacological tools for research.

Initial studies using Caco-2 human intestinal epithelial cells incubated in the presence of the nucleoside analog 2-chloroadenosine (2ClAdo) suggested that the adenine nucleotide inhibitory pathway may be a target to develop anti-secretory therapy for enterotoxigenic diarrhea [112, 113]. Similarly, a small molecule CFTR blocker markedly reduced intestinal ion and fluid secretion caused by cAMP/cGMP-dependent bacterial enterotoxins, demonstrating that CFTR inhibition may thus reduce fluid secretion in infectious secretory diarrheas [114].

Since adenine nucleotides can regulate the guanylyl cyclase activity of GC-C by binding to the intracellular KHD, the effect of several protein kinase inhibitors was tested on GC-C activity, and tyrphostins, which are tyrosine kinase inhibitors, were capable of inhibiting GC-C activity *in vitro*. Inhibition was reversible but noncompetitive and nonselective, since also adenylyl cyclase and soluble guanylyl cyclase were inhibited [115].

In another experimental study, a pyrido-pyrimidine derivative (BPIPP) was found to inhibit chloride-ion transport stimulated by activation of guanylyl (both membrane-bound and soluble forms) or adenylyl

cyclases and suppressed STa-induced fluid accumulation in an *in vivo* rabbit intestinal loop model, suggesting that this drug could potentially be used for therapy of toxin-induced secretory diarrhea. It was suggested that BPIPP exerted this effect through an indirect mechanism possibly involving phospholipase C and tyrosine-specific protein phosphorylation [116].

From 50,000 small molecules, the 2-(acylamino)-3-thiophenecarboxylates were found to strongly suppress cAMP and cGMP in multiple cell lines in response to different agonists acting on G-protein-coupled receptors, adenylyl cyclase, and guanylyl cyclase. The 2-(acylamino)-3-thiophenecarboxylates functioned as nonselective phosphodiesterase activators, although it was not determined whether their action was direct or indirect. Besides other effects, the 2-(acylamino)-3-thiophenecarboxylates suppressed CFTR-mediated Cl^- current in T84 colonic cells in response to cholera and *Escherichia coli* (STa) toxins, and prevented intestinal fluid accumulation in a closed-loop mouse model of secretory diarrhea [117].

More recently, 4 N-2-(propylamino)-6-phenylpyrimidin-4-one-substituted piperidines were similarly found to be effective in T84 cells, as well as in ligated intestinal loops in piglets and rectal biopsy specimens. Interestingly, the new molecules tested blocked STa/GC-C-dependent, but not forskolin/adenylyl cyclase-dependent, CFTR activity. It was suggested that these GC-C inhibitors may form the basis for development of future therapeutics for (infectious) diarrheal disease [118].

Finally, it is worth mentioning that mouse and human intestinal enteroids have recently been developed as a new model to explore the pathophysiology of, and develop platforms for, high-throughput drug screening to identify novel compounds to prevent and treat diarrheal disease associated to enterotoxigenic *E. coli* infection. These enteroids express the components of the GUCY2C secretory signaling axis in three-dimensional *ex vivo* cultures. In these systems, STa and its structural analog, linaclotide, induced fluid accumulation, through intestinal secretion. Enteroid secretion depended on canonical molecular signaling events responsible for STa-induced diarrhea, including cyclic GMP (cGMP) produced by GC-C, activation of PKG, and opening of the CFTR. Importantly, pharmacological inhibition of CFTR abrogated enteroid fluid secretion, providing proof of concept for the utility of this model to screen for antidiarrheal agents [119].

GC-C AND ITS ROLE IN INTESTINAL FUNCTIONS

Expression of GC-C and its endogenous ligands in the intestine

GC-C is found at the apical level of the intestinal epithelium, from the duodenum to the rectum in adult placental mammals [2, 120] (Fig. 4). In neonates, in which STa diarrhea is more common and severe, higher expressions of GC-C have been detected [121] while its expression decreases with aging in humans, rodents and pigs [122-125].

In the rodent intestine, the expression of guanylin and uroguanylin mRNA follows a graded pattern, showing a higher level of uroguanylin in proximal sections whereas guanylin expression occurs more

often at distal gut [126]. It seems that species differences exist concerning the expression of both endogenous agonists.

Guanylin mRNA is expressed in the human intestinal epithelium, especially at distal small intestine and colon [126, 127]. More specifically, in humans it has been detected in colonic goblet cells and colonocytes while in duodenum it is expressed in Paneth cells and scarcely in villous epithelial cells [127].

In rat duodenum and jejunum uroguanylin is located in goblet but not Paneth cells [127]. While in rat duodenum uroguanylin mRNA expression was widespread, in colon it was seldom present and it seemed to be located in colonocytes and occasionally goblet cells. In human duodenum, in contrast, it was less expressed and limited to occasional epithelial cells. In human colon, it was strongly expressed in solitary superficial epithelial cells [127].

In mice, guanylin and uroguanilyn mRNA are expressed in the crypts of Lieberkühn and in the villus-surface region. In jejunum and ileum, both mRNA are detected in enterocytes but in colon only guanylin transcript is expressed in colonocytes. In duodenum, guanylin mRNA is restricted to secretory cells whereas uroguanylin mRNA is found in columnar cells [128].

Protein expression of guanylin and uroguanilyn remains controversial due to the lack of specific antibodies [5]. Concerning guanylin protein, it may be expressed in mucus-producing goblet cells and colonic columnar epithelial cells in rat small intestine and colon [129]. Uroguanylin protein has been detected in enterochromaffin cells (EC) in rat small intestine [130] although other authors have not identified its mRNA in cells that co-expressed an EC marker [127].

Interestingly, the high expression of uroguanylin in the duodenum and its pH-dependent mechanism of action indicate a significant role of this peptide in the neutralization of acidic stomach secretion in the duodenal lumen after gastric emptying. Uroguanylin action results in pH increase in the duodenum due to inhibition of H^+ secretion and activation of HCO_3^-secretion (in addition to pancreatic secretion) [131]. Of note, uroguanylin action is more marked after gastric emptying when pH of the duodenal lumen is acidic [132].

Intestinal fluid and electrolyte homeostasis

As already mentioned, the role of GC-C in intestinal homeostasis has been recognized for a long time. First, it was discovered to be the receptor for STa leading to secretory diarrhea associated to alimentary toxi-infections. More recently, several genetic mutations have been found in different parts of the molecule with different consequences. Finally, with this background behind, it was only a question of time that ligands were developed to treat constipation.

In humans, the gene for GC-C (*GUCY2C*) is located in chromosome 12 (12p12) [133]. Recently, its role in gastrointestinal physiology has been reinforced with the discovery of an intersection of human GC-C

gene mutations and digestive pathologies. Figure 1 shows the site of the mutations affecting human GUCY2C described so far [134].

Thus, in two consanguineous Bedouin families, an inactivating mutation of GUCY2C (thereby reducing cGMP) was described to produce meconium ileus [135]. One of the findings was an amino acid substitution (p.Asp387Gly) in a well conserved area affecting the extracellular domain of GC-C, interfering with ligand binding. When the mutation was transfected into a cell line, STa-mediated activation of GC-C cells expressing the mutant protein produced 60% less cGMP than normal GC-C expressing cells. The second family had a single affected person, which suffered from a severe meconium ileus that needed surgery. In this case, a homozygous insertion mutation was detected (p.Asn757Lysfs*2) resulting in a premature stop codon, which fully abolished the guanylate cyclase catalytic domain of GC-C, thereby preventing cGMP production [66, 135]. A third family of Lebanese origin showing GC-C gene mutations has been reported. The parents were first cousins and 2 out of their 4 children were affected. Two mutations were found, p.(Ala670Thr) and p.(Cys928Arg), in conserved regions of the protein. One of them led to the change of a hydrophobic amino acid for a hydrophilic one, resulting in a conformational change of the receptor. The other mutation led to the substitution of an amino acid residue that created disulfide bonds to another one. Once again, these changes made the receptor less functional [136]. The authors speculate that these genetic mutations, found in desert-living population, essentially children, may be a selective adaptation to a dry, hostile environment [135, 136].

In contrast, a Norwegian family suffering from periodic diarrhea was identified to carry a mutation in GUCY2C (p.Ser840Ile), which led to a hyper-active receptor that produced high levels of cGMP. In the Norwegian kindred, the predominant phenotype of affected family members mimicked IBS, resulting in 3-4 loose stools/day. One fourth of the affected family members (8 out of 32) already presented in infancy with severe dehydration. IBD and small-bowel obstruction were found in 20-25% of these patients [137]. More recently, four additional activating germline mutations in GUCY2C have been identified to be the cause of severe congenital secretory diarrhea [134]. As opposed to the relatively mild type of diarrhea in the Norwegian family previously mentioned, a more severe phenotype (which uniformly began prenatally) was found. It included maternal polyhydramnios, prominent abdominal distension due to dilated fluid-filled loops of the intestine (increasing the risk of intestinal obstruction at birth), and high fecal losses of sodium and chloride. One of the patients developed severe IBD at 4 years of age. All four patients harbored different heterozygous *de novo* mutations in GUCY2C. Postnatally, these patients required total parenteral nutrition to treat dehydration for at least several months, as well as sodium supplementation to treat severe dehydration and to maintain normal body growth by preventing total body sodium depletion [134].

In the Norwegian family, no increase in basal activity of the receptor was observed [137]. In contrast, constitutive activity was identified as the cause of the diarrhea in one of the mutations described by Müller *et al* [134]. Interestingly, the enhanced activity of the remaining newly-described mutant receptors and that described in the Norwegian family, was not a consequence of increased affinity, but was due to the fact that ligands were more potent in terms of enhancing cGMP production. Thus, normal

concentrations of uroguanylin and guanylin would elicit supraphysiological accumulation of intracellular cGMP, leading to diarrhea [134, 137].

Regulation of the intestinal barrier and anti-inflammatory effects

In humans, the gastrointestinal tract has a surface area of approximately 100 m^2 and is integrally linked via effector systems (epithelium, secretory epithelium and endocrine cells and vasculature) to the enteric nervous system (gut-brain axis) [138]. The cells lining the intestinal tract include enterocytes, secretory cells, and goblet and enteroendocrine cells. Tight junctions working with actin and cytoskeletal proteins hold the enterocytes in place, and the mucus layer containing bioactive compounds, hormones such as gastric inhibitory peptide, serotonin and gastrin, and the immune cells provide both a chemical and physical barrier. Intestinal homeostasis may be altered in different conditions, including IBD. In IBD patients, a chronic and increasingly aberrant inflammatory response occurs, which is thought to be triggered by a break down in immune homeostasis, exposure to intestinal flora in the gastrointestinal tract as a consequence of a disruption to the mucus barrier [139], and autophagy of breakdown proteins as well as microbial attack.

One of the putative mechanisms underlying colitis is related to the disruption of the intestinal epithelial barrier. In that sense, GC-C may play a role in preserving the barrier integrity, as a disruption of this has been detected in GC-C KO mice [140]. This may be due to the loss of tight junctions such as the junctional adhesion molecule A (JAM-A), occludin, claudin 2 and claudin 4 [140, 141]. However, the disruption in GC-C signaling has also been associated with a reduced number of colonic goblet cells, resulting in decreased production of mucin and intestinal trefoil factor [142], which are the principal components of the gut coating mucus layer for maintenance of epithelial barrier protection and for post-injury restitution. Furthermore, it has been speculated that the underlying mechanism may be a change in microbiota composition caused by ion or water secretion [8]. As described above, GC-C-induced inhibition of NHE3 may be involved in the physiopathology of diarrheic conditions. In fact, NHE3 KO mice show diarrhea [143], develop distal colitis and are more susceptible to DSS-colitis [144]. Furthermore, NHE3, as well as the chloride/bicarbonate exchanger DRA (downregulated in adenoma) are less expressed in the mucosa of IBD patients [145-149]. Importantly, alterations in sodium and chloride transport may lead to changes in microbiota composition. Thus, a decrease in phylogenetic diversity of microbiota has been detected in the colon of NHE3 KO in comparison to WT mice [150] [. The infection with *Clostridium difficile* induces mucosal NHE3 downregulation and increase in stool sodium content that collaborates to dysbiosis [151]. Consequently, NHE3 may play a role in the composition of microbiota, leading to dysbiosis in case of downregulation as in IBD or bacterial infections [150, 151].

In addition to these factors, there is evidence supporting that activation of GC-C receptors have anti-inflammatory actions in the gut. First, pharmacologic inhibitors of phosphodiesterases, which degrade cGMP, have beneficial effects in mouse models of IBD [152, 153]. Also, the uroguanylin analogue, plecanatide, might down-regulate cyclooxygenase-2 (COX-2) production of the inflammatory mediator prostaglandin E2 (PGE2), through a decrease in intracellular arachidonic acid levels. Arachidonic acid is

released from membrane phospholipids via cleavage by phospholipase A2 (PLA2), which is phosphorylated and inhibited by PKG [72, 154].

Importantly, the deletion of GC-C gene results in a more severe colitis in several experimental models of IBD, like colitis induced by DSS or 2-4-6 trinitrobenzene sulfonic acid (TNBS) [141, 155]. Moreover, genes involved in GC-C signaling are downregulated in IBD and TNBS-treated rats [155]. GC-C deficiency increases DSS susceptibility in mice and makes these mice more prone to lipopolysaccharide-mediated pro-inflammatory gene expression [156] whereas enhancing GC-C signaling protects against colitis [141]. However, there are controversial data, since GC-C deficiency has also been postulated by other researchers as a protective factor against DSS-induced colitis [157]. These discrepancies are difficult to explain but it seems clear that correct GC-C expression and function is important for intestinal homeostasis.

There is evidence also in humans for a role of GC-C in gut inflammation. Patients with IBD have lower expression of the receptor and its agonists [155, 158], as well as several transcription factors and genes involved in GC-C signaling in comparison to healthy controls [155]. Furthermore, an inverse correlation between expression and the severity of ulcerative colitis has been shown [158]. However, this study was not accompanied by the determination of cGMP levels, which may have strengthened the data. Interestingly, in a proteomic study, the level of a peptide from pro-guanylin (VTVQDGNFSFSLESVK) was found to be elevated in the serum of ulcerative colitis patients in comparison with the serum of patients suffering from Crohn's disease [159], suggesting that this could be a relatively non-invasive marker to differentiate between these two types of IBD.

IBD is a risk factor in the development of colorectal cancer (CRC). Very recently, it has been shown in mice that the activation of GC-C by plecanatide reduced inflammation-related colonic dysplasia and significantly increased uroguanylin expression in the proximal small intestine and proximal colon. Expression was also higher at the distal colon, but the value did not reach statistical significance, maybe due to its minimal expression at this location even at normal conditions. Interestingly, no changes in GC-C expression were observed in plecanatide-treated colitic mice [160]. Further research is needed to determine if the loss of uroguanylin and guanylin in the colonic mucosa is a predictor of the risk for developing CRC in IBD patients.

Epithelial proliferation and tumorigenesis

There is an increasingly recognized inverse relationship between GC-C signaling and CRC, as has been extensively reviewed elsewhere. Among other evidences, this relationship was suggested from epidemiologic studies showing that in geographic areas where enterobacterial infections leading to diarrhea are more frequent, there is an inverse correlation between these and CRC incidence [62]. Infectious diarrhea caused by ST-producing bacteria is a major morbidity factor in areas of poor sanitation and crowded conditions, and remains a principal cause of travelers' diarrhea and infant mortality in developing nations [161]. Provocatively, the worldwide risk of travelers' diarrhea inversely

correlates with the incidence of colon cancer, and developing countries appear to be protected from colorectal transformation [62].

Indeed, GC-C may have anti-proliferative or cytostatic actions in the intestine, as suggested in both *in vitro* and *in vivo* studies. At the molecular level, different mechanisms have been proposed (Fig. 2). In general, it can be said that GC-C-activating ligands and downstream mediators suppress oncogenic drivers (e.g. pRb, cyclin D1, β-catenin, pAKT) and increase tumor suppressors (e.g. p21, p27) [162]. The activation of GC-C in T84 and Caco-2 cell cultures inhibited proliferation [163] and suppressed the formation of tumors and its metabolism in mice via the inhibition of AKT (PKB) pathways [164]. In p53-deficient human colorectal carcinoma cells, prolonged activation of the PKGII/p38/MAPK/p21 pathway lead to cellular senescence and reduced tumorigenic potential [165]. Some other studies suggest different mechanisms, such as the regulation of calcium levels through a CNG channel [162, 166, 167]. Other actions exerted by GC-C that have been proposed to be involved in its anticancer effects are the regulation of crypt growth [142], maintenance of genomic integrity [168] and the preservation of the epithelial barrier [140, 141]. Furthermore, GC-C may prevent metastasis by regulating cell matrix metalloproteinase 9 produced by CRC cells [169].

GC-C regulates proliferation by modulating AKT signaling [170, 171]. PTEN-PI3K/AKT signaling is one of the most frequently altered pathways in CRC. This pathway constitutes a central node integrating mitogenic, pro-oncogenic, and tumor suppressing signals to coordinate programs, including the cell cycle, metabolism, DNA repair, and apoptosis, at the intersection of tissue homeostasis and tumorigenesis [171, 172]. In fact, GC-C signaling regulates all the processes typically involved in tumorigenesis [173]. Silencing GC-C and cGMP production potentiates tumorigenesis in genetic and carcinogen-induced mouse models of CRC [142, 164], and is associated to:

- Corruption of normal mechanisms regulating proliferation, with enhanced expression of the drivers, and reduced expression of inhibitors, of the cell cycle, and expansion of the proliferating crypt compartment [62, 142, 163, 164, 168],
- Disruption of the DNA damage sensing and repair machinery, with promotion of mutations in key tumor suppressors like APC, and in oncogenes like β-catenin [164, 168],
- Contraction of the differentiated epithelial cell compartment, with specific loss of the secretory lineage, including goblet cells [168],
- Blockade of metabolic plasticity, with imposition of glycolytic programming across the entire crypt-surface axis, thus mimicking the Warburg metabolic phenotype pathognomonic of neoplasia [164],
- Reprogramming of bidirectional interactions between epithelial and mesenchymal compartments, with creation of maladaptive circuits that drive the formation of desmoplasia, a defining feature of tumorigenesis [174].

Interestingly, dysregulated GC-C alone does not induce spontaneous transformation, underscoring the significance of the integrity of compensatory apoptotic mechanisms in the context of hyperproliferation,

metabolic reprogramming and loss of genomic integrity [72]. Paradoxically, in CRC tissue, GC-C is overexpressed, but guanylin is reduced or absent and it has been shown that the lack of action of endogenous activators of GC-C, guanylin and uroguanylin, contributes to CRC susceptibility [175]. Adenoma or adenocarcinoma colon cells show less expression of these endogenous agonists than normal colonic tissue [126, 176]. Thus, a model of colorectal preneoplasia was suggested in which pro-oncogenic signaling by the PTEN-PI3K/AKT axis was engaged, in the absence of component mutations, through a lineage-specific mechanism involving silencing of the intestinal gene product GUCY2C by loss of paracrine hormone expression [72]. It is now admitted that GC-C acts as an intestinal tumor suppressor with the potential to prevent the initiation and progression of CRC [160, 177, 178] and its functional silencing through loss of paracrine hormone expression is a universal mechanistic step in sporadic colorectal carcinogenesis [179].

Thus, CRC might initiate as a disease of paracrine hormone insufficiency [3, 171]. In future studies, it will be important to define the molecular mechanisms silencing hormone expression, in order to determine their reversibility to prevent tumorigenesis [179]. One possible mechanism has already been proposed that links the development of CRC with endoplasmic reticulum stress, reduced satiety and development of obesity [180, 181].

Whatever the case may be, like other diseases of endocrine insufficiency reflecting hormone loss, but preservation of receptor expression, silencing GC-C might be prevented by therapeutic replacement of GC-C ligands. In fact, oral administration of guanyline inhibited intestinal tumorigenesis in mice [160], and cancer resistance in developing countries has been suggested to reflect, in part, longitudinal exposure of endemic populations to enterotoxigenic infections [182] and the ability of STa to regulate the cell cycle transition, and suppress proliferation of intestinal epithelial cells [62, 163]. Since linaclotide is an approved drug for chronic constipation, with a good safety profile, its use might soon give light on whether it is capable of reducing the risk for developing CRC in non-cancerous patients. In fact, linaclotide and its synthetic analogues have been proposed for treatment of ulcerative colitis, which is associated to an increased risk of CRC [183]. Furthermore, a clinical program to explore its utility in preventing colorectal transformation in humans has been recently initiated (ClinicalTrials.gov Identifier: NCT01950403) [184]. However, it has very recently been shown that daily oral administration of 0.87 milligrams of linaclotide for 7 days to healthy volunteers was associated with homeostatic GUCY2C signaling, including increase of cGMP, phosphorylation of vasodilator-stimulated phosphoprotein and inhibition of proliferation quantified by reduced Ki67-positive epithelial cells, but only after oral colon preparation with polyethylene glycol solution (MoviPrep), suggesting that the effect was related to the laxative preparation. Thus, the FDA approved formulation for small bowel delivery to treat CIC seems to be inadequate for reliably regulating GUCY2C in the colorectum to prevent tumorigenesis and it could be necessary to develop a novel GC-C agonist formulated for release and activity targeted to the large intestine for CRC prevention [185].

Whether or not linaclotide or its analogues could be useful to treat CRC patients is not yet known, but several aspects makes GC-C a potentially useful therapeutic target. First, GC-C signaling has been shown

to revert the tumorigenic phenotype of human CRC cells, due to regulation of proliferation and metabolism [62, 71, 163, 164, 168]. Second, it inhibits MMP-9 produced by the CRC cells, thus reducing invasion and metastasis [169]. Third, it suppresses epithelial secretion of TGF-β, activation of submucous fibroblasts and the desmoplasic reaction leading to invasion and progression of CRC [174].

Finally, since CRC cells overexpress GC-C, it has been proposed to be a good and selective marker of liver and other metastasis of colorectal tumor [186]. Thus, different strategies may be developed aimed at localizing this receptor (or its specific mRNA), which is normally confined to the gut, in extra intestinal sites throughout the body, i.e., using antibodies against GC-C labeled with radionucleotides, for detection and monitoring of treatment progress, or attached to chemotherapeutic drugs. Indeed, immunotoxin therapy or T cell-mediated immunotherapies might be efficient to eliminate metastases [162, 187].

Visceral sensitivity

GC-C may modulate colonic sensitivity. Antinociceptive effects of linaclotide were first reported by Eutamene *et al* [188], who used animal models of inflammatory (TNBS) and non-inflammatory (stress models) pain. These authors measured sensitivity electromiographically, during colorectal distension through an intracolonic balloon. This technique is based in the principle that visceral pain evokes the reflex contraction of abdominal muscles. In this study, oral linaclotide did not affect basal sensitivity but elicited analgesia in all the models tested [188]. GC-C activation was involved in linaclotide effects, as demonstrated in TNBS-induced hypersensitive mice, expressing or not the receptor, in which linaclotide was effective only in WT mice [188]. Not only linaclotide-mediated GC-C activation has antinociceptive effects, endogenous agonists have also been implicated. In rats or mice subjected to TNBS-induced colitis, uroguanylin reversed visceral hypersensitivity [189, 190].

Interestingly, no expression of GC-C was detected in neuronal structures such as dorsal root ganglia or spinal cord neurons, and therefore it was concluded that this effect of uroguanylin depended exclusively on activation of epithelial receptors [190]. Furthermore, the removal of colonic mucosa attenuated linaclotide inhibition of nociceptors [190]. Thus, GC-C activation might decrease afferent pain fiber firing, presumably through the release of specific soluble mediators, which stimulate surrounding dendritic nerve endings and alter neuronal firing rates. Evidence strongly suggests that the analgesic mechanism depends on cGMP produced by epithelial cells and later transported outside the cells through the basolateral membrane [67]. In rats, oral cGMP reproduced linaclotide and uroguanilyn analgesic effects in a dose-dependent manner [189]. Besides, Caco-2 cell cultures exposed to linaclotide or uroguanylin increased cGMP extracellular apical and basolateral transport, which was blocked by probenecid, an inhibitor of a cGMP pump [189, 190]. Multidrug resistance-associated protein 4 (MRP4) was found to potentiate linaclotide-induced electrolyte secretion and intracellular cGMP accumulation in colonic tissue mounted in Ussing chambers, but MK571 (an MRP4 inhibitor) was only capable of blocking cGMP efflux from the apical (not the basolateral) membrane [191]. Interestingly, high levels of MRP5 expression have been reported in different segments of the rat colon, and predominantly basolateral expression of MRP5 in human colonic mucosal biopsies, implying a prominent role for this

cGMP efflux pump [189, 192]. Remarkably, cGMP production following activation of GC-C occurs locally in close proximity to the apical membrane, but the mechanisms potentially involved in the translocation of cGMP to the basolateral cell membrane to drive extracellular transport, such as encapsulation into transport vesicles and/or passive diffusion, remain unknown [191].

Two randomized, double-blind placebo-controlled Phase III multicenter clinical trials with linaclotide have validated its use for the symptomatic treatment of IBS-C patients [193-195]. In one of the studies, 804 IBS-C patients received daily linaclotide (290 μg) or placebo for 26 weeks. Linaclotide-treated patients showed an improvement of pain from the first week of therapy [195]. In the other study, 800 patients were recruited for a 12 week randomized trial, in which they received linaclotide (290 μg) or placebo followed by a 4 week randomized withdrawal period, in which patients who had received linaclotide were re-randomized to oral linaclotide or placebo. As in the other mentioned study, pain was improved in linaclotide patients during the 12-week period, starting from the first week. Concerning the withdrawal period, patients re-randomized to placebo showed a worsening of abdominal pain while in linaclotide patients pain relief continued [194]. However, other studies have questioned the efficacy and safety of linaclotide to treat constipation and have described the occurrence of abdominal adverse events, possibly due to intestinal fluid accumulation [196].

IMPLICATION OF GC-C IN THE PHYSIOLOGY OF NON-DIGESTIVE ORGANS

The GC-C receptor is not only involved in regulating intestinal function and homeostasis. It has also been found to be expressed in other organs like brain, kidney, adrenal gland, lung, pancreas, and male and female reproductive organs, although at lower levels than in the intestine [5]. Furthermore, uroguanylin mRNA is expressed in the kidney, heart, reproductive system, and brain [197]. It is then supposed that uro/guanylin would act not only as local paracrine factors in the intestine, but also as endocrine hormones regulating processes like satiety, sodium regulation, hyperactivity, Clara cells function and NO-related effects. We will next concentrate in some of these activities.

GC-C as an endocrine receptor regulating feeding behavior

As described above, the binding of GC-C and its ligands activates the intracellular accumulation of the second messenger cGMP. This plays an essential role in feeding satiation according to an evolutionary-conserved circuit [198, 199] that mimics other known gut-brain endocrine axes involving hormones like peptide YY, leptin, and ghrelin [200]. This signaling program mediated by cGMP was first described in invertebrates, where it regulates feeding behavior and accumulation of fat by means of neurons controlling satiety that express GC-C. Uroguanylin appeared to be the key ligand in this process [201]. Interestingly, the sequence homology shared by invertebrate GC-C with mammalian guanylyl cyclases gave a clue of its role in vertebrates [202, 203]. Details of the situation in mammals came after the generation of GC-C KO mice [199]. These animals exhibited hyperphagia and diminished satiation, independently of caloric intake, as it was observed when they were put on many different diets, ranging

from a standard low-calorie to a high-calorie diet. The increase in their body weight (26% heavier than WT*)* was associated with greater accumulation of adipose mass and adiposity index (percentage of body weight contributed by fat) in visceral and subcutaneous fat compartments. Similarly, mice displayed amplified metabolic syndrome characteristics like hepatic steatosis, cardiac hypertrophy, hyperleptinemia, hyperinsulinemia, and impaired glycemic control. As expected, GC-C KO mice were resistant to diarrhea induced by heat-stable enterotoxins [199]. Interestingly, the expression of *Gucy2c* mRNA and protein occurred not only in the intestinal tube but also, although to a lower extent, in the hypothalamus. However, orally administered STa, which is not absorbed, did not alter food consumption in WT mice, eliminating a role for digestive GC-C in mechanisms regulating appetite. In contrast, iv administration of STa induced dose-dependent satiation in WT mice, but not GC-C KO mice [199]. Finally, the central mechanism for this effect became clear when GC-C agonists were injected directly into the third ventricle of the brain and feeding was reduced. Looking for endogenous ligands for this pathway, differences between guanylin and uroguanylin have been found. Both endogenous ligands are produced by the intestine, from which they are secreted into the circulation as inactive pro-hormones that require proteolytic hydrolysis to become active [83, 204]. However, like in invertebrates, only pro-uroguanylin induced satiation in mice, reflecting a hormone-specific proteolytic activation in hypothalamus. Similarly, only pro-uroguanylin-neutralizing antibodies could block this process [199].

These findings indicate that there is a central mechanism of GC-C signaling in feeding, with little effect of orally administered ligands as shown with STa administration over a 12 hour period [199]. However, a role of peripheral neurons should not be discarded, given that they are increasingly recognized as important regulators of food intake, and that occurrence of STa in the intestine is very frequent [66, 200].

Finally, the production of uroguanylin itself depends on diet. It has been shown that diet-induced obesity suppressed intestinal uroguanylin expression in mice through endoplasmic reticulum (ER) stress [205] and its associated unfolded protein response, a mechanism common to other hormones like insulin [206]. This suppression depended on consumed calories since calorie restriction restored uroguanylin in obese mice, whereas a diet with high carbohydrates eliminated ligand synthesis in lean animals. Again, the iv administration of STa agonists restored hypothalamic GC-C function even in obese animals, demonstrating that endocrine sensitivity in the hypothalamus is preserved in the context of a chronic high-fat diet [205]. This is in contrast with other endocrine hormones like leptin and insulin, whose serum concentrations rise in obese animals, leading to chronic overstimulation and receptor desensitization [207]. These experiments clearly show a novel mechanism to generate obesity in which calorie-induced suppression of intestinal uroguanylin impairs hypothalamic axis regulating satiety through loss of endocrine signaling. Thus, in a situation of hormone insufficiency but preservation of receptor sensitivity, obesity might be prevented or treated by GC-C agonists.

However, the precise central mechanisms and efferent pathways mediating uroguanylin effects have still not been well defined. It has been recently shown that chronic infusion of uroguanylin reduces weight gain in obese mice independently of food intake, by means of brown adipose tissue thermogenesis and a certain browning in white adipose tissue through stimulation of the sympathetic nervous system. At the

same time, brain uroguanylin increased fecal output through the vagus nerve with no involvement of gastrointestinal adverse effects, such as diarrhea [208]. Opposite to these results, another study found that uroguanylin or STa directly administered in the third ventricule failed to regulate appetite, concluding that the modest effects of uroguanylin on energy homeostasis is not centrally mediated [209]. It is difficult to explain the disparities of this striking result with the previous papers cited, although methodological differences may have contributed to it. For example, authors used both mice and rats, when uroguanylin pharmacology is not fully characterized in this last rodent species [205]. On the other hand, animals were only fed a high-fat diet when they were adults, not at earlier stages [209], which could have also contributed to alter their response.

There might be several ways for GC-C signaling to regulate satiety pathways. First, it could be mediated through the modulation of action potentials via depolarization or hyperpolarization of neuronal membrane potentials. Interestingly, whereas in the intestine, cGMP produced by GC-C activation affects ion channels of the epithelial cells, in the brain it regulates neurotransmitter release [210]. It is then conceivable that GC-C might control neuronal activation in the hypothalamus through the regulation of electrolyte flux via cGMP-regulated channels [170]. Second, the increase of cGMP after GC-C activation in neurons may induce nuclear translocation of cGMP-dependent protein kinases (PKG) to regulate gene expression of satiety neurotransmitters. Also, increasing of intracellular calcium concentration is a known regulator of vesicle secretion in neurons that may lead to the release of neurotransmitters that regulate appetite [170]. Finally, GC-C may regulate appetite interacting with other signaling pathways since pro-uroguanylin shares endocrine axis with other hormones. Besides, receptors for leptin, insulin, and ghrelin are expressed not only in the hypothalamus, but also in dopaminergic neurons of the midbrain, which is another key regulatory site for energy homeostasis via reward circuits [211]. The detection of GC-C transcripts in the midbrain [212] suggested a role for GC-C receptors in two fundamental areas for appetite regulation, as other known receptors essential for energy regulation [170].

Related to its function regulating feeding, GC-C receptors have also been found in adipose tissue. After performing microarray assays to identify specific genes in the mesenteric fat of diet-resistant non-obese rats, a high expression of guanylin and GC-C was found in macrophages [213]. Transgenic rats overexpressing guanylin and GC-C were generated to study this. They resulted resistant to the effects of high-fat diets, downregulated apoptosis-related genes and upregulated genes involved in fatty acid oxidation. Similarly, mesenteric macrophages overexpressing guanylin and GC-C inhibited lipid accumulation in adipocytes *in vitro* [213]. The same researchers used these double transgenic mice to study the inflammatory state of mesenteric fat of rats fed with a high-fat diet. They found that transgenic animals had high concentrations of both PKG and one of its targets, the vasodilator-stimulated phosphoprotein (VASP). On the contrary, mRNA levels of pro-inflammatory factors (TNF-α, COX_2, iNOS, MCP-1 and CD11c) were lower than in WT animals [214]. Interestingly, the expression of inflammatory cytokines is increased significantly in the macrophages of VASP KO mice [215]. The authors of this study concluded that the GC-C system in macrophages regulates the cGMP–PKG–VASP pathway and controls obesity through the downregulation of pro-inflammatory factors [214].

Overall, the role of GC-C receptors in the hypothalamus, the ventral tegmental area, the *substantia nigra*, the adipose tissue, and the dynamics of synthesis of its ligands represent novel therapeutic strategies for the understanding and treatment of obesity. On the other hand, the association between obesity and colon cancer has been well established [170] and the loss of intestinal uroguanylin expression with chronic overnutrition mimics the universal loss of intestinal GC-C hormones in CRC. Similarly, GC-C plays a role in intestine as a tumor suppressor since GC-C signaling reverses the tumorigenic phenotype of human colon cancer cells by regulating proliferation and metabolism [177]. This has opened the suggestion of considering CRC as a disease of paracrine hormone insufficiency that can be prevented or treated by oral hormone replacement therapy employing GC-C ligands [167, 170, 180, 205]. Thus, the reconstitution of GC-C signaling could restore anorexigenic responses corrupted by diet-induced obesity, reducing adiposity and its comorbidities. In turn, the reduction of caloric intake could restore endogenous GC-C ligand expression, promoting GC-C tumor suppressor activity and epithelial homeostasis agonism, opposing both obesity and intestinal tumorigenesis [170, 175, 180, 216].

GC-C and attention deficits

Apart from its role as an endocrine receptor regulating satiety, GC-C expression in neurons located in the ventral tegmental area and *substantia nigra* of the midbrain have been shown to be related to hyperactivity and attention deficits [217]. This single and interesting report showed that GC-C protein was expressed on the soma and dendrites of dopaminergic neurons, although application of neither guanylin nor uroguanylin (G/UG) affected the physiological properties of these neurons since they did not produce any effect on synaptic responses mediated by ionotropic glutamate or GABA receptors. However, G/UG increased the responses evoked by (*S*)-3,5-Dihydroxyphenylglycine (DHPG), a ligand of metabotropic glutamate receptors (mGluRs). Similarly, activation of GC-C signaling amplified the excitatory responses mediated by an agonist of muscarinic acetylcholine receptors (mAChRs). The potentiating effect of GC-C was mediated by PKG in both routes. Importantly, this effect was lost in GC-C KO mice. Long-term monitoring revealed that the locomotor activity of GC-C KO mice was higher than that of WT mice. These animals also manifested higher levels of novelty-seeking behavior in olfactory habituation tests and an impaired olfactory habituation after repetitive presentation of the same odorant. Similarly, GC-C KO mice showed an impaired behavioral inhibition to signals with times of reaction three times longer than their WT counterparts [217]. Hyperactivity and reduced response habituation is associated with impaired attention in humans and animals. Thus, the behavioral phenotypes of GC-C KO mice mimic the core symptoms of Attention Deficit Hyperactivity Disorder (ADHD). In fact, it is considered that ADHD involves a dysfunction in the dopaminergic system and GC-C KO mice happened to have significantly lower levels of basal extracellular dopamine than WT mice [217]. Treatment with amphetamines at a dose comparable to that used for treating human ADHD significantly reduced the hyperactivity of GC-C KO mice. Finally, normal behavior of GC-C KO mice was restored after infusion of cGMP-dependent protein kinases and PKG activators in the ventral tegmental area and *substantia nigra* [195]. It would then be possible to consider GC-C KO mice as an ADHD model, and pathways to stimulate cGMP signaling as new tools for the treatment of neuropsychiatric disorders associated with the dopaminergic system without undesirable amphetamine-like effects [217]. Indirectly,

given the association between diet and ADHD [218, 219] some authors have speculated that a lack of pro-uroguanylin release in response to a poor diet (associated to malnutrition) might be a contributing factor to the symptoms of ADHD, via reduced GC-C action in midbrain dopaminergic neurons. Therefore, an improved diet or the use of GC-C agonists to target these pathways would be of benefit in treating symptoms associated with ADHD [66].

GC-C and kidney function

The concept of a gastrointestinal-renal axis regulating sodium excretion independently of aldosterone was raised after finding that an oral sodium load induced in rabbits a greater natriuretic response than the same load given intravenously [220]. Uroguanylin has been proposed to be the responsible natriuretic factor since the intravenous infusion of pro-uroguanylin increased renal sodium excretion in a dose-dependent manner [221, 222]. Similarly, uroguanylin mRNA levels increased in mice kidney after high-salt intake [223] as well as in plasma and urine of rats [224]. Even the architecture of proximal convoluted tubules changed in uroguanylin KO after salt intake with vacuolization and altered distribution of sodium transporters [5].

This gastrointestinal-renal natriuretic signaling axis has however raised several questions regarding both the ligand and the receptor. It was first considered that the endogenous ligand uroguanylin would be secreted mainly in the intestine, since very low levels of pro-uroguanylin mRNA were detected in the kidney. However, it has been shown that the rat kidney contains enough amounts of pro-uroguanylin (16% of the level in the intestine) expressed in the distal tubules [225]. It is then considered that both sources of uroguanylin could be contributing to the renal pool of the ligand. The second question refers to the GC-C receptor since GC-C KO mice still exhibit significant uroguanylin-induced natriuresis in the absence of GC-C expression [226]. Other authors have described certain levels of GC-C differentially expressed depending on the renal localization. Thus, in proximal tubules GC-C would mediate a canonical cGMP-dependent pathway [227], but in the cortical collecting ducts uroguanylin would function in a GC-C- and cGMP-independent signaling pathway [228, 229]. Therefore, it seems that the effects of uroguanylin in the kidney may be mostly independent of GC-C, although its involvement should be considered under conditions of high salt ingestion, when the receptor is actually up-regulated [230, 231].

However, a major objection aroused against this model after testing in humans the role of pro-uroguanylin and pro-guanylin as mediators of this intestinal-renal axis under low and high sodium intakes [232]. Authors found neither differences in sodium excretion nor changes in pro-uroguanylin or pro-guanylin concentrations in response to oral versus intravenous sodium loads. In fact, they even observed a decrease in pro-uroguanylin concentration [232]. This is in high contrast with previous reports, which considered that the essential role for guanylin/uroguanylin in the entero-renal axis is to maintain salt homeostasis [228].

Besides its action in the kidney under normal conditions, the role of these ligands should also be considered under pathological situations. Uroguanylin dysfunction has been correlated with several diseases: KO mice lacking uroguanylin showed higher blood pressure [233]; patients with chronic renal failure presented higher uroguanylin plasma levels [234]; congestive heart failure was associated with an increase in uroguanylin excretion in the urine [235]; and nephrotic syndrome was related to higher uroguanylin plasma levels and low urine excretion of uroguanylin [236]. These elevated plasma pro-uroguanylin levels were probably a result of impaired renal clearance of the circulating peptide [237].

Taken together, these results support the idea of the existence of a gastrointestinal–renal natriuretic axis mediated by GC-C. However, its relevance in humans has not been definitely demonstrated. Thus, the role of GC-C and uroguanylin in the regulation of renal function needs to be clarified and could be more complex than previously indicated.

Other locations of GC-C receptors: reproductive and respiratory systems

Several studies have demonstrated an effect of uroguanylin on male and female reproductive organs of rodents [228] and in human penis [238].

Besides the nitric oxide (NO)-induced relaxation of myometrium, which occurs in a cGMP-independent manner, it has been found that uroguanylin generates contractions in the pregnant myometrium of guinea pigs via a cGMP–dependent mechanism [239].

Regarding males, uroguanylin has been found to induce concentration-dependent relaxation of the cavernous body of the penis in humans. This was mediated by a guanylate cyclase and K_{ca}-channel-dependent mechanism, independent but additive to the classical NO-activated soluble guanylyl cyclase-cGMP pathway, making GC-C receptors potential targets for the treatment of male erectile dysfunction [238].

Finally, GC-C and its ligands have also been demonstrated to be expressed in airway epithelium, where they play a role in airway function. They have been identified in the apical membrane and secretory granules of Clara cells of humans [240]. Similarly, they were found to activate chloride conductance in the human airway cells from cystic fibrosis patients through activation of unusual channels [241], and animal models of asthma treated with uroguanylin improved their respiratory function [242], which opens new possibilities for these bioactive peptides.

CONCLUSIONS

Herein, we have deeply described the current knowledge about GC-C and its ligands. GC-C is found mainly in the mucosa of small and large intestine where it plays an essential role to regulate water and electrolyte luminal secretion, as well as more recently uncovered functions, including regulation of

proliferation, inflammation and visceral sensitivity. These actions have allowed some synthetic ligands to be developed based on the molecular structure of the endogenous ligands uroguanylin and guanylin, as well as the heat-stable enterotoxin from bacteria like *E. coli*. In fact, available ligands are approved or being assayed for treatment of chronic constipation (idiopathic, or associated to irritable bowel syndrome), inflammatory bowel disease and colorectal cancer.

However, GC-C has also been found in other locations where new, surprising roles have been suggested related to satiety, attention, obesity, natriuresis and others, which are exerted by the endogenous ligands secreted from the intestinal cells to the blood stream as pro-hormones. Considering the increasingly available information and the current interest in this target, it is expected that new roles will soon be described for GC-C. For example, taking into account its location related to central dopaminergic pathways, new data might link it with Parkinson's disease and/or psychosis.

Thus, it is a question of time that new indications are approved for the currently available ligands, and that new ligands capable of activating/inhibiting GC-C and its downstream pathways are developed.

FIGURE LEGENDS

Figure 1: Structure of the guanylate cyclase C (GC-C) receptor. GC-C receptor is a membrane-bound protein with 8 domains (A-H). The following features of GC-C receptor are graphically shown: the position, length (in amino acid residues), and function of each domain, and the different mutations described so far, according to Müller *et al* [134].

Figure 2: Molecular mechanisms of action of guanylate cyclase C (GC-C) receptors in intestinal mucosal cells. The **left cell** shows the mechanisms of action related to regulation of intestinal fluid balance and pain perception. Ligand binding to GC-C on the apical membrane of the cells, lead to intracellular cGMP accumulation, which activates PKGII and inactivates PDE_3, which in turn leads to cAMP accumulation. Then, cyclic nucleotides activate PKGII (in the proximal small intestine) and PKA (in the colon), which activate CFTR, provoking chloride secretion. This activates also bicarbonate (through an unknown transporter) and water secretion. cGMP transported to the submucosa through the basolateral membrane via (maybe) MRP5 attenuates visceral sensation. The action of GC-C is terminated when cGMP is converted to GMP by PDE_5. The **right cell** shows some mechanisms suggested to be involved in regulation of cell proliferation. In this case, the increase in cGMP after GC-C ligand binding, in addition to activating PKGII, activates CNG, which allows translocation of CaR to the apical membrane. Activated PKGII activates PTEN and p38/MAPK pathways, and reduces the levels of β-catenin and other key proteins promoting cell proliferation and cell cycle progression, and this inhibits cell proliferation and induces cytostasis via a G1/S delay in the cell cycle. PTEN inhibits AKT pathways, leading to suppressed cell proliferation and protection of tight junctional protein concentrations and intestinal barrier integrity. GC-C-induced AKT inhibition also prevents the degradation of the tumor suppressor p53 and promotes DNA damage repair and genomic stability. p38/MAPK increases phosphorilation of the Sp1 transcription factor, leading to intranuclear accumulation of p21, which facilitates senescence and quiescence (cytostasis), thereby reducing colorectal tumorigenesis. Arrows indicate activation. Bar-headed lines indicate inhibition. AKT: protein kinase B. CaR: calcium-sensing receptor. cGMP, cAMP: cyclic GMP, cyclic AMP. CFTR: cystic fibrosis transmembrane conductance regulator. CNG: cyclic nucleotide-gated cation channel. GC-C: guanylate cyclase C. MAPK: mitogen-activated protein-kinase. MRP5: multidrug resistance-associated protein 5. NHE3: Na^+/H^+ exchanger type 3. PDE: phosphodiesterase. PKA: protein kinase A. PKGII: protein kinase G type II. PTEN: phosphatase and tensin homolog. Sp1: specific protein 1. Adapted from Pitari [73], Basu*et al*, [165] and Jiang*et al*, [98].

Figure 3. Guanylate cyclase C (GC-C) agonists. All GC-C natural (guanylin and uroguanylin from mammals including *Homo sapiens*, renoguanylin and lymphoguanylin from non-mammals, and ST from bacteria) and synthetic (plecanatide, dolcanatide and linaclotide) ligands are peptides whose primary structures are shown as chains of single letter abbreviations for amino acids (letters in bold represent the amino acids changed in the synthetic derivatives). With the exception of lymphoguanylin, all of them have at least 4 cysteines (C), enabling the formation of 2 (guanylin, uroguanylin, renoguanylin, plecanatide, dolcanatide) or 3 (ST, linaclotide: superagonists) intramolecular disulfide bonds, represented by solid lines (in lymphoguanlyin only 1 disulfide bond is formed). The amino acids at the N and C

termini are represented by the first (left) letter in the upper and bottom raws of each structure, respectively. Because of the existence of these disulfide bonds, the chain is folded at the Alanine (A) represented in all structures in the middle raw of letters to the right. The amino acid sequence for ST reflects that of the heat-stable enterotoxin isoform STh (STa) produced by *Escherichia coli*.

Figure 4: Expression of the endogenous guanylate cyclase-C (GC-C) agonists guanylin and uroguanylin mRNA in human, rat and mouse intestine according to Brenna *et al* [127] and Ikpa *et al* [128]. Guanylin and uroguanylin transcripts have been located in different intestinal epithelial cell types depending on the species. GC-C agonists expression in rodents is graded along the intestinal tube, guanylin being more abundant in the colon, whereas uroguanylin is predominantly expressed in the duodenum. V: villous. ↑: High levels of expression. ↓: Low levels of expression.

List of Abbreviations

2ClAdo: 2-chloroadenosine
ADHD: attention deficit hyperactivity disorder
AKT: protein kinase B
APC: adenomatous polyposis coli
BPIPP: pyridopyrimidine derivative
cAMP: cyclic adenosine monophosphate
CaR: calcium-sensing receptor
CFTR: cystic fibrosis transmembrane conductance regulator
cGMP: cyclic guanosine monophosphate
CIC: chronic idiopathic constipation
CNG: cyclic nucleotide-gated cation channel
COX-2: cyclooxygenase-2
CRC: colorectal cancer
DHPG: (*S*)-3,5-Dihydroxyphenylglycine
DOLC: dolcanatide
DRA: intestinal anion exchanger downregulated in adenoma
DSS: dextran sodium sulfate
EC: enterochromaffin cells
ERK: extracellular signal-regulated kinase
ETEC: enterotoxigenic *Escherichia coli*
FDA: US Food and Drug administration
GABA: gamma-aminobutyric acid
GC-A (-B, -C, -D, -E, -F, -G): guanylate cyclase A (-B, -C, -D, -E, -F, -G) receptor
GI: gastrointestinal
GPs: guanylin peptides
GUCA2A: guanylin
GUCA2B: uroguanylin
IBD: inflammation bowel disease
IBS-C: irritable bowel syndrome with constipation
iNOS: inducible nitric oxide synthase
JAM-A: junctional adhesion molecule A
KHD: kinase homology domain
KO: knock out
LGN: lymphoguanylin
LINA: linaclotide
mAChRs: muscarinic acetylcholine receptors
MAPK: mitogen-activated protein kinase
MCP-1: monocyte chemoattractant protein-1
mGluRs: metabotropic glutamate receptors
MRP4 (5): multidrug resistance-associated protein 4 (or 5)
NHE3: sodium-hydrogen exchanger 3
NO: nitric oxide
PDE: phosphodiesterase
pGC: particulate (membrane-bound) guanylate cyclase
PGE2: prostaglandin E2
PKA (-C, -G): protein kinase A (-C, -G)
PKG I, -II: protein kinase G type I, -II
PLA2: phospholipase A2
PLEC: plecanatide
pRb: retinoblastoma protein
PTEN-PI3K: phosphatase and tensin homolog-phosphoinositide 3-kinase
RGN: renoguanylin
SNPs: single nucleotide polymorphisms
Sp1: specific protein 1
STa (b): heat-stable enterotoxin a or b
TGF-β: transforming growth factor beta
TNF-α: tumor necrosis factor alpha
TNBS: 2-4-6 trinitrobenzene sulfonic acid

UC: ulcerative colitis
VASP: vasodilator-stimulated phosphoprotein
WT: wild type

Conflict of Interest

The authors have declared no conflict of interest.

Author contribution

J.A.U., M.C. and R.A. wrote the paper. All authors approved the manuscript

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Fig. 1

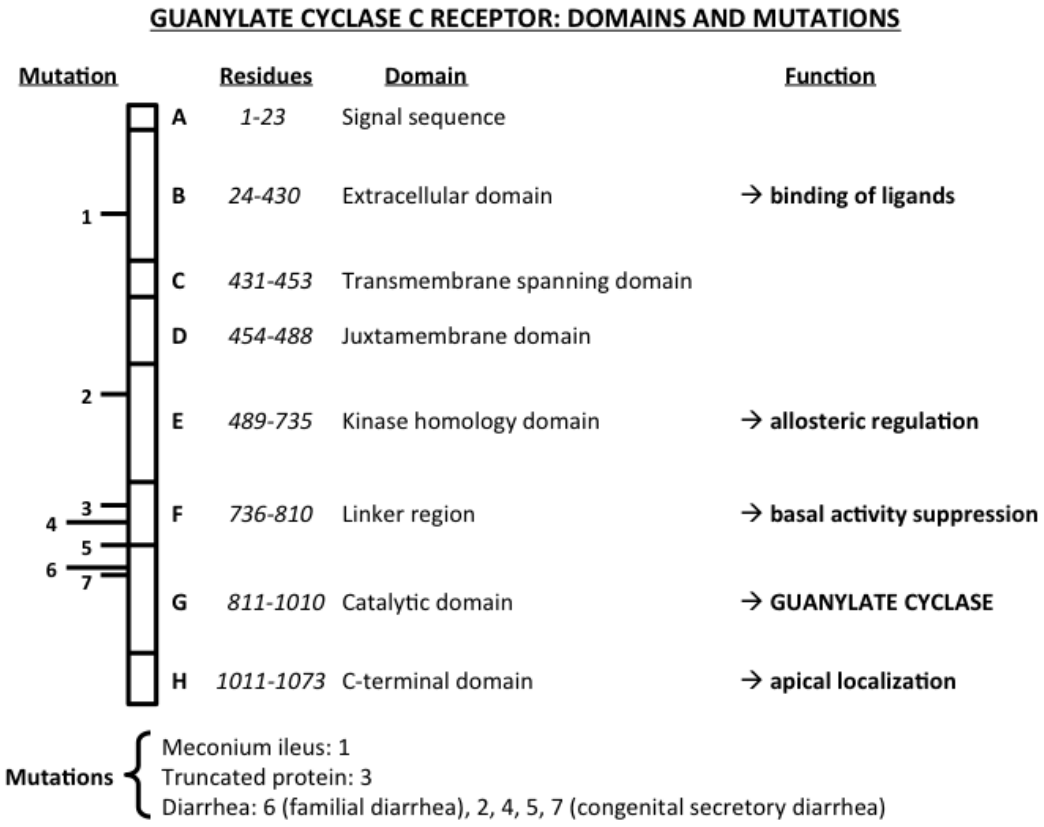
GUANYLATE CYCLASE C RECEPTOR: DOMAINS AND MUTATIONS
Mutation
Residues
Domain
Function
A 1-23 Signal sequence
B 24-430 Extracellular domain → binding of ligands
C 431-453 Transmembrane spanning domain
D 454-488 Juxtamembrane domain
E 489-735 Kinase homology domain → allosteric regulation
F 736-810 Linker region → basal activity suppression
G 811-1010 Catalytic domain → GUANYLATE CYCLASE
H 1011-1073 C-terminal domain → apical localization
1
2
3
4
5
6
7
Mutations
Meconium ileus: 1
Truncated protein: 3
Diarrhea: 6 (familial diarrhea), 2, 4, 5, 7 (congenital secretory diarrhea)

Fig. 2

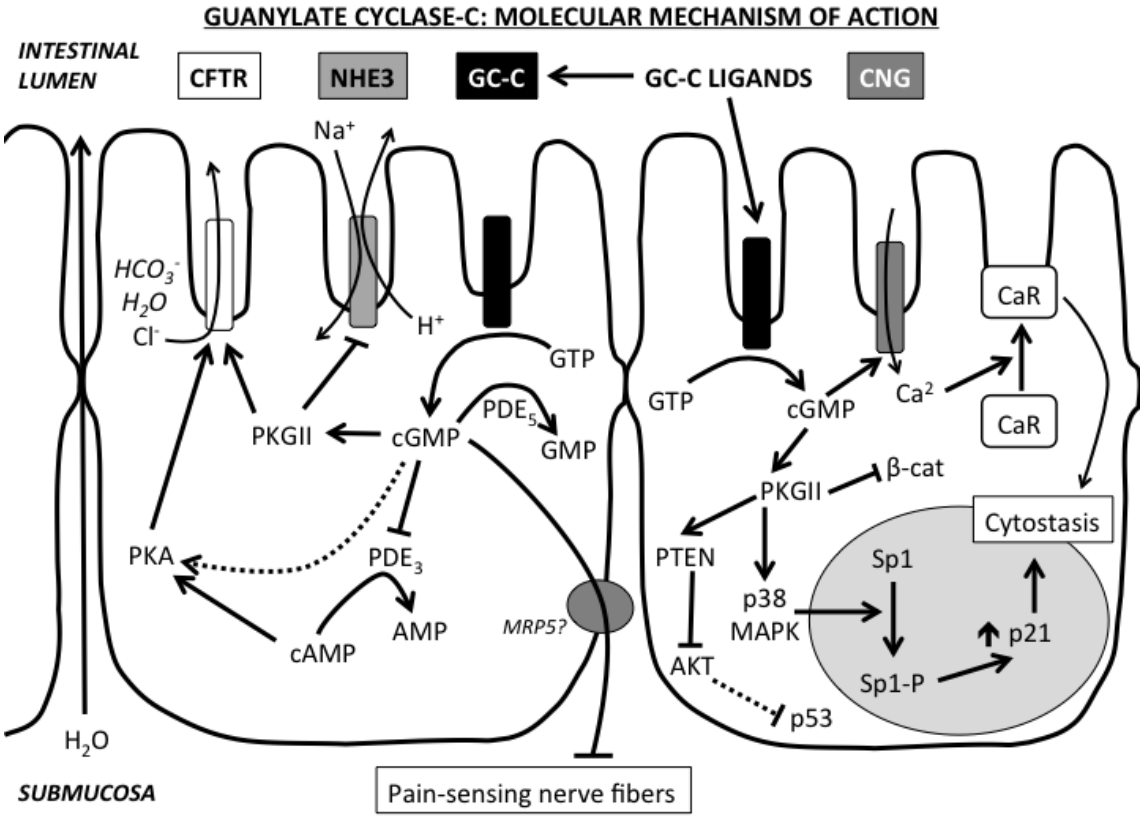
GUANYLATE CYCLASE-C: MOLECULAR MECHANISM OF ACTION
INTESTINAL LUMEN
CFTR
NHE3
GC-C
GC-C LIGANDS
CNG
Na+
HCO3-
H2O
Cl-
H+
GTP
PKGII
cGMP
PDE5
GMP
PKA
PDE3
cAMP
AMP
MRP5?
H2O
SUBMUCOSA
Pain-sensing nerve fibers
GTP
cGMP
Ca2
CaR
CaR
β-cat
PKGII
Cytostasis
PTEN
Sp1
p38 MAPK
p21
AKT
Sp1-P
p53

Fig. 3

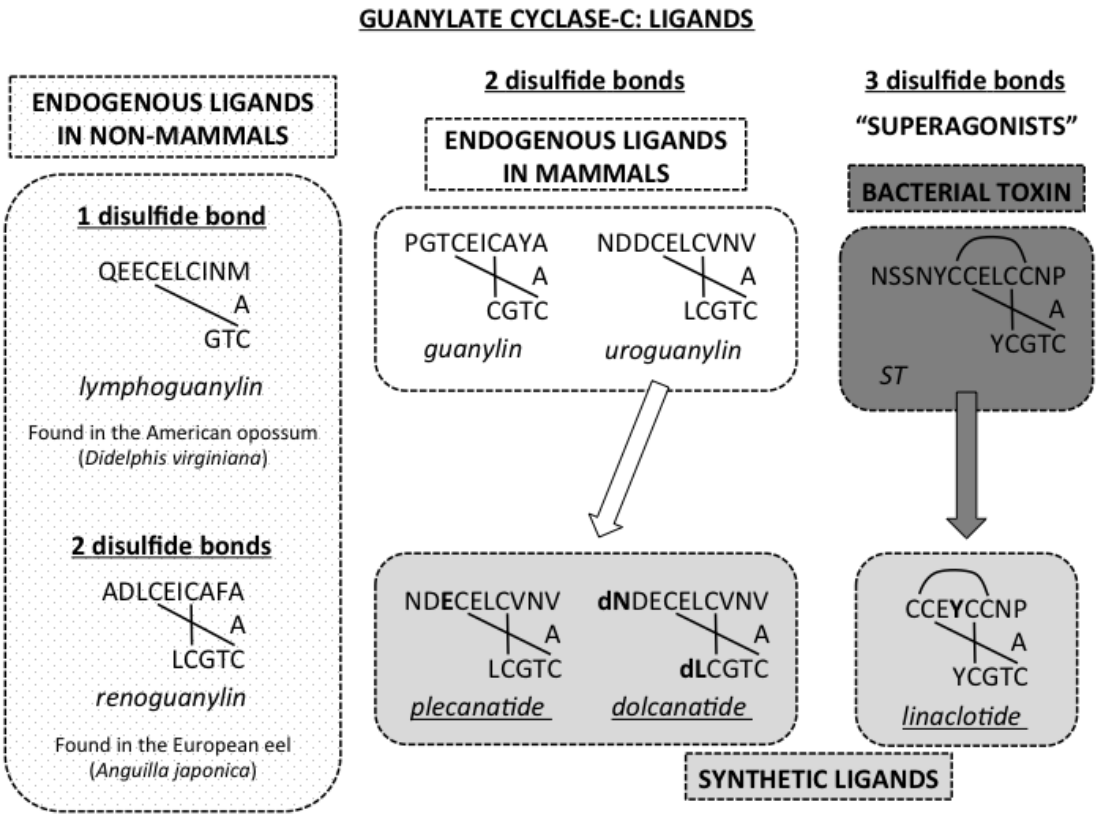
GUANYLATE CYCLASE-C: LIGANDS
ENDOGENOUS LIGANDS IN NON-MAMMALS
1 disulfide bond
QEECELCINM
A
GTC
lymphoguanylin
Found in the American opossum (Didelphis virginiana)
2 disulfide bonds
ADLCEICAFA
A
LCGTC
renoguanylin
Found in the European eel (Anguilla japonica)
2 disulfide bonds
ENDOGENOUS LIGANDS IN MAMMALS
PGTCEICAYA
A
CGTC
guanylin
NDDCELCVNV
A
LCGTC
uroguanylin
3 disulfide bonds
"SUPERAGONISTS"
BACTERIAL TOXIN
NSSNYCCELCCNP
A
YCGTC
ST
NDECELCVNV
A
LCGTC
plecanatide
dNDECELCVNV
A
dLCGTC
dolcanatide
CCEYCCNP
A
YCGTC
linaclotide
SYNTHETIC LIGANDS

Fig. 4

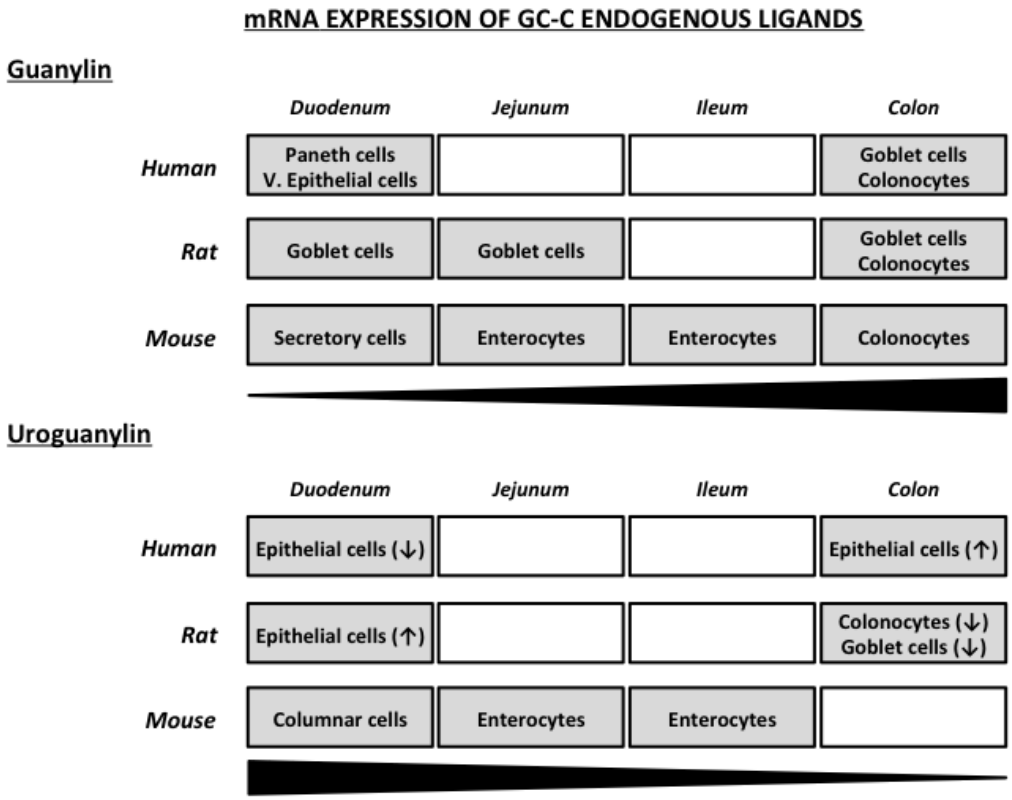
mRNA EXPRESSION OF GC-C ENDOGENOUS LIGANDS
Guanylin
Duodenum
Jejunum
Ileum
Colon
Human
Paneth cells
V. Epithelial cells
Goblet cells
Colonocytes
Rat
Goblet cells
Goblet cells
Goblet cells
Colonocytes
Mouse
Secretory cells
Enterocytes
Enterocytes
Colonocytes
Uroguanylin
Duodenum
Jejunum
Ileum
Colon
Human
Epithelial cells (↓)
Epithelial cells (↑)
Rat
Epithelial cells (↑)
Colonocytes (↓)
Goblet cells (↓)
Mouse
Columnar cells
Enterocytes
Enterocytes

Table I

Table I. Main characteristics of guanylate cyclase C (GC-C) ligands.

	LGN	RGN	GN (GUCA2A)	UGN (GUCA2B)	STa	LINA	PLEC	DOLC
Origin	Opossum	Eel	Mammals	Mammals	Bacteria	STa	UGN	UGN
Prepro-peptide (n. of amino acid residues)	109		115	112		-	-	-
Pro-peptide (n. of amino acid residues)			94	86	> 53	-	-	-
Peptide (n. of amino acid residues)	14	16	15	16	19	14	16	16
Disulfide bonds (n.)	1	2	2	2	3	3	2	2
Optimal pH value for effect			8.0	5.0	pH-independent	pH-independent	5.0	
GC-C binding potency					10xUGN 100xGN	Ki=1.23-1.64 mM (in T84 cells) >STa	8xUGN	
Metabolites						MM419447 (equally active to parental drug)		
Oral bioavailability			Negligible	Negligible	Negligible	Negligible	Negligible	Negligible
Implication for intestinal health			Intestinal fluid and electrolyte homeostasis	Intestinal fluid and electrolyte homeostasis	Traveller's diarrhea Counteracts CRC?	Treatment of IBS-C, CIC Treatment of CRC? Diarrhea as main adverse effect	Treatment of IBS-C, CIC Treatment of CRC?	Treatment of IBD Treatment of CRC?
Others			Hormone	Hormone				

LGN=lymphoguanylin. RGN=renoguanylin. GN=guanylin. UGN=uroguanylin. STa=exogenous het-stable enterotoxin from diarrhoeagenic enterotoxigenic bacteria (*E. coli*…). LINA=linaclotide. PLEC=plecanatide. DOLC=dolcanatide.