BAUSCH Health

Mylan Pharmaceuticals Inc. et al. v. Bausch Health Ireland Limited

Patent Owner's Demonstratives | June 14, 2023

Case IPR2022-00722[1]
U.S. Pat. No. 7,041,786

[1] IPR2023-00016 has been joined with this preceding.

Outline

- Claim 1 Would Not Have Been Obvious Over Currie and Li
- A POSA Would Not Have Selected Human Uroguanylin as a Lead Compound
- A POSA Would Not Have Been Motivated to Substitute Asp^3 with Glu^3 with Any Reasonable Expectation of Success
- Objective Evidence Supports the Nonobviousness of the Claims

Claim 1 Would Not Have Been Obvious Over Currie and Li

X. GROUND 1: CLAIM 1 WAS OBVIOUS OVER CURRIE AND LI

Currie teaches the human uroguanylin peptide activates the GC-C receptor, which is useful for providing laxative effect in the intestines (by drawing water into the intestines). EX1002, ¶¶126-29, 153. From Li, a skilled artisan would have known that making a Glu^3 analog of human uroguanylin using known peptide synthesis methods was reasonably likely to result in an active peptide analog. *Id.*, ¶¶147-50, 154. Hence, a skilled artisan had good reason to make [Glu^3]-human uroguanylin with a reasonable expectation of success. Claim 1 was obvious over Currie in view of Li. EX1002, ¶179.

BLAKE ROBERT PETERSON , Ph.D.

Q. In your opinion, Li cannot be used to make cross-peptide activity comparisons, correct?

A. That's correct.

Q. In your opinion, the experiments in Li do not provide dose response curves and do not permit comparison of the affinity or potency of rat uroguanylin to human uroguanylin or opossum uroguanylin, correct?

A. Correct.

Pet. at 37; Ex. 2069 at 84:18-20, 81:20-24
Sur-Reply at 13

Obviousness of a New Chemical Compound Ordinarily Follows a Two-Part Analysis

First, Petitioner must establish that a POSA would have selected the asserted prior-art compound as a lead compound, or starting point, **"that would be most promising to modify in order to improve upon its ... activity and obtain a compound with better activity."**

- "In determining whether a chemist would have selected a prior art compound as a lead, the analysis is guided by evidence of the **compound's pertinent properties**."
- "Absent a reason or motivation based on such prior art evidence, **mere structural similarity** between a prior art compound and the claimed compound **does not inform the lead compound selection**."

Second, Petitioner must establish that **"the prior art would have supplied [a POSA] with a reason or motivation to modify a lead compound to make the claimed compound with a reasonable expectation of success."**

Otsuka Pharm. Co. v. Sandoz, Inc., 678 F.3d 1280, 1291-93 (Fed. Cir. 2012)
Patent Owner's Resp. at 26-27; *see also* Sur-Reply at 2-3; Ex. 2024 ¶¶ 25-26

A POSA Would Not Have Selected Human Uroguanylin as a Lead Compound

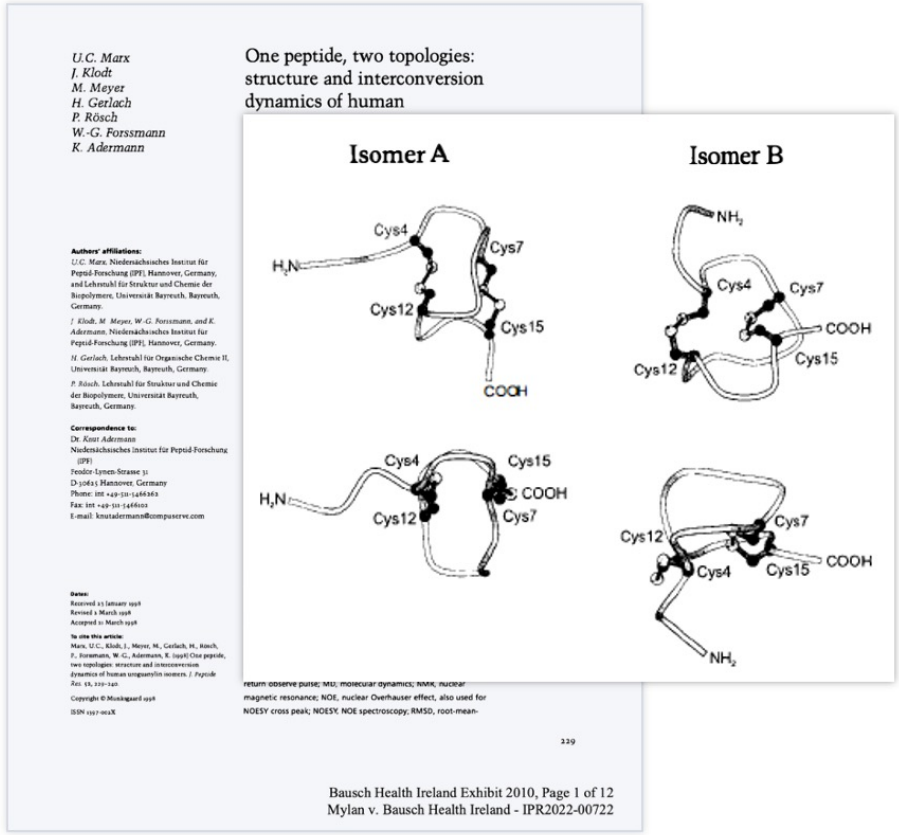
Federal Circuit: Lead Compound Selection

"[T]he analysis still requires the challenger to demonstrate . . . that one of ordinary skill in the art would have had a reason to select a proposed lead compound or compounds **over other compounds in the prior art**."

"Potent and promising activity in the prior art trumps **mere structural relationships**."

Daiichi Sankyo Co. v. Matrix Lab'ys, Ltd., 619 F.3d 1346, 1354 (Fed. Cir. 2010)
Patent Owner's Resp. at 26-29, 35; Ex. 2024 ¶ 26

Human Uroguanylin Is Plagued by Topoisomerism



U.C. Marx
J. Klodt
M. Meyer
H. Gerlach
P. Rösch
W.-G. Forssmann
K. Adermann

One peptide, two topologies: structure and interconversion dynamics of human

Isomer A

Isomer B

Cys4
Cys7
Cys12
Cys15
COOH
H_2N
NH_2

Bausch Health Ireland Exhibit 2010, Page 1 of 12
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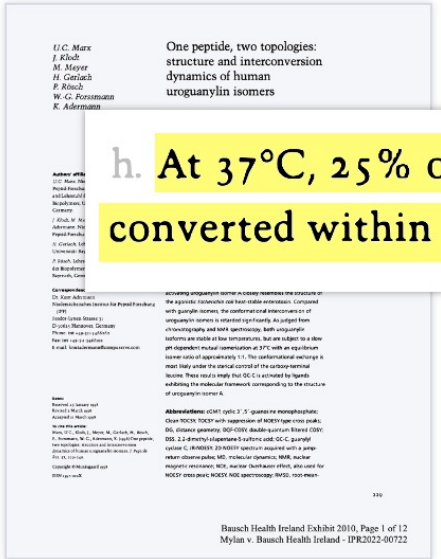
oisomers of uroguanylin are present in the body. In addition to the possible existence of other specific receptors for the GC-C-active isomer of uroguanylin, nothing is known about the structure and function of the peptide's B form that does not cause an increase of intracellular cGMP.

multiple-cysteine peptide. Our results demonstrate that, up to now, all published functional studies with bioactive guanylin reported in the literature have not been carried out with a single defined molecule but with a mixture of two peptides, one of these a ligand of GC-C, the other one with completely unknown biological properties. Because stereoisomers are present in syn-

structures (Fig. 1). Both peptides are characterized by two disulfide bonds in relative positions 1 3 and 2 4, which are crucial for biological activity (2, 14, 15). The disulfides are

Ex. 2010 at 230, 235 (Fig. 4); Ex. 2020 at 229
Patent Owner's Resp. at 9-10; *see also* Sur-Reply at 4-5; Ex. 2024 ¶¶ 63-66

Topoisomerism Is Temperature and pH Dependent



U.C. Marx
J. Kloth
M. Mayer
H. Gerlach
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One peptide, two topologies: structure and interconversion dynamics of human uroguanylin isomers

Bausch Health Ireland Exhibit 2010, Page 1 of 12
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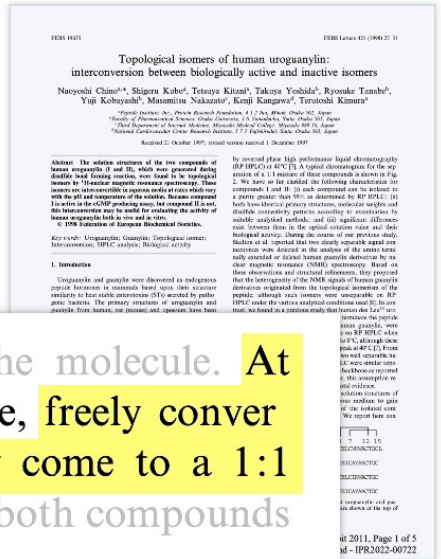
h. At 37°C, 25% of both uroguanylin-16 isomers are interconverted within 24 h. This and conversion experiments



Topological isomers of human uroguanylin: interconversion between biologically active and inactive isomers

Naoyoshi Chino[a,*], Shigeru Kubo[a], Tetsuya Kitani[a], Takuya Yoshida[b], Ryosuke Tanabe[b], Yuji Kobayashi[b], Masamitsu Nakazato[c], Kenji Kangawa[d], Terutoshi Kimura[a]

ionization state of functional group(s) in the molecule. At acidic pH, both compounds are, in one sense, freely convertible (same conversion rates) and eventually come to a 1:1 equilibrium ratio. In contrast, conversions of both compounds

MICHAEL SAMUEL EPSTEIN, M.D.

Q. And so if we're talking about the transit time from the time the patient ingests something all the way until the time they have a bowel movement, you agree that that transit time could be longer in a constipated patient?

***

THE WITNESS: It's -- it can be, yes. It can be longer if it's in a patient who's constipated primarily due to colonic transit.

Ex. 2010 at 236; Ex. 2011 at 30; Ex. 2070 at 45:18-46:4
Patent Owner's Resp. at 10; *see also* Sur-Reply at 19; Ex. 2024 ¶¶ 67-70.

Petitioner's Approach to Topoisomerism Leads Away from Plecanatide

BLAKE ROBERT PETERSON , Ph.D.

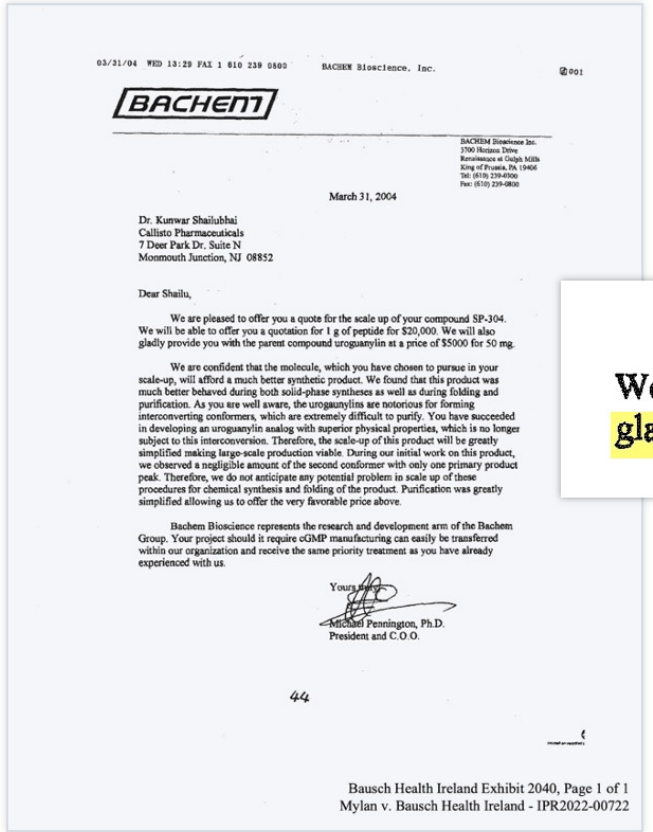
Q. In your opinion, in vivo topoisomeric conversion of human uroguanylin would have been easily avoided by administering it in a dosage form to time its release specifically for the intestines rather than the stomach, correct?

A. That's what's written here, yes.

- This approach **would not have resulted in plecanatide**

Ex. 2069 at 33:24-34:4
Sur-Reply at 10

Topoisomerism Is More than an *In-Vivo* Problem



03/31/04 WED 13:29 FAX 1 610 239 0800 BACHEM Bioscience, Inc. 001

BACHEM

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March 31, 2004

Dr. Kunwar Shailubhai
Callisto Pharmaceuticals
7 Deer Park Dr. Suite N
Monmouth Junction, NJ 08852

Dear Shailu,

We are pleased to offer you a quote for the scale up of your compound SP-304. We will be able to offer you a quotation for 1 g of peptide for $20,000. We will also gladly provide you with the parent compound uroguanylin at a price of $5000 for 50 mg.

We are confident that the molecule, which you have chosen to pursue in your scale-up, will afford a much better synthetic product. We found that this product was much better behaved during both solid-phase syntheses as well as during folding and purification. As you are well aware, the uroguanylins are notorious for forming interconverting conformers, which are extremely difficult to purify. You have succeeded in developing an uroguanylin analog with superior physical properties, which is no longer subject to this interconversion. Therefore, the scale-up of this product will be greatly simplified making large-scale production viable. During our initial work on this product, we observed a negligible amount of the second conformer with only one primary product peak. Therefore, we do not anticipate any potential problem in scale up of these procedures for chemical synthesis and folding of the product. Purification was greatly simplified allowing us to offer the very favorable price above.

Bachem Bioscience represents the research and development arm of the Bachem Group. Your project should it require cGMP manufacturing can easily be transferred within our organization and receive the same priority treatment as you have already experienced with us.

Yours sincerely,

Michael Pennington, Ph.D.
President and C.O.O.

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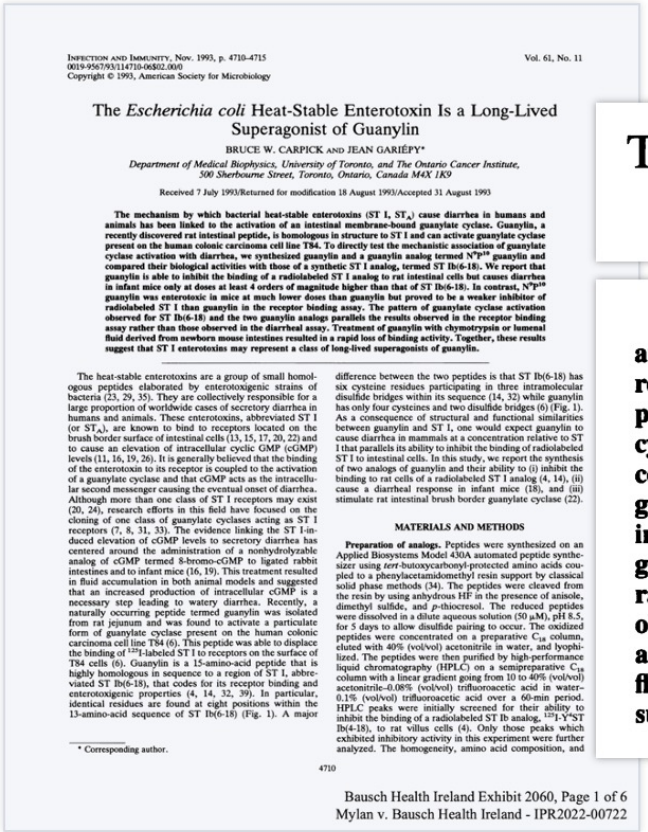
Bausch Health Ireland Exhibit 2040, Page 1 of 1
Mylan v. Bausch Health Ireland - IPR2022-00722

We are pleased to offer you a quote for the scale up of your compound SP-304. We will be able to offer you a quotation for 1 g of peptide for $20,000. We will also gladly provide you with the parent compound uroguanylin at a price of $5000 for 50 mg.

Ex. 2040
Patent Owner's Resp. at 32; *see also* Ex. 2024 ¶¶ 128-129

Carpick: STs Are "Long-Lived Superagonists"



INFECTION AND IMMUNITY, Nov. 1993, p. 4710–4715 Vol. 61, No. 11
0019-9567/93/114710-06$02.00/0

The *Escherichia coli* Heat-Stable Enterotoxin Is a Long-Lived Superagonist of Guanylin

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Received 7 July 1993/Returned for modification 18 August 1993/Accepted 31 August 1993

The mechanism by which bacterial heat-stable enterotoxins (ST I, ST_A) cause diarrhea in humans and animals has been linked to the activation of an intestinal membrane-bound guanylate cyclase. Guanylin, a recently discovered rat intestinal peptide, is homologous in structure to ST I and can activate guanylate cyclase present on the human colonic carcinoma cell line T84. To directly test the mechanistic association of guanylate cyclase activation with diarrhea, we synthesized guanylin and a guanylin analog termed N^9P^{10} guanylin and compared their biological activities with those of a synthetic ST I analog, termed ST Ib(6-18). We report that guanylin is able to inhibit the binding of a radiolabeled ST I analog to rat intestinal cells but causes diarrhea in infant mice only at doses at least 4 orders of magnitude higher than that of ST Ib(6-18). In contrast, N^9P^{10} guanylin was enterotoxic in mice at much lower doses than guanylin but proved to be a weaker inhibitor of radiolabeled ST I than guanylin in the receptor binding assay. The pattern of guanylate cyclase activation observed for ST Ib(6-18) and the two guanylin analogs parallels the results observed in the receptor binding assay rather than those observed in the diarrheal assay. Treatment of guanylin with chymotrypsin or lumenal fluid derived from newborn mouse intestines resulted in a rapid loss of binding activity. Together, these results suggest that ST I enterotoxins may represent a class of long-lived superagonists of guanylin.

The heat-stable enterotoxins are a group of small homologous peptides elaborated by enterotoxigenic strains of bacteria (23, 29, 35). They are collectively responsible for a large proportion of worldwide cases of secretory diarrhea in humans and animals. These enterotoxins, abbreviated ST I (or ST_A), are known to bind to receptors located on the brush border surface of intestinal cells (13, 15, 17, 20, 22) and to cause an elevation of intracellular cyclic GMP (cGMP) levels (11, 16, 19, 26). It is generally believed that the binding of the enterotoxin to its receptor is coupled to the activation of a guanylate cyclase and that cGMP acts as the intracellular second messenger causing the eventual onset of diarrhea. Although more than one class of ST I receptors may exist (20, 24), research efforts in this field have focused on the cloning of one class of guanylate cyclases acting as ST I receptors (7, 8, 31, 33). The evidence linking the ST I-induced elevation of cGMP levels to secretory diarrhea has centered around the administration of a nonhydrolyzable analog of cGMP termed 8-bromo-cGMP to ligated rabbit intestines and to infant mice (16, 19). This treatment resulted in fluid accumulation in both animal models and suggested that an increased production of intracellular cGMP is a necessary step leading to watery diarrhea. Recently, a naturally occurring peptide termed guanylin was isolated from rat jejunum and was found to activate a particulate form of guanylate cyclase present on the human colonic carcinoma cell line T84 (6). This peptide was able to displace the binding of ^{125}I-labeled ST I to receptors on the surface of T84 cells (6). Guanylin is a 15-amino-acid peptide that is highly homologous in sequence to a region of ST I, abbreviated ST Ib(6-18), that codes for its receptor binding and enterotoxigenic properties (4, 14, 32, 39). In particular, identical residues are found at eight positions within the 13-amino-acid sequence of ST Ib(6-18) (Fig. 1). A major difference between the two peptides is that ST Ib(6-18) has six cysteine residues participating in three intramolecular disulfide bridges within its sequence (14, 32) while guanylin has only four cysteines and two disulfide bridges (6) (Fig. 1). As a consequence of structural and functional similarities between guanylin and ST I, one would expect guanylin to cause diarrhea in mammals at a concentration relative to ST I that parallels its ability to inhibit the binding of radiolabeled ST I to intestinal cells. In this study, we report the synthesis of two analogs of guanylin and their ability to (i) inhibit the binding to rat cells of a radiolabeled ST I analog (4, 14), (ii) cause a diarrheal response in infant mice (18), and (iii) stimulate rat intestinal brush border guanylate cyclase (22).

* Corresponding author.

MATERIALS AND METHODS

Preparation of analogs. Peptides were synthesized on an Applied Biosystems Model 430A automated peptide synthesizer using *tert*-butoxycarbonyl-protected amino acids coupled to a phenylacetamidomethyl resin support by classical solid phase methods (34). The peptides were cleaved from the resin by using anhydrous HF in the presence of anisole, dimethyl sulfide, and *p*-thiocresol. The reduced peptides were dissolved in a dilute aqueous solution (50 μM), pH 8.5, for 5 days to allow disulfide pairing to occur. The oxidized peptides were concentrated on a preparative C_{18} column, eluted with 40% (vol/vol) acetonitrile in water, and lyophilized. The peptides were then purified by high-performance liquid chromatography (HPLC) on a semipreparative C_{18} column with a linear gradient going from 10 to 40% (vol/vol) acetonitrile–0.08% (vol/vol) trifluoroacetic acid in water–0.1% (vol/vol) trifluoroacetic acid over a 60-min period. HPLC peaks were initially screened for their ability to inhibit the binding of a radiolabeled ST Ib analog, ^{125}I-Y-ST Ib(4-18), to rat villus cells (4). Only those peaks which exhibited inhibitory activity in this experiment were further analyzed. The homogeneity, amino acid composition, and

The *Escherichia coli* Heat-Stable Enterotoxin Is a Long-Lived Superagonist of Guanylin

The mechanism by which bacterial heat-stable enterotoxins (ST I, ST_A) cause diarrhea in humans and animals has been linked to the activation of an intestinal membrane-bound guanylate cyclase. Guanylin, a recently discovered rat intestinal peptide, is homologous in structure to ST I and can activate guanylate cyclase present on the human colonic carcinoma cell line T84. To directly test the mechanistic association of guanylate cyclase activation with diarrhea, we synthesized guanylin and a guanylin analog termed N^9P^{10} guanylin and compared their biological activities with those of a synthetic ST I analog, termed ST Ib(6-18). We report that guanylin is able to inhibit the binding of a radiolabeled ST I analog to rat intestinal cells but causes diarrhea in infant mice only at doses at least 4 orders of magnitude higher than that of ST Ib(6-18). In contrast, N^9P^{10} guanylin was enterotoxic in mice at much lower doses than guanylin but proved to be a weaker inhibitor of radiolabeled ST I than guanylin in the receptor binding assay. The pattern of guanylate cyclase activation observed for ST Ib(6-18) and the two guanylin analogs parallels the results observed in the receptor binding assay rather than those observed in the diarrheal assay. Treatment of guanylin with chymotrypsin or lumenal fluid derived from newborn mouse intestines resulted in a rapid loss of binding activity. Together, these results suggest that ST I enterotoxins may represent a class of long-lived superagonists of guanylin.

Ex. 2060 at 4710
Patent Owner's Resp. at 14; Ex. 2025 ¶ 64

STs Are Not Plagued by Topoisomerism

U.C. Marx
J. Klodt
M. Meyer
H. Gerlach
P. Rösch
W.-G. Forssmann
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One peptide, two topologies: structure and interconversion dynamics of human uroguanylin isomers

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ISSN 1397-002X

Key words: guanylin; heat-stable enterotoxin; isomerization; solution structure; topological stereoisomer; uroguanylin

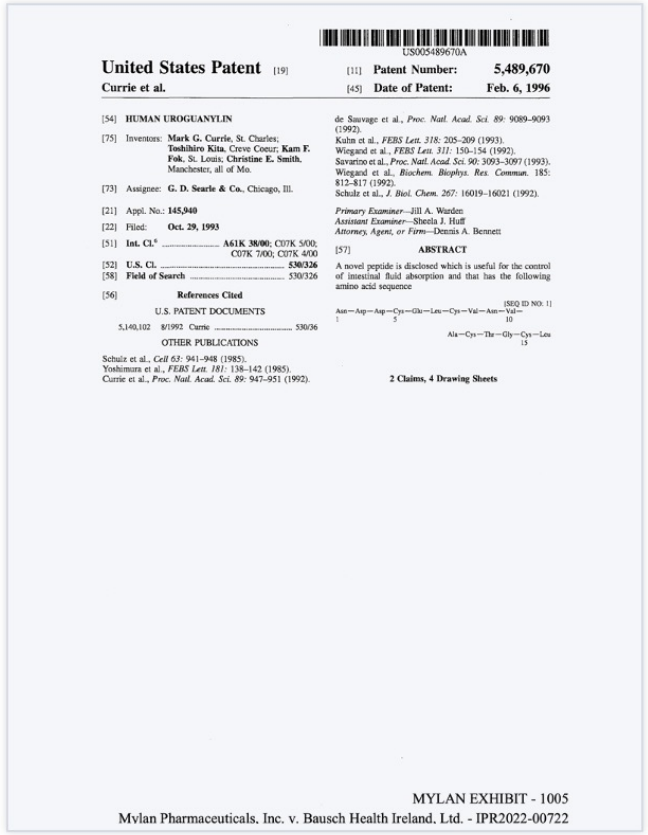
Abstract: The peptide hormone uroguanylin stimulates chloride secretion via activation of intestinal guanylyl cyclase C (GC-C). It is characterized by two disulfide bonds in a 1–3/2–4 pattern that causes the existence of two topological stereoisomers of which only one induces intracellular cGMP elevation. To obtain an unambiguous structure-function relationship of the isomers, we determined the solution structure of the separated uroguanylin isoforms using NMR spectroscopy. Both isomers adopt well-defined structures that correspond to those of the isomers of the related peptide guanylin. Furthermore, the structure of the GC-C-activating uroguanylin isomer A closely resembles the structure of the agonistic *Escherichia coli* heat-stable enterotoxin. Compared with guanylin isomers, the conformational interconversion of uroguanylin isomers is retarded significantly. As judged from chromatography and NMR spectroscopy, both uroguanylin isoforms are stable at low temperatures, but are subject to a slow pH-dependent mutual isomerization at 37°C with an equilibrium isomer ratio of approximately 1:1. The conformational exchange is most likely under the sterical control of the carboxy-terminal leucine. These results imply that GC-C is activated by ligands exhibiting the molecular framework corresponding to the structure of uroguanylin isomer A.

Abbreviations: cGMP, cyclic 3′,5′-guanosine monophosphate; Clean-TOCSY, TOCSY with suppression of NOESY-type cross peaks; DG, distance geometry; DQF-COSY, double-quantum filtered COSY; DSS, 2,2-dimethyl-silapentane-5-sulfonic acid; GC-C, guanylyl cyclase C; JR-NOESY, 2D NOESY spectrum acquired with a jump-return observe pulse; MD, molecular dynamics; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect, also used for NOESY cross peak; NOESY, NOE spectroscopy; RMSD, root-mean-

0.45 nm. The known higher activation potency of ST may be related to the additional disulfide bond which causes a higher rigidity of its three-dimensional structure and, thus, a possibly more efficient interaction with the receptor. Structure calculations of uroguanylin-16 with an ad-

Ex. 2010 at 235
Patent Owner's Resp. at 14, 33, 39; *see also* Ex. 2024 ¶¶ 145-146, 215; Ex. 2025 ¶¶ 46-47

STs Outperform Human Uroguanylin: Potency and Affinity



US005489670A

United States Patent [19]
Currie et al.

[11] **Patent Number: 5,489,670**
[45] **Date of Patent: Feb. 6, 1996**

[54] **HUMAN UROGUANYLIN**

[75] Inventors: **Mark G. Currie**, St. Charles; **Toshihiro Kita**, Creve Coeur; **Kam F. Fok**, St. Louis; **Christine E. Smith**, Manchester, all of Mo.

[73] Assignee: **G. D. Searle & Co.**, Chicago, Ill.

[21] Appl. No.: **145,940**

[22] Filed: **Oct. 29, 1993**

[51] **Int. Cl.**[6] **A61K 38/00**; C07K 5/00; C07K 7/00; C07K 4/00

[52] **U.S. Cl.** **530/326**

[58] **Field of Search** 530/326

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Primary Examiner—Jill A. Warden
Assistant Examiner—Sheela J. Huff
Attorney, Agent, or Firm—Dennis A. Bennett

[57] **ABSTRACT**

A novel peptide is disclosed which is useful for the control of intestinal fluid absorption and that has the following amino acid sequence

(SEQ ID NO: 1)
Asn—Asp—Asp—Cys—Glu—Leu—Cys—Val—Asn—Val—Ala—Cys—Thr—Gly—Cys—Leu
1 5 10 13

2 Claims, 4 Drawing Sheets

MYLAN EXHIBIT - 1005
Mylan Pharmaceuticals, Inc. v. Bausch Health Ireland, Ltd. - IPR2022-00722

Potency

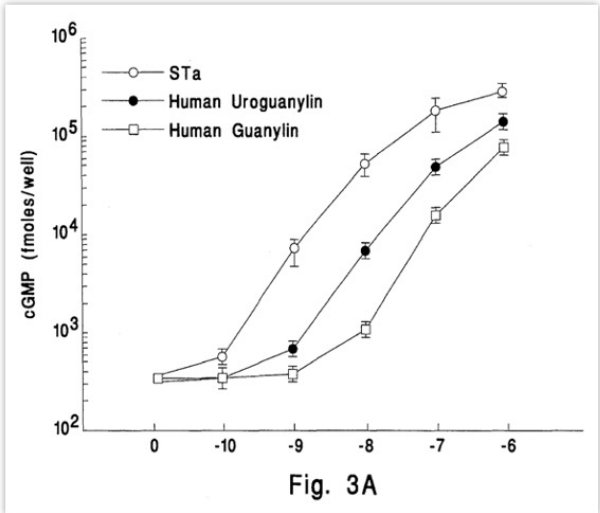


Fig. 3A

Binding Affinity

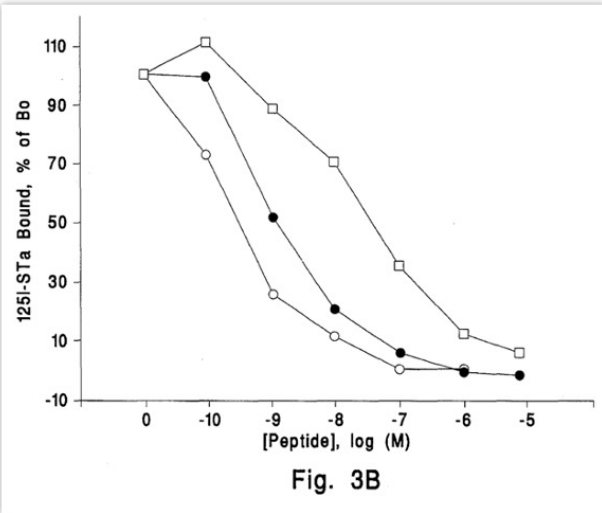


Fig. 3B

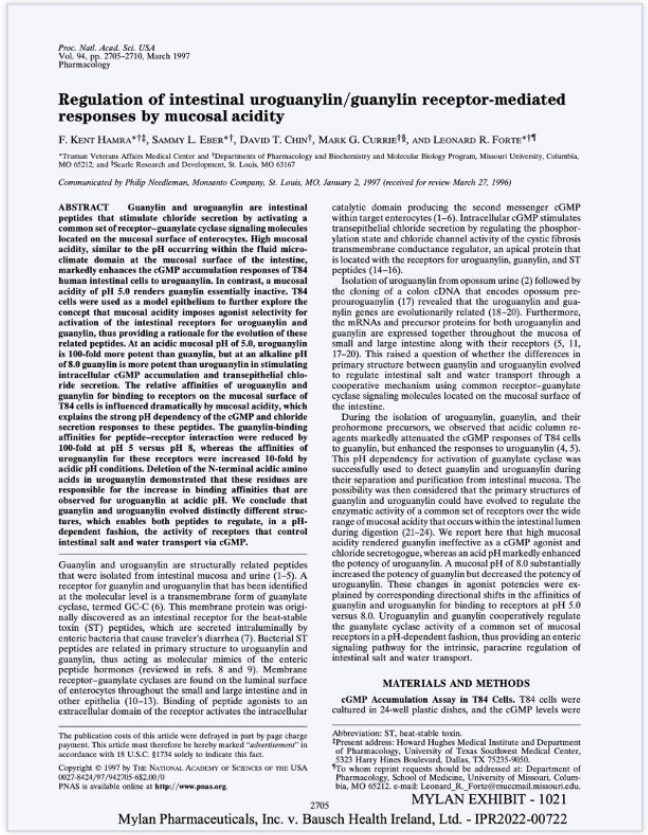
BLAKE ROBERT PETERSON, Ph.D.

Q. Based upon Currie's disclosure, quantitatively, how much less effective is human uroguanylin than STa at stimulating cyclic GMP?

A. In this particular experiment, it appears to be about tenfold less potent.

Ex. 1005 at 6:13-15, Fig. 3A, 6:15-19, Fig. 3B; Ex. 2026 at 59:17-21
Patent Owner's Resp. at 15-17, 34; *see also* Sur-Reply at 25; Ex. 2024 ¶¶ 93-94; Ex. 2025 ¶¶ 74-75

STs Outperform Human Uroguanylin: Potency and Affinity



Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 2705–2710, March 1997
Pharmacology

Regulation of intestinal uroguanylin/guanylin receptor-mediated responses by mucosal acidity

F. KENT HAMRA*†‡, SAMMY L. EBER*†, DAVID T. CHIN†, MARK G. CURRIE†§, AND LEONARD R. FORTE*†¶

*Truman Veterans Affairs Medical Center and †Departments of Pharmacology and Biochemistry and Molecular Biology Program, Missouri University, Columbia, MO 65212; and §Searle Research and Development, St. Louis, MO 63167

Communicated by Philip Needleman, Monsanto Company, St. Louis, MO, January 2, 1997 (received for review March 27, 1996)

ABSTRACT **Guanylin and uroguanylin are intestinal peptides that stimulate chloride secretion by activating a common set of receptor–guanylate cyclase signaling molecules located on the mucosal surface of enterocytes. High mucosal acidity, similar to the pH occurring within the fluid microclimate domain at the mucosal surface of the intestine, markedly enhances the cGMP accumulation responses of T84 human intestinal cells to uroguanylin. In contrast, a mucosal acidity of pH 5.0 renders guanylin essentially inactive. T84 cells were used as a model epithelium to further explore the concept that mucosal acidity imposes agonist selectivity for activation of the intestinal receptors for uroguanylin and guanylin, thus providing a rationale for the evolution of these related peptides. At an acidic mucosal pH of 5.0, uroguanylin is 100-fold more potent than guanylin, but at an alkaline pH of 8.0 guanylin is more potent than uroguanylin in stimulating intracellular cGMP accumulation and transepithelial chloride secretion. The relative affinities of uroguanylin and guanylin for binding to receptors on the mucosal surface of T84 cells is influenced dramatically by mucosal acidity, which explains the strong pH dependency of the cGMP and chloride secretion responses to these peptides. The guanylin-binding affinities for peptide–receptor interaction were reduced by 100-fold at pH 5 versus pH 8, whereas the affinities of uroguanylin for these receptors were increased 10-fold by acidic pH conditions. Deletion of the N-terminal acidic amino acids in uroguanylin demonstrated that these residues are responsible for the increase in binding affinities that are observed for uroguanylin at acidic pH. We conclude that guanylin and uroguanylin evolved distinctly different structures, which enables both peptides to regulate, in a pH-dependent fashion, the activity of receptors that control intestinal salt and water transport via cGMP.**

Guanylin and uroguanylin are structurally related peptides that were isolated from intestinal mucosa and urine (1–5). A receptor for guanylin and uroguanylin that has been identified at the molecular level is a transmembrane form of guanylate cyclase, termed GC-C (6). This membrane protein was originally discovered as an intestinal receptor for the heat-stable toxin (ST) peptides, which are secreted intraluminally by enteric bacteria that cause traveler's diarrhea (7). Bacterial ST peptides are related in primary structure to uroguanylin and guanylin, thus acting as molecular mimics of the enteric peptide hormones (reviewed in refs. 8 and 9). Membrane receptor–guanylate cyclases are found on the luminal surface of enterocytes throughout the small and large intestine and in other epithelia (10–13). Binding of peptide agonists to an extracellular domain of the receptor activates the intracellular catalytic domain producing the second messenger cGMP within target enterocytes (3–6). Intracellular cGMP stimulates transepithelial chloride secretion by regulating the phosphorylation state and chloride channel activity of the cystic fibrosis transmembrane conductance regulator, an apical protein that is located with the receptors for uroguanylin, guanylin, and ST peptides (14–16).

Isolation of uroguanylin from opossum urine (2) followed by the cloning of a colon cDNA that encodes opossum prouroguanylin (17) revealed that the uroguanylin and guanylin genes are evolutionarily related (18–20). Furthermore, the mRNAs and precursor proteins for both uroguanylin and guanylin are expressed together throughout the mucosa of small and large intestine along with their receptors (5, 11, 17–20). This raised a question of whether the differences in primary structure between guanylin and uroguanylin evolved to regulate intestinal salt and water transport through a cooperative mechanism using common receptor–guanylate cyclase signaling molecules located on the mucosal surface of the intestine.

During the isolation of uroguanylin, guanylin, and their prohormone precursors, we observed that acidic column reagents markedly attenuated the cGMP responses of T84 cells to guanylin, but enhanced the responses to uroguanylin (4, 5). This pH dependency for activation of guanylate cyclase was successfully used to detect guanylin and uroguanylin during their separation and purification from intestinal mucosa. The possibility was then considered that the primary structures of guanylin and uroguanylin could have evolved to regulate the enzymatic activity of a common set of receptors over the wide range of mucosal acidity that occurs within the intestinal lumen during digestion (21–24). We report here that high mucosal acidity rendered guanylin ineffective as a cGMP agonist and chloride secretogogue, whereas an acid pH markedly enhanced the potency of uroguanylin. A mucosal pH of 8.0 substantially increased the potency of guanylin but decreased the potency of uroguanylin. These changes in agonist potencies were explained by corresponding directional shifts in the affinities of guanylin and uroguanylin for binding to receptors at pH 5.0 versus 8.0. Uroguanylin and guanylin cooperatively regulate the guanylate cyclase activity of a common set of mucosal receptors in a pH-dependent fashion, thus providing an enteric signaling pathway for the intrinsic, paracrine regulation of intestinal salt and water transport.

MATERIALS AND METHODS

cGMP Accumulation Assay in T84 Cells. T84 cells were cultured in 24-well plastic dishes, and the cGMP levels were

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Abbreviation: ST, heat-stable toxin.

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pared with the stimulation observed at pH 7.8 (Fig. 3). The rank order of potencies for agonist-mediated stimulation of chloride secretion was ST > uroguanylin > guanylin at acidic pH and ST > guanylin > uroguanylin at an alkaline pH (Fig. 3). The relative potencies of uroguanylin, guanylin, and ST-(5–17) in the stimulation of transepithelial chloride secretion across monolayers of T84 cells at acidic versus alkaline pH matched their relative potencies for stimulation of cGMP levels under these conditions.

E. coli ST-(5–17) binds with extraordinarily high affinities to the uroguanylin/guanylin receptors on the apical surface of T84 cells and potently stimulates cGMP production and chloride secretion at both alkaline and acidic pH. The interactions

of the intestinal hormones, uroguanylin and guanylin. The remarkable potencies of ST peptides compared with the potencies of the enteric hormones is caused by higher affinities for ST binding to the intestinal receptors for uroguanylin and guanylin. Bacteria have created superagonist peptide toxins

Ex. 1021 at 2706-07, 2710
Patent Owner's Resp. at 16-17, 34; Ex. 2024 ¶¶ 96-98; Ex. 2025 ¶ 76

Petitioner's ST Diarrhea Concerns Are Unfounded

Pathogenic strains of E. coli and other bacteria produce a family of heat stable entertoxins (STs) that activate intestinal guanylate cyclase. STs are acidic peptides 18–19 amino acids in length with six cysteines and three disulfide bridges that are required for full expression of bioactivity (7). The increase of intestinal epithelial cyclic GMP elicited by STs is thought to cause a decrease in water and sodium absorption and an increase in chloride secretion (8,9). These changes in intestinal fluid and electrolyte transport then act to cause secretory diarrhea. In developing countries, the diarrhea due to STs is the cause of many deaths, particularly in the infant population (10). STs are also considered to be a major cause of traveler's diarrhea in developed countries (11). STs have also been reported to be a leading cause of morbidity in domestic animals (12).

strains of *Escherichia coli* [3–5]. Exposure to high levels of toxin, as occurs during acute bacterial infections, triggers non-physiological movement of electrolytes, and produces a watery diarrhea that can lead to dehydration and death.

time if it is incubated at 37°C (Fig. 3). Such an increase is not observed when synthetic guanylin, synthetic uroguanylin, or commercially-purified STa are incubated under similar conditions (Fig. 3). We do not yet know the

BLAKE ROBERT PETERSON , Ph.D.

```
Q. Chemotherapy at a high dose will destroy
   normal cells, correct?
                         ***
   THE WITNESS:  It -- it depends incredibly
   on the nature of the chemotherapy you are
   describing. At a high dose, water can be
   toxic. Any, any compound can be toxic at a
   high enough dose.
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Q. Well, lowering the dose of an active
   ingredient generally decreases side
   effects, correct?
                         ***
   THE WITNESS: That can be true.
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Ex. 1005 at 1:31-44; Ex. 1006 at 45, 51; Ex. 2069 at 17:25-18:6, 19:4-7
Patent Owner's Resp. at 36; *see also* Sur-Reply at 7; Ex. 2024 ¶ 139; Ex. 2025 ¶¶ 70-73

Currie Selected a Prior Art ST for Commercial Development

BLAKE ROBERT PETERSON , Ph.D.

Q. Are you aware that Dr. Currie selected a prior art enterotoxin and modified it to make linaclotide which became marketed as Linzess?

A. Yes, I'm aware of that.

Ex. 2069 at 16:3-6
Patent Owner's Resp. at 24, 37; Sur-Reply at 7; Ex. 2024 ¶¶ 92, 99, 141

A POSA Would Not Have Been Motivated to Substitute Asp^3 with Glu^3 with Any Reasonable Expectation of Success

Marx: Isomerism Not Affected by *N*-Terminal Region

U.C. Marx
J. Klodt
M. Meyer
H. Gerlach
P. Rösch
W.-G. Forssmann
K. Adermann

One peptide, two topologies: structure and interconversion dynamics of human uroguanylin isomers

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Key words: guanylin; heat-stable enterotoxin; isomerization; solution structure; topological stereoisomer; uroguanylin

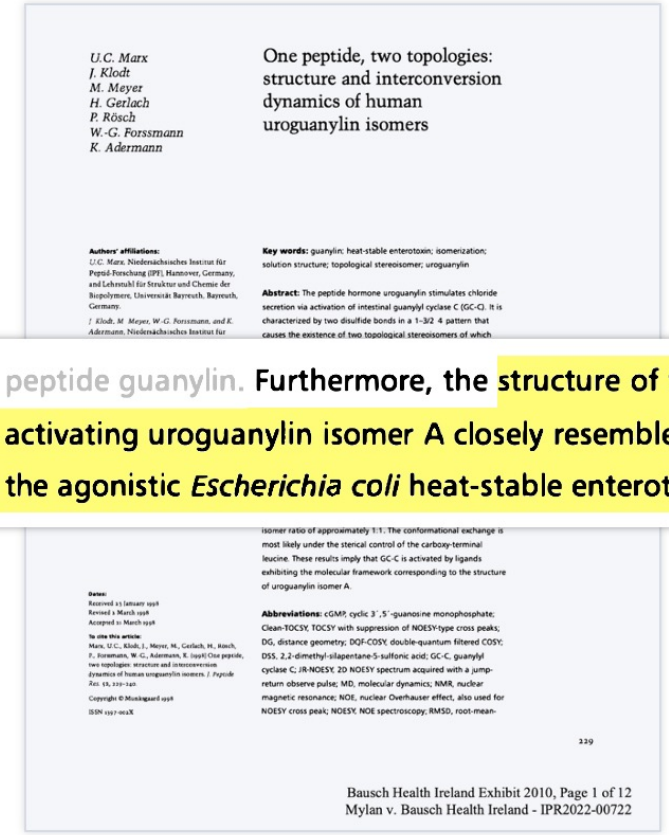
Abstract: The peptide hormone uroguanylin stimulates chloride secretion via activation of intestinal guanylyl cyclase C (GC-C). It is characterized by two disulfide bonds in a 1–3/2 4 pattern that causes the existence of two topological stereoisomers of which only one induces intracellular cGMP elevation. To obtain an unambiguous structure-function relationship of the isomers, we determined the solution structure of the separated uroguanylin isoforms using NMR spectroscopy. Both isomers adopt well-defined structures that correspond to those of the isomers of the related peptide guanylin. Furthermore, the structure of the GC-C-activating uroguanylin isomer A closely resembles the structure of the agonistic *Escherichia coli* heat-stable enterotoxin. Compared with guanylin isomers, the conformational interconversion of uroguanylin isomers is retarded significantly. As judged from chromatography and NMR spectroscopy, both uroguanylin isoforms are stable at low temperatures, but are subject to a slow pH-dependent mutual isomerization at 37°C with an equilibrium isomer ratio of approximately 1:1. The conformational exchange is most likely under the sterical control of the carboxy-terminal leucine. These results imply that GC-C is activated by ligands exhibiting the molecular framework corresponding to the structure of uroguanylin isomer A.

Abbreviations: cGMP, cyclic 3′,5′-guanosine monophosphate; Clean-TOCSY, TOCSY with suppression of NOESY-type cross peaks; DG, distance geometry; DQF-COSY, double-quantum filtered COSY; DSS, 2,2-dimethyl-silapentane-5-sulfonic acid; GC-C, guanylyl cyclase C; JR-NOESY, 2D NOESY spectrum acquired with a jump-return observe pulse; MD, molecular dynamics; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect, also used for NOESY cross peak; NOESY, NOE spectroscopy; RMSD, root-mean-

The slow development of the equilibrium between uroguanylin isomers at alkaline pH indicates that the ion ization state of the isomeric molecules strongly influences the kinetics of transition between the isomers of uroguanylin 16 and uroguanylin 24. Thus, the terminal carboxyl, ionizable side-chains of Asp2, Asp3 and Glu5, or those groups able to form intrachain hydrogen bonds, may be involved in the control of stabilization of the two isomers. After 3 days at alkaline pH, both isoforms decomposed because of disulfide exchange. Comparison of the conversion of uroguanylin 16 isomers with the isomers of the N-terminally extended uroguanylin-24 resulted in identical kinetics for isoforms A and B at a pH of 4.5 and 7.7 for both peptides (Fig. 6C). This result clearly demonstrates that the isomerization is not affected by the amino-terminal region of uroguanylin. Corresponding to the

Ex. 2010 at 236
Patent Owner's Resp. at 39, 59; *see also* Sur-Reply at 26; Ex. 2024 ¶ 147

Marx: Disulfide Bonds Address Topoisomerism



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One peptide, two topologies: structure and interconversion dynamics of human uroguanylin isomers

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J. Klodt, M. Meyer, W.-G. Forssmann, and K. Adermann, Niedersächsisches Institut für

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isomer ratio of approximately 1:1. The conformational exchange is most likely under the sterical control of the carboxy-terminal leucine. These results imply that GC-C is activated by ligands exhibiting the molecular framework corresponding to the structure of uroguanylin isomer A.

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Bausch Health Ireland Exhibit 2010, Page 1 of 12
Mylan v. Bausch Health Ireland - IPR2022-00722

peptide guanylin. Furthermore, the structure of the GC-C-activating uroguanylin isomer A closely resembles the structure of the agonistic *Escherichia coli* heat-stable enterotoxin. Compared

0.45 nm. The known higher activation potency of ST may be related to the additional disulfide bond which causes a higher rigidity of its three-dimensional structure and, thus, a possibly more efficient interaction with the receptor. Structure calculations of uroguanylin-16 with an additional distance restraint between protons that occupy the positions of fictitious sulfur atoms of a third disulfide bridge between residues 3 and 8 show that a third disulfide bridge is possible for the A form structure without distortion of the peptide backbone. The same calculation for the B form resulted in a higher overall energy of these structures and a slight violation of the additional fictitious distance restraint, indicating that a third disulfide bridge for the B form structure could be possible but needs a higher distortion of the peptide backbone (data not shown). A third disulfide bond apparently would lead to a preference of a structure similar to the A form isomer that was found for ST (22).

Ex. 2010 at 229, 235
Patent Owner's Resp. at 14, 39; *see also* Sur-Reply at 11; Ex. 2024 ¶¶ 90-91; Ex. 2025 ¶¶ 66-67

Petitioner's Conservative Substitution Theory Fails

promising modification. EX1002, ¶¶132-38, 142, 155. As Professor Peterson explains, a skilled artisan would have expected this particular conservative substitution to retain excellent receptor-activating activity. EX1002, ¶¶139-52

137. When preparing synthetic analogs to human uroguanylin, a skilled artisan would have had good reason to look first to making conservative changes to the peptide. The target receptor, as Currie discloses, is part of a "group of proteins that share structural characteristics relative to the enzymatic function of producing cyclic GMP, but differ quite remarkably in their selective activation by ligands." EX1005, 1:7-11. Thus, as Currie notes, the guanylate cyclases are only selectively activated by their ligands. Moreover, as described above in Section VII, skilled artisans routinely began analog synthesis with conservative substitutions to avoid causing immunogenicity or ablating activity altogether.

A lead compound is "'a compound in the prior art that would be most promising to modify in order to **improve upon its . . . activity** and **obtain a compound with better activity.**'"

Pet. at 36; Ex. 1002 ¶ 137; *Otsuka Pharm. Co. v. Sandoz, Inc.*, 678 F.3d 1280, 1291 (Fed. Cir. 2012)
Patent Owner's Resp. at 3, 41-42; *see also* Sur-Reply at 12

Conservative Substitution Would Not Enhance Functionality

BLAKE ROBERT PETERSON, Ph.D.

Q. Sure. Why would a person of ordinary skill in the art not expect a conservative substitution to enhance functionality?

***

THE WITNESS: A conservative substitution, generally speaking, has a minimal effect on biological activity, but in some cases can enhance biological activity, or reduce biological activity; but typically a person of ordinary skill in the art would make a conservative substitution to modulate biological activity.

- Dr. Peterson also admits that "**screening approaches…are not considered as rational**"

Ex. 2026 at 47:3-14; Ex. 2069 at 15:5-8
Patent Owner's Resp. at 22, 42; *see also* Sur-Reply at 12; Ex. 2024 ¶ 153

Jonson: Asp-Glu Is Not a Conservative Substitution

Protein Engineering vol.14 no.6 pp.397–402, 2001

A critical view on conservative mutations

Per Harald Jonson and Steffen B.Petersen[1]

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By analysing the surface composition of a set of protein 3D structures, complemented with predicted surface compositional information for homologous proteins, we have found significant evidence for a layer composition of protein structures. In the innermost and outermost parts of proteins there is a net negative charge, while the middle has a net positive charge. In addition, our findings indicate that the concept of conservative mutation needs substantial revision, e.g. very different spatial preferences were found for glutamic acid and aspartic acid. The alanine screening often used in protein engineering projects involves the substitution of residues to alanine, based on the assumption that alanine is a 'neutral' residue. However, alanine has a high negative correlation with all but the non-polar residues. We therefore propose the use of, for example, serine as a substitute for the residues that are negatively correlated with alanine.

Keywords: amino acid properties/protein engineering/solvent accessibility/spatial contacts/structural preference

Introduction

Upon folding of a peptide chain into a 3D protein structure, some residues are transferred from a polar environment to a more non-polar environment in the interior of the folded protein. This transfer is driven by the thermodynamic properties of the amino acids and the solvent. Throughout molecular evolution nature has selected for suitable function and stability of the resulting protein. For small to medium sized proteins—in the folded structure—only a few residues are totally buried (Chothia, 1976; Miller *et al.*, 1987; Petersen *et al.*, 1998), whereas most residues are only partially buried. The variation in solvent accessibility is dependent on the properties of the residue in question and is reflected in the amino acid composition throughout the protein structure. These differences in the solvent accessibility profile have found wide applications in various structure prediction methods (Holbrook *et al.*, 1990; Rost and Sander, 1994; Thompson and Goldstein, 1996). Also, the use of environment specific substitution matrices (Donnelly *et al.*, 1994; Wako and Blundell, 1994) have proven valuable. The sequential neighbourhood of amino acids has been investigated previously (Vondervisztˊ *et al.*, 1986) and its use has been found in, for example, loop prediction (Wojcik *et al.*, 1999) and secondary structure prediction (Chou and Fasman, 1978; Chandonia and Karplus, 1999; Jones, 1999). No significant correlation between residues sequential neighbour preference was discovered.

The spatial neighbourhood around individual residues has also been previously investigated (Burley and Petsko, 1985; Bryant and Amzel, 1987; Miyazawa and Jernigan, 1993; Petersen *et al.*, 1999). Further, spatial contacts have been studied to derive contact potentials for the different amino acid interactions (Brocchieri and Karlin, 1995; Miyazawa and Jernigan, 1996, 1999). The common strategy is to study the number of contacts within a given distance cut-off. However, the literature seems devoid of investigations of distance-dependent contacts and also of reports utilizing the embedded information of the solvent accessibility of the residues involved.

A two-state prediction of solvent accessibility correlation between hydrophobicity, buried contact propensity and the location in the prediction window has been reported (Mucchielli-Giorgi *et al.*, 1999). However, it does not describe any correlation between individual residue distributions.

It is important to be able to discriminate between correctly folded and misfolded model structures. It has been pointed out that potential energy-based methods do not discriminate well between folded and misfolded structures. However, structural features such as buried polar surface (Overington *et al.*, 1992) and number of polar contacts (Bryant and Amzel, 1987; Golovanov *et al.*, 1999) have proven valuable.

In protein engineering the concept of conservative mutations is frequently used. The general idea is that a substitution of an amino acid with another amino acid with similar physico-chemical properties will not influence the stability and function of the protein. The present paper shows that the spatial preferences for similar residues can be dramatically different in protein structures under similar circumstances (in this context solvent accessibility).

The results of the neighbour analysis will be valuable in model validation, as a tool for structure prediction and especially as a guide in the search for stability enhancing mutations.

Methods

The sequences used are a subset of the 25% sequence identity set of non-homologous structures (Hobohm *et al.*, 1992; Hobohm and Sander, 1994) derived from the protein structure databank PDB (Bernstein *et al.*, 1977). Only single-chain protein sequences were used. The resulting dataset consisted of 336 single-chain sequences with a maximum pairwise sequence identity of 25%. The subset was expanded through the use of the corresponding HSSP-files (Dodge *et al.*, 1998). The total data set contained 8379 aligned sequences and 1 415 986 residues. This corresponds to 6.7% of all residues in version 34 of SWISS-PROT (Bairoch and Apweiler, 1997). The length of the sequences was between 64 and 1017 residues. The resolution of the X-ray structures used varied between 1.0 and 3.0 Å, with an average of 2.0 Å. Further, the subset contained 31 structures solved by NMR. However, all hydrogen-atom co-ordinates were discarded. To check for a possible bias introduced by the use of the homologous sequences the complete analysis was done with and without the aligned sequences. No significant differences were observed,

positive charge. **In addition, our findings indicate that the concept of conservative mutation needs substantial revision, e.g. very different spatial preferences were found for glutamic acid and aspartic acid.** The alanine screening

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tryptophan–aspartic acid are shown. The common belief that a glutamic acid to aspartic acid mutation is conservative is contrary to the observations shown. The tryptophan–glutamic

Ex. 2035 at 397 (Abstract), 400
Patent Owner's Resp. at 20; *see also* Sur-Reply at 12; Ex. 2024 ¶¶ 153-156

Jonson Reflects a Systematic Study of Over 1.4M Residues

Protein Engineering vol.14 no.6 pp.397–402, 2001

A critical view on conservative mutations

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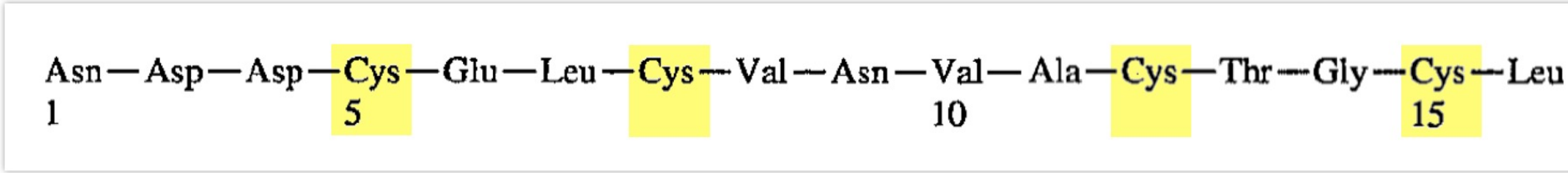
Ex. 2035 at 397
Sur-Reply at 12

The Enormous Number of Potential Substituting Options for Human Uroguanylin



- Even utilizing only the 20 naturally-occurring amino acids at each position, the number of potential substituting options is approximately 20^{16}
- Even leaving the disulfide bonds undisturbed, the number of potential substituting options is still approximately 20^{12}

Patent Owner's Resp. at 16; *see also* Ex. 2024 ¶ 158

Petitioner's Own Argument Leads Away from Plecanatide

BLAKE ROBERT PETERSON, Ph.D.

Q. Based upon Li's teachings with respect to opossum uroguanylin, what, if any, modification to human uroguanylin would a person of ordinary skill in the art have been motivated to make?

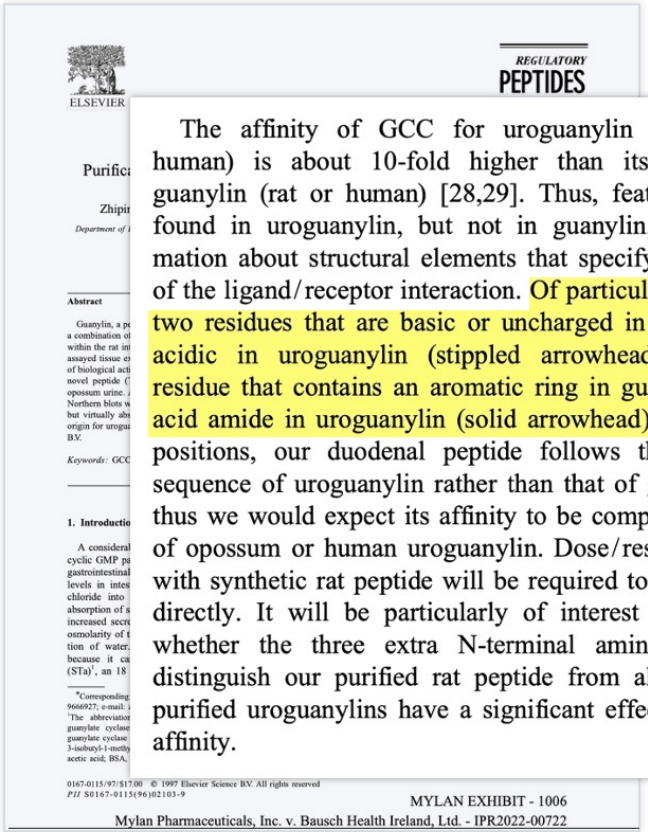
A. The person of ordinary skill in the art would have been motivated to study changing those aspartic acids at positions 2 and 3, the glutamic acids, based on the homology to a opossum and rat uroguanylin.

- This compound is the **unclaimed SP-302** in the '786 patent

Ex. 2026 at 67:23-68:6
Patent Owner's Resp. at 45

Li Teaches Modifications to Rat, Not Human, Uroguanylin

Purification, cDNA sequence, and tissue distribution of rat uroguanylin



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REGULATORY PEPTIDES

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Department of

Abstract

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B.V.

Keywords: GCC

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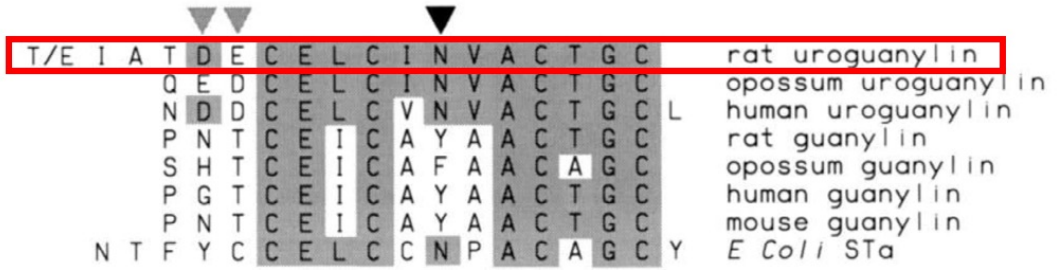
acetic acid; BSA,

0167-0115/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved
PII S0167-0115(96)02103-9

The affinity of GCC for uroguanylin (opossum or human) is about 10-fold higher than its affinity for guanylin (rat or human) [28,29]. Thus, features that are found in uroguanylin, but not in guanylin, offer information about structural elements that specify the strength of the ligand/receptor interaction. Of particular interest are two residues that are basic or uncharged in guanylin but acidic in uroguanylin (stippled arrowheads), and one residue that contains an aromatic ring in guanylin but an acid amide in uroguanylin (solid arrowhead). At all three positions, our duodenal peptide follows the consensus sequence of uroguanylin rather than that of guanylin, and thus we would expect its affinity to be comparable to that of opossum or human uroguanylin. Dose/response curves with synthetic rat peptide will be required to test this idea directly. It will be particularly of interest to determine whether the three extra N-terminal amino acids that distinguish our purified rat peptide from all previously-purified uroguanylins have a significant effect on binding affinity.

MYLAN EXHIBIT - 1006
Mylan Pharmaceuticals, Inc. v. Bausch Health Ireland, Ltd. - IPR2022-00722



directly. It will be particularly of interest to determine whether the three extra N-terminal amino acids that distinguish our purified rat peptide from all previously-purified uroguanylins have a significant effect on binding affinity.

Ex. 1006 at 45 (Title), 52, 54
Patent Owner's Resp. at 46-47; Ex. 2024 ¶¶ 74-78

Li: Rat Uroguanylin Is Far Less Potent than Opossum Uroguanylin

ELSEVIER

Regulatory Peptides 68 (1997) 45–56

REGULATORY PEPTIDES

Purification, cDNA sequence, and tissue distribution of rat uroguanylin

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Received 29 June 1996; revised 17 October 1996; accepted 31 October 1996

Abstract

Guanylin, a peptide purified from rat jejunum, is thought to regulate water and electrolyte balance in the intestine. We show here, using a combination of Northern blots, Western blots, and functional assays, that guanylin and its receptor (GCC) are not distributed in parallel within the rat intestine. To investigate the possibility that there might be a second intestinal peptide that serves as a ligand for GCC, we assayed tissue extracts for the ability to stimulate cyclic GMP synthesis in a GCC-expressing cell line. Duodenal extracts display a peak of biological activity that is not present in colon and that does not comigrate with guanylin or proguanylin. The activity co-purifies with a novel peptide (TIATDECELCINVACTGC) that has high homology with uroguanylin, a peptide initially purified from human and opossum urine. A rat uroguanylin cDNA clone was found to encode a propeptide whose C-terminus corresponds to our purified peptide. Northern blots with probes generated from this clone reveal that prouroguanylin mRNA is strongly expressed in proximal small intestine, but virtually absent from colon, corroborating our biochemical measurements. Taken together, these studies demonstrate an intestinal origin for uroguanylin, and show that within the intestine its distribution is complementary to that of guanylin. © 1997 Elsevier Science B.V.

Keywords: GCC; STa receptor; CFTR; Guanylin; Uroguanylin

1. Introduction

A considerable body of evidence supports a role for the cyclic GMP pathway in the control of ion transport in the gastrointestinal tract. Elevation of intracellular cyclic GMP levels in intestinal epithelial cells enhances secretion of chloride into the intestinal lumen [1], and diminishes absorption of sodium and chloride [2]. The combination of increased secretion and decreased absorption elevates the osmolarity of the lumen, and drives the luminal accumulation of water. This mechanism was initially identified because it can be induced by heat-stable enterotoxin (STa)[1], an 18 amino acid peptide secreted by pathogenic strains of *Escherichia coli* [3–5]. Exposure to high levels of toxin, as occurs during acute bacterial infections, triggers non-physiological movement of electrolytes, and produces a watery diarrhea that can lead to dehydration and death.

When the STa receptor was cloned from a small intestinal cDNA library [6], it was found to belong to a family of receptors that contain endogenous guanylate cyclase (GC) activity. Two other members of this family are the natriuretic peptide receptors, GCA and GCB [7]. Because the STa receptor was the third such receptor cloned, it was named GCC. All members of this family contain: (a) an intracellular catalytic domain responsible for the conversion of GTP to cyclic GMP, (b) an intracellular regulatory domain that controls the activity of the catalytic domain, (c) a single transmembrane domain, and (d) an extracellular receptor domain that provides an agonist binding site [7].

These findings led to the hypothesis that GCC serves as a receptor for one or more endogenous ligands in the GI

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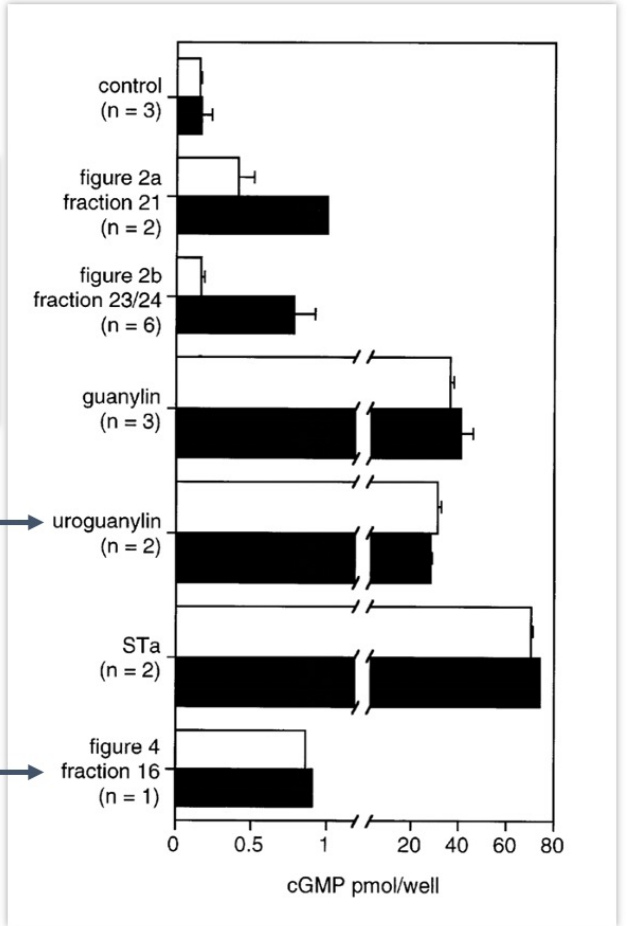
[1]The abbreviations used are: STa, heat-stable enterotoxin; GCA, guanylate cyclase type A; GCB, guanylate cyclase type B; GCC, guanylate cyclase type C; HBSS, Hanks' buffered salt solution; IBMX, 3-isobutyl-1-methylxanthine; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; BSA, bovine serum albumin; RIA, radioimmunoassay.

PII S0167-0115(96)02103-9

Fig. 3. Preincubation at 37°C enhances the activity of HPLC column fractions, but not of synthetic guanylin or uroguanylin. The bars show the mean level of cyclic GMP (±SEM or range) in T84 cells after exposure to the indicated stimuli. Each stimulus was either held at 4°C (□) or incubated at 37°C (■) prior to applying it to the cells. The numbers used to identify the HPLC fractions correspond to (i) the figure in which the pertinent HPLC run is shown and (ii) the appropriate fraction number(s) from that column run.

opossum uroguanylin →

rat uroguanylin →



Ex. 1006 at 49 (Fig. 3)
Patent Owner's Resp. at 46-47; *see also* Sur-Reply at 13, fn. 3; Ex. 2024 ¶¶ 75, 79

Rat Uroguanylin Is Plagued by Topoisomerism

FEBS 19675 FEBS Letters 421 (1998) 27–31

Topological isomers of human uroguanylin: interconversion between biologically active and inactive isomers

Naoyoshi Chino[a,*], Shigeru Kubo[a], Tetsuya Kitani[a], Takuya Yoshida[b], Ryosuke Tanabe[b], Yuji Kobayashi[b], Masamitsu Nakazato[c], Kenji Kangawa[d], Terutoshi Kimura[a]

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[b]Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan
[c]Third Department of Internal Medicine, Miyazaki Medical College, Miyazaki 889-16, Japan
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Received 21 October 1997; revised version received 1 December 1997

Abstract The solution structures of the two compounds of human uroguanylin (I and II), which were generated during disulfide bond forming reaction, were found to be topological isomers by ^{1}H-nuclear magnetic resonance spectroscopy. These isomers are interconvertible in aqueous media at rates which vary with the pH and temperature of the solution. Because compound I is active in the cGMP producing assay, but compound II is not, this interconversion may be useful for evaluating the activity of human uroguanylin both in vivo and in vitro.

Key words: Uroguanylin; Guanylin; Topological isomer; Interconversion; HPLC analysis; Biological activity

1. Introduction

Uroguanylin and guanylin were discovered as endogenous peptide hormones in mammals based upon their structure similarity to heat stable enterotoxins (STs) secreted by pathogenic bacteria. The primary structures of uroguanylin and guanylin from human, rat (mouse) and opossum have been reported as being comprised of 15 or 16 amino acid residues [1–5]; the human and rat peptide sequences are shown in Fig. 1. The sequence similarity among them is high and four Cys residues in all the peptides are conserved. These Cys residues participate in the formation of the two intramolecular disulfide linkages, one between Cys^4 and Cys^{12} and the other between Cys^7 and Cys^{15}. Uroguanylin and guanylin, as well as ST, are reported to be involved in the regulation of salt and water transport in the intestinal tract and kidney. In addition, these peptides are known to stimulate cGMP production by activating the guanylyl cyclase C in both enterocytes and T84 colon cancer cells. Therefore, endogenous uroguanylin and guanylin are suggested to play important roles in intestinal and renal dysfunction and salt dependent hypertension [6].

In our previous paper on the chemical synthesis of human uroguanylin using a two step selective disulfide forming method, two compounds (I and II) were found to be generated upon analyzing the second disulfide bond forming reaction by reversed phase high performance liquid chromatography (RP HPLC) at 40°C [7]. A typical chromatogram for the separation of a 1:1 mixture of these compounds is shown in Fig. 2. We have so far clarified the following characteristics for compounds I and II: (i) each compound can be isolated to a purity greater than 99% as determined by RP HPLC; (ii) both have identical primary structures, molecular weights and disulfide connectivity patterns according to examination by suitable analytical methods; and (iii) significant differences exist between them in the optical rotation value and their biological activity. During the course of our previous study, Skelton et al. reported that two clearly separable signal connectivities were detected in the analysis of the amino terminally extended or deleted human guanylin derivatives by nuclear magnetic resonance (NMR) spectroscopy. Based on these observations and structural refinements, they proposed that the heterogeneity of the NMR signals of human guanylin derivatives originated from the topological isomerism of the peptide, although such isomers were unseparable on RP HPLC under the various analytical conditions used [8]. In contrast, we found in a previous study that human des Leu^{15} uroguanylin and rat guanylin, both of which terminate the peptide chains at the fourth Cys residue like human guanylin, were detected as two base line separable peaks on RP HPLC when the analytical temperature was decreased to 8°C, although these peptides were eluted in a broad but single peak at 40°C [7]. From these observations, we assumed that the two well separable human uroguanylin compounds on RP HPLC were similar topological isomers with respect to the peptide backbone as reported for human guanylin derivatives. However, this assumption required definite confirmation by experimental evidence.

In the present study, we analyzed the solution structures of both compounds by NMR in an aqueous medium to gain further insight into the characteristics of the isolated compounds I and II of human uroguanylin. We report here con

4 7 12 15
Human Uroguanylin: NDDCELCVN/ACTGCL
Human Guanylin: PGTCEICAYAACTGC
Rat Uroguanylin: TDECELCIN/ACTGC
Rat Guanylin: PNTCEICAYAACTGC

Fig. 1. Primary structures of human and rat uroguanylin and guanylin. Two intramolecular disulfide linkages are shown at the top of the sequences.

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E-mail: chino@prf.or.jp

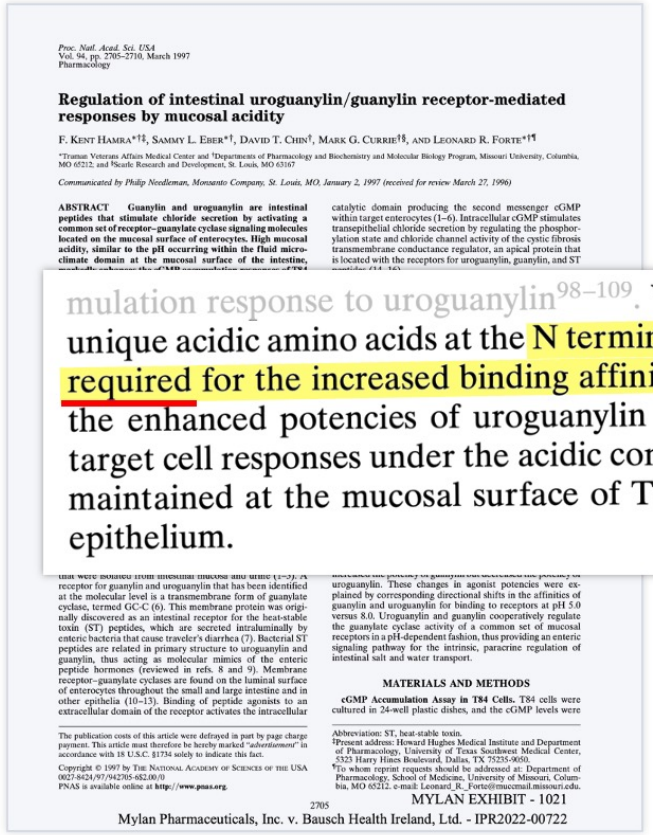
Abbreviations: ST, heat stable enterotoxin; cGMP, cyclic 3′,5′-guanosine monophosphate; RP HPLC, reversed phase high performance liquid chromatography; CD, circular dichroism; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; MD, molecular dynamics; RMSD, root mean square deviation; GdnHCl, guanidine hydrochloride; NEM, *N*-ethylmaleimide; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide

PII S0014 5793(97)01527 5

In the RP HPLC analyses of the two human uroguanylin isomers, we had already established that they are separable at 40°C. However, separations of the human des Leu^{16} uroguanylin isomers, as well as the rat guanylin isomers, were possible only at lower temperatures such as 8°C [7]. This separation characteristic has also been observed for a recently disclosed member of the uroguanylin and guanylin peptide family, rat uroguanylin 15 (unpublished result). Considering

Ex. 2011 at 30
Patent Owner's Resp. at 47; Ex. 2024 ¶ 164

N-terminal Amino Acids of Human Uroguanylin Are Required



Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 2705–2710, March 1997
Pharmacology

Regulation of intestinal uroguanylin/guanylin receptor-mediated responses by mucosal acidity

F. KENT HAMRA*†‡, SAMMY L. EBER*†, DAVID T. CHIN†, MARK G. CURRIE†§, AND LEONARD R. FORTE*†¶

Communicated by Philip Needleman, Monsanto Company, St. Louis, MO, January 2, 1997 (received for review March 27, 1996)

ABSTRACT **Guanylin and uroguanylin are intestinal peptides that stimulate chloride secretion by activating a common set of receptor–guanylate cyclase signaling molecules located on the mucosal surface of enterocytes. High mucosal acidity, similar to the pH occurring within the fluid microclimate domain at the mucosal surface of the intestine,**

mulation response to uroguanylin$^{98–109}$. We conclude that the unique acidic amino acids at the N terminus of uroguanylin are required for the increased binding affinities, and accordingly, the enhanced potencies of uroguanylin in the stimulation of target cell responses under the acidic conditions of pH 5.0–5.5 maintained at the mucosal surface of T84 cells in this model epithelium.

catalytic domain producing the second messenger cGMP within target enterocytes (1–6). Intracellular cGMP stimulates transepithelial chloride secretion by regulating the phosphorylation state and chloride channel activity of the cystic fibrosis transmembrane conductance regulator, an apical protein that is located with the receptors for uroguanylin, guanylin, and ST

receptor for guanylin and uroguanylin that has been identified at the molecular level is a transmembrane form of guanylate cyclase, termed GC-C (6). This membrane protein was originally discovered as an intestinal receptor for the heat-stable toxin (ST) peptides, which are secreted intraluminally by enteric bacteria that cause traveler's diarrhea (7). Bacterial ST peptides are related in primary structure to uroguanylin and guanylin, thus acting as molecular mimics of the enteric peptide hormones (reviewed in refs. 8 and 9). Membrane receptor–guanylate cyclases are found on the luminal surface of enterocytes throughout the small and large intestine and in other epithelia (10–13). Binding of peptide agonists to an extracellular domain of the receptor activates the intracellular

uroguanylin. These changes in agonist potencies were explained by corresponding directional shifts in the affinities of guanylin and uroguanylin for binding to receptors at pH 5.0 versus 8.0. Uroguanylin and guanylin cooperatively regulate the guanylate cyclase activity of a common set of mucosal receptors in a pH-dependent fashion, thus providing an enteric signaling pathway for the intrinsic, paracrine regulation of intestinal salt and water transport.

MATERIALS AND METHODS

cGMP Accumulation Assay in T84 Cells. T84 cells were cultured in 24-well plastic dishes, and the cGMP levels were

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0027-8424/97/942705-6$2.00/0
PNAS is available online at http://www.pnas.org.

Abbreviation: ST, heat-stable toxin.
‡Present address: Howard Hughes Medical Institute and Department of Pharmacology, University of Texas Southwest Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-9050.
¶To whom reprint requests should be addressed at: Department of Pharmacology, School of Medicine, University of Missouri, Columbia, MO 65212. e-mail: Leonard_R_Forte@mucemail.missouri.edu.

2705

MYLAN EXHIBIT - 1021
Mylan Pharmaceuticals, Inc. v. Bausch Health Ireland, Ltd. - IPR2022-00722

BLAKE ROBERT PETERSON , Ph.D.

Q. Based on Hamra 1997, it is your opinion that the N-terminal amino acid residues of human uroguanylin are required for the increased binding affinities and enhanced potency for activation of receptors under acidic conditions, correct?

A. Correct.

Ex. 1021 at 2708, 2709, Ex. 2069 at 89:13-18
Patent Owner's Resp. at 49; Sur-Reply at 14; Ex. 2024 ¶ 167

Fiser: Asp Preferred Over Glu

FEBS 17787 FEBS Letters 397 (1996) 225-229

Conservation of amino acids in multiple alignments: aspartic acid has unexpected conservation

András Fiser[a,b], István Simon[b], Geoffrey J. Barton[a,*]

[a]University of Oxford, Laboratory of Molecular Biophysics, The Rex Richards Building, South Parks Road, Oxford OX1 3QU, UK
[b]Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, PO Box 7, Budapest H-1518, Hungary

Received 18 July 1996; revised version received 25 September 1996

Abstract Analysis of the relationship between surface accessibility and amino acid conservation in multiple sequence alignments of homologous proteins confirms expected trends for hydrophobic amino acids, but reveals an unexpected difference between the conservation of Asp, Glu and Gln. Even when not in an active site, Asp is more highly conserved than Glu. There is a clear preference for conserved and buried Asp to be present in coil, but there is no tendency for Asp to conserve φ/ψ in the ++ region of the Ramachandran map. Glu does not show any preference to be conserved in a particular secondary structure. Analysis of recently derived substitution matrices (e.g. BLOSUM) confirms that Glu tends to substitute more frequently with other amino acids than does Asp. Analysis of relative accessibility versus relative conservation for individual amino acid positions in alignments shows a negative correlation for all amino acid types. With the exception of Arg, Lys, Gly, Glu, Asp and Tyr, a relative conservation of > 2 suggests the amino acid will have a relative accessibility of < 50%. Observation of conserved Cys, Gly or Asp in a reliable multiple alignment suggests a position important for the structure of the protein. Furthermore, the Asp is likely to be involved in polar interactions through its side chain oxygen atoms. In contrast, Gln is the least conserved amino acid overall.

Key words: Conservation analysis; Multiple sequence alignment; Protein structure prediction

1. Introduction

Knowledge of protein sequences is growing much faster than knowledge of either three-dimensional structure or function. Accordingly, the interpretation of sequence data to identify structurally or functionally important residues is essential if the data are to be effective in furthering understanding of biological systems. Multiple sequence alignments of families of protein sequences are now used routinely to indicate residues of key importance to the function of the protein. A position in an alignment that has identical residues in all members of a protein family may have a key catalytic role. A position where similar physico-chemical properties (e.g. hydrophobicity) are shared may suggest importance in stabilising the native conformation of the protein [1,2]. Identification of such conserved features in multiple alignments has been used to good effect to improve the accuracy of prediction of secondary structure and buried residues (α-helix and β-strand) (e.g. [3–7]).

Here we report a systematic study of residue conservation in multiple alignments where at least one protein is of known tertiary structure. Our analysis complements that of Overington et al. [8] who considered only pairwise substitution frequencies for amino acids in structurally aligned families.

2. Materials and methods

2.1. Data base

A non-redundant set of 81 proteins was generated from the April 1993 release of the Brookhaven Protein Data Bank (PDB) [9]. The set was chosen in a two-step procedure. First, all pairs of chains (over 50 residues and resolution better than 2.5 Å) in the data bank were compared by calculating correlation coefficients between the dipeptide frequencies in each protein. A set of 101 protein chains was selected such that all pairs had a correlation of < 0.4. All pairs in this set were then compared by a rigorous sequence comparison method [10,11] followed by cluster analysis. This reduced the set to 81 protein chains that show no obvious sequence similarity (PDB code and chain identifiers: 1SSC 1ACX 1ALC 1BRP_A 1CC5 1ECA 1FKF 1FNR 1GCR 1GP1_A 1HDSB 1HIP 1HOE 1LRD_4 1PAZ 1PCY 1PHH 1PRC_C 1RBP 1RHD 1RNH 1SN3 1TGS 1TPK_A 1WSY_B 256B_A 2ALP 2AZA_A 2CAB 2CD4 2CDV 2CPP 2FXB 2GN5 2LHT 2LIV 2LTN_A 2ORI_L 2PAB_A 2RNT 2RSP_A 2SEC_I 2SNL_E 2SNS 2SOD_B 2SSI 2STV 2TSI 2UTG_A 3ADK 3BSC 3CLA 3FXC 3GAP_B 3LZM 3SGB_I 451C 4BP2 4FD1 4FXN 4HHB_A 4PEP 4PFK 4PTP 4TNC 5CTS 5CYT_R 5EBX 5RUB_A 5RXN 6LDH 6TMN_E 7PTI 8ADH 8ATC_B 8CAT_A 8DFR 9PAP 9RSA_A 9WGA_A).

Each protein in the set was compared by the Smith-Waterman algorithm [11,12] to the NBRF-PIR sequence data bank (Release 38) and all sequences that gave probability values of $< 10^{-5}$ by a length-dependent scoring scheme (program SCANPS ftp://geoff.biop.ox.ac.uk/programs/scanps) were multiply aligned with the query sequence by the algorithm of Barton and Sternberg [13]. This gave 81 alignments with between 3 and 499 sequences in each (median of 28 sequences).

2.2. Calculation of conservation and accessibility

Conservation scores based upon the physico-chemical properties of the amino acids were calculated for each position in each alignment according to Livingstone and Barton [2]. Conservation scores range from 0 to 10 and represent the number of the properties: Hydrophobic, Positive, Negative, Polar, Charged, Small, Tiny, Aliphatic, Aromatic, Proline and their negations (e.g. *not* positive) that are shared at a position. The program AMAS [2], which calculates conservation values from a multiple alignment, may be run over the World Wide Web (http://geoff.biop.ox.ac.uk/servers/amas-server.html).

Although conservation scores are absolute, the relative importance of a conservation score is dependent on the overall similarity between the sequences in the multiple alignment. For example, in an alignment of 20 sequences that all share > 90% pairwise identity conservation scores above 8 may be interesting. In contrast, if the pairwise identity is below 30% then lower conservation scores will be informative. Accordingly, in this study we normalised conservation scores by the average conservation for each alignment to give relative conservation scores C_r. We refer to a position as 'conserved' if $C_r > 1$.

Accessible surface areas were calculated by the program DSSP [14] and converted to relative accessibilities by dividing by the accessibility of the residue in a Gly-X-Gly tripeptide [15]. Two relative accessibility classes were considered $0 \le A \le 0.25$ (buried) and $0.25 < A \le max(A)$ (exposed).

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PII S0014-5793(96)01181-7

Abstract Analysis of the relationship between surface accessibility and amino acid conservation in multiple sequence alignments of homologous proteins confirms expected trends for hydrophobic amino acids, but reveals an unexpected difference between the conservation of Asp, Glu and Gln. Even

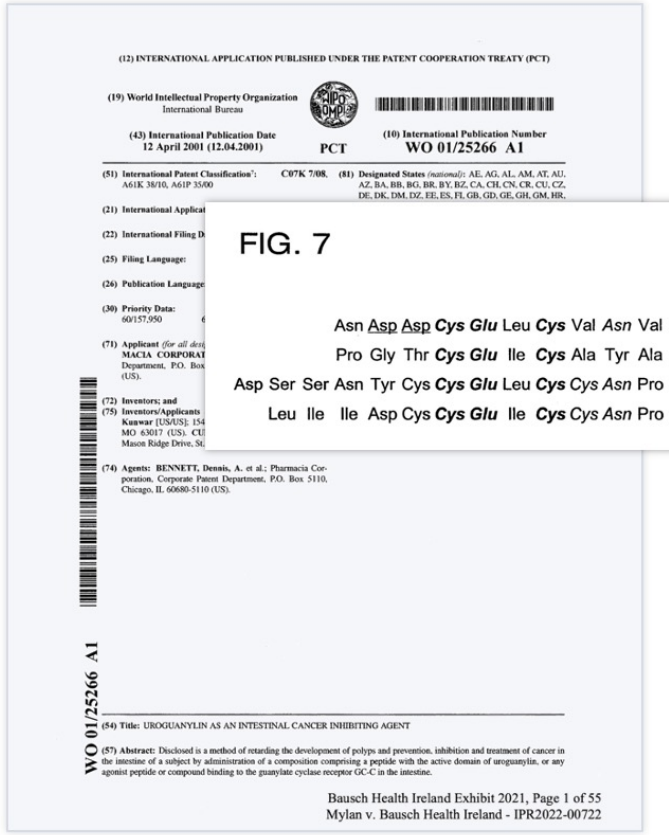
Since our data do not suggest a significant preference for ++ φ/ψ, the preferred conservation of Asp is likely to be due to differing side-chain interactions. The most obvious hypoth-

to differing side-chain interactions. The most obvious hypothesis is that since Glu has a higher proportion of non-polar atoms than Asp it can make more non-specific interactions and so there are fewer constraints on its environment. In

Why, then, is Asp most highly conserved when buried in coil? The short Asp side chain is restricted in mobility yet able to make strong polar interactions. It is possible that Asp may form a 'pin' that stabilises non-regular structures in loops.

Ex. 2036 at 225 (Abstract), 227, 228
Patent Owner's Resp. at 20-21, 50; Ex. 2024 ¶¶ 103, 147, 153, 214

WO ’266: Retain Asp at 2 and 3 Positions of Human Uroguanylin



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WO 01/25266 A1

(51) International Patent Classification[7]: A61K 38/10, A61P 35/00 C07K 7/08

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(74) Agents: BENNETT, Dennis, A. et al.; Pharmacia Corporation, Corporate Patent Department, P.O. Box 5110, Chicago, IL 60680-5110 (US).

WO 01/25266 A1

(54) Title: UROGUANYLIN AS AN INTESTINAL CANCER INHIBITING AGENT

(57) Abstract: Disclosed is a method of retarding the development of polyps and prevention, inhibition and treatment of cancer in the intestine of a subject by administration of a composition comprising a peptide with the active domain of uroguanylin, or any agonist peptide or compound binding to the guanylate cyclase receptor GC-C in the intestine.

Bausch Health Ireland Exhibit 2021, Page 1 of 55
Mylan v. Bausch Health Ireland - IPR2022-00722

FIG. 7

		Asn	Asp	Asp	***Cys***	***Glu***	Leu	***Cys***	Val	*Asn*	Val	Ala	***Cys***	Thr	***Gly***	***Cys***	Leu	h UroG.
		Pro	Gly	Thr	***Cys***	***Glu***	Ile	***Cys***	Ala	Tyr	Ala	Ala	***Cys***	Thr	***Gly***	***Cys***		h Gua
Asp	Ser	Ser	Asn	Tyr	Cys	***Cys***	***Glu***	Leu	***Cys***	*Cys*	*Asn*	Pro	Ala	***Cys***	Thr	***Gly***	***Cys***	Tyr	E. *coli*
Leu	Ile	Ile	Asp	Cys	***Cys***	***Glu***	Ile	***Cys***	*Cys*	*Asn*	Pro	Ala	***Cys***	Phe	***Gly***	***Cys***	Leu	Asn	V. *cholerae*

intestinal pH. Two underlined (Asp-Asp) residues are believed to be important for regulating the functional activity of uroguanylin only at the acidic environment of the intestinal mucosa.

X_8-Asp- Asp- Cys- X_1- X_2- Cys- X_3- Asn- X_4- X_5- Cys- X_6- X_7- Cys-X_9

species. The functionally active domain in most of these peptides are highly conserved. Therefore, the physiological

agents as well. Thus, as long as the functionally active domains of these peptides are conserved, substitutions in the non-active domains may be achieved with no change in the activity of the peptides.

BLAKE ROBERT PETERSON, Ph.D.

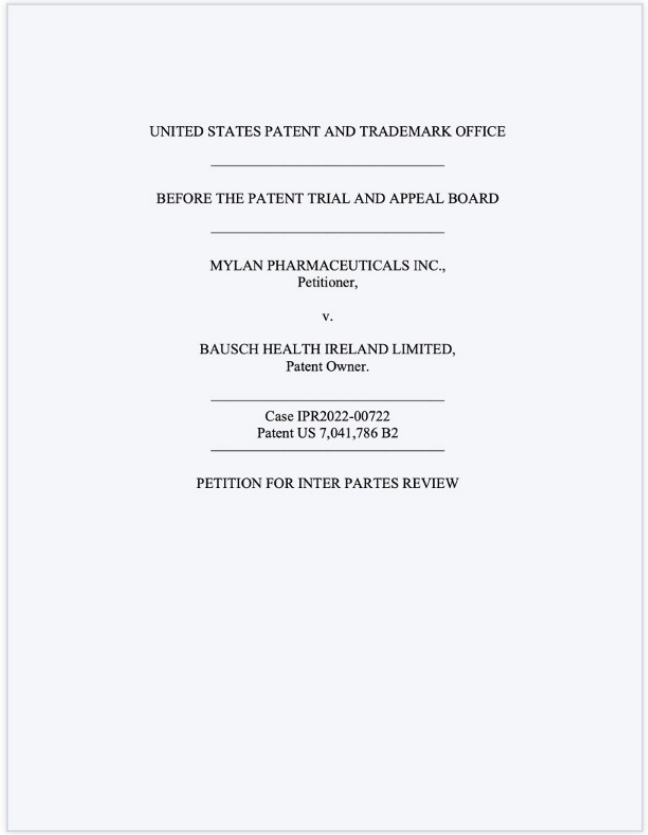
Q. WO 266 identifies the aspartate residues at positions 2 and 3 in human uroguanylin as important for regulating the functional activity of uroguanylin in the acidic environment of intestinal mucosa, correct?

***

THE WITNESS: I mean those amino acids are important for regulating the functional activity of uroguanylin in the acidic environment of intestinal mucosa, that's, that's correct.

Ex. 2021 at 4:23-34, 7:37-8:3, Fig. 7, 17:29-30, 17:33-36; Ex. 2069 at 89:24- 90:7
Patent Owner’s Resp. at 23-24; Sur-Reply at 15; Ex. 2024 ¶¶ 111-113

Petitioner's Flawed pH Argument



UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

MYLAN PHARMACEUTICALS INC.,
Petitioner,

v.

BAUSCH HEALTH IRELAND LIMITED,
Patent Owner.

Case IPR2022-00722
Patent US 7,041,786 B2

PETITION FOR INTER PARTES REVIEW

Moreover, Professor Peterson explains that the [Glu^3]-substitution would have been expected to result in a protonated glutamate at a higher pH (pKa = 4.25 rather than aspartic acid's 3.65) for better activity in the less acidic environment of the intestinal lumen further away from the stomach. EX1002, ¶¶157-61; EX1012, 118, Table 5-1. In this way, the substitution would have been expected to apply the enhanced activity of human uroguanylin (relative to guanylin) more broadly to the intestines instead of being localized only proximate to the more acidic environment near the stomach. EX1002, ¶¶162-170; *see also* EX1002, ¶¶61-65; EX1016, E960,

Pet. at 36
Patent Owner's Resp. at 52; *see also* Sur-Reply at 8; Ex. 2024 ¶¶ 173-186

Nelson: Free pKa Values Cannot Be Strictly Applied to Peptides

peptide R groups, and other environmental factors can affect the pK_a. The pK_a values for R groups listed in Table 5–1 can be a useful guide to the pH range in which a given group will ionize, but they cannot be strictly applied to peptides.

Lehninger **Principles of Biochemistry**

David L. Nelson
Professor of Biochemistry
University of Wisconsin–Madison

Michael M. Cox

BLAKE ROBERT PETERSON, Ph.D.

Q. Nelson teaches that the pKa values for the R groups listed in Table 5-1 of Nelson cannot be strictly applied to peptides, correct?

***

THE WITNESS: So, as I mentioned, it's not accurate to compare a free amino acid pKa necessarily with a peptide amino acid pKa, unless one does a systematic study where we're looking at systematic differences, such as aspartic acid versus glutamic acid in a given peptide sequence. If that makes sense.

Mylan Pharmaceuticals, Inc. v. Bausch Health Ireland, Ltd. - IPR2022-00722

table 5–1

Properties and Conventions Associated with the Standard Amino Acids

Amino acid	Abbreviated names		M_r	pK_a values: pK_1 (—COOH)	pK_2 ($—NH_3^+$)	pK_R (R group)	pI	Hydropathy index*	Occurrence in proteins (%)†
Nonpolar, aliphatic R groups									
Glycine	Gly	G	75	2.34	9.60		5.97	−0.4	7.2
Alanine	Ala	A	89	2.34	9.69		6.01	1.8	7.8
Valine	Val	V	117	2.32	9.62		5.97	4.2	6.6
Leucine	Leu	L	131	2.36	9.60		5.98	3.8	9.1
Isoleucine	Ile	I	131	2.36	9.68		6.02	4.5	5.3
Methionine	Met	M	149	2.28	9.21		5.74	1.9	2.3
Aromatic R groups									
Phenylalanine	Phe	F	165	1.83	9.13		5.48	2.8	3.9
Tyrosine	Tyr	Y	181	2.20	9.11	10.07	5.66	−1.3	3.2
Tryptophan	Trp	W	204	2.38	9.39		5.89	−0.9	1.4
Polar, uncharged R groups									
Serine	Ser	S	105	2.21	9.15		5.68	−0.8	6.8
Proline	Pro	P	115	1.99	10.96		6.48	1.6	5.2
Threonine	Thr	T	119	2.11	9.62		5.87	−0.7	5.9
Cysteine	Cys	C	121	1.96	10.28	8.18	5.07	2.5	1.9
Asparagine	Asn	N	132	2.02	8.80		5.41	−3.5	4.3
Glutamine	Gln	Q	146	2.17	9.13		5.65	−3.5	4.2
Positively charged R groups									
Lysine	Lys	K	146	2.18	8.95	10.53	9.74	−3.9	5.9
Histidine	His	H	155	1.82	9.17	6.00	7.59	−3.2	2.3
Arginine	Arg	R	174	2.17	9.04	12.48	10.76	−4.5	5.1
Negatively charged R groups									
Aspartate	Asp	D	133	1.88	9.60	3.65	2.77	−3.5	5.3
Glutamate	Glu	E	147	2.19	9.67	4.25	3.22	−3.5	6.3

*A scale combining hydrophobicity and hydrophilicity of R groups; it can be used to measure the tendency of an amino acid to seek an aqueous environment (− values) or a hydrophobic environment (+ values). See Chapter 12. From Kyte, J. & Doolittle, R.F. (1982) *J. Mol. Biol.* **157,** 105–132.

†Average occurrence in over 1150 proteins. From Doolittle, R.F. (1989) Redundancies in protein sequences. In *Prediction of Protein Structure and the Principles of Protein Conformation* (Fasman, G.D., ed) Plenum Press, NY, pp. 599–623.

Ex. 1012 at 127, 118, Ex. 2026 at 101:15-102:2
Patent Owner's Resp. at 52-53; Ex. 2024 ¶¶ 176-177

Side Chain pKa Requires Accounting for Peptide Environment

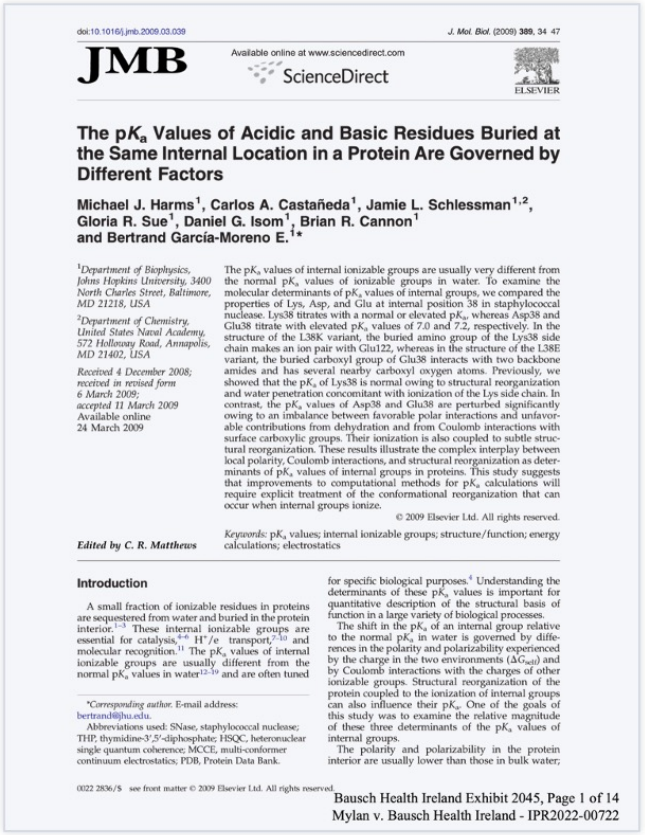
BLAKE ROBERT PETERSON , Ph.D.

Q. Do you agree with Dr. Davies that a prediction as to the pKa of the carboxylic acid on the side chain of the aspartate or glutamate at the third position would require accounting for the environment in which these residues are found when incorporated into a peptide chain, correct?

A. That's correct.

Ex. 2069 at 90:16-22
Sur-Reply at 16; Ex. 2024 ¶¶ 181-182

When in a Peptide, Asp's pKa May Be Higher than Glu's pKa



doi:10.1016/j.jmb.2009.03.039 J. Mol. Biol. (2009) 389, 34–47

JMB Available online at www.sciencedirect.com ScienceDirect ELSEVIER

The pK_a Values of Acidic and Basic Residues Buried at the Same Internal Location in a Protein Are Governed by Different Factors

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Available online 24 March 2009

The pK_a values of internal ionizable groups are usually very different from the normal pK_a values of ionizable groups in water. To examine the molecular determinants of pK_a values of internal groups, we compared the properties of Lys, Asp, and Glu at internal position 38 in staphylococcal nuclease. Lys38 titrates with a normal or elevated pK_a, whereas Asp38 and Glu38 titrate with elevated pK_a values of 7.0 and 7.2, respectively. In the structure of the L38K variant, the buried amino group of the Lys38 side chain makes an ion pair with Glu122, whereas in the structure of the L38E variant, the buried carboxyl group of Glu38 interacts with two backbone amides and has several nearby carboxyl oxygen atoms. Previously, we showed that the pK_a of Lys38 is normal owing to structural reorganization and water penetration concomitant with ionization of the Lys side chain. In contrast, the pK_a values of Asp38 and Glu38 are perturbed significantly owing to an imbalance between favorable polar interactions and unfavorable contributions from dehydration and from Coulomb interactions with surface carboxylic groups. Their ionization is also coupled to subtle structural reorganization. These results illustrate the complex interplay between local polarity, Coulomb interactions, and structural reorganization as determinants of pK_a values of internal groups in proteins. This study suggests that improvements to computational methods for pK_a calculations will require explicit treatment of the conformational reorganization that can occur when internal groups ionize.

Keywords: pK_a values; internal ionizable groups; structure/function; energy calculations; electrostatics

Edited by C. R. Matthews

Introduction

A small fraction of ionizable residues in proteins are sequestered from water and buried in the protein interior.[1–3] These internal ionizable groups are essential for catalysis,[4–6] H^+/e^- transport,[7–10] and molecular recognition.[11] The pK_a values of internal ionizable groups are usually different from the normal pK_a values in water[12–19] and are often tuned for specific biological purposes.[4] Understanding the determinants of these pK_a values is important for quantitative description of the structural basis of function in a large variety of biological processes.

The shift in the pK_a of an internal group relative to the normal pK_a in water is governed by differences in the polarity and polarizability experienced by the charge in the two environments (ΔG_{wcl}) and by Coulomb interactions with the charges of other ionizable groups. Structural reorganization of the protein coupled to the ionization of internal groups can also influence their pK_a. One of the goals of this study was to examine the relative magnitude of these three determinants of the pK_a values of internal groups.

The polarity and polarizability in the protein interior are usually lower than those in bulk water;

*Corresponding author. E-mail address: bertrand@jhu.edu.

Abbreviations used: SNase, staphylococcal nuclease; THP, thymidine-3′,5′-diphosphate; HSQC, heteronuclear single quantum coherence; MCCE, multi-conformer continuum electrostatics; PDB, Protein Data Bank.

Table 1. Comparison of pK_a values determined by linkage analysis and NMR spectroscopy

Variant	Residue	Linkage analysis pK_a	±	NMR pK_a	±
Δ+PHS/L38E	Glu38	7.0	0.3	7.0	0.1
Δ+PHS/L38E/E122Q	Glu38	—	—	6.2	0.1
Δ+PHS/L38D	Asp38	6.8	0.3	7.2	0.1
Δ+PHS/L38D/E122Q	Asp38	6.9	0.3	6.6	0.1

—, pK_a could not be determined using linkage analysis because variant unfolds in an apparent three-state manner.

Ex. 2045 at 37 (Table 1)
Patent Owner's Resp. at 53-54; Sur-Reply at 16; Ex. 2024 ¶ 185

Intrachain Hydrogen Bonding Can Affect pKa

BLAKE ROBERT PETERSON , Ph.D.

Q. How does the formation of intrachain hydrogen bonds affect the pKa of the involved amino acids?

***

THE WITNESS: Intrachain amino acids can -- intrachain interactions, I should say, can affect pKas, as they describe.

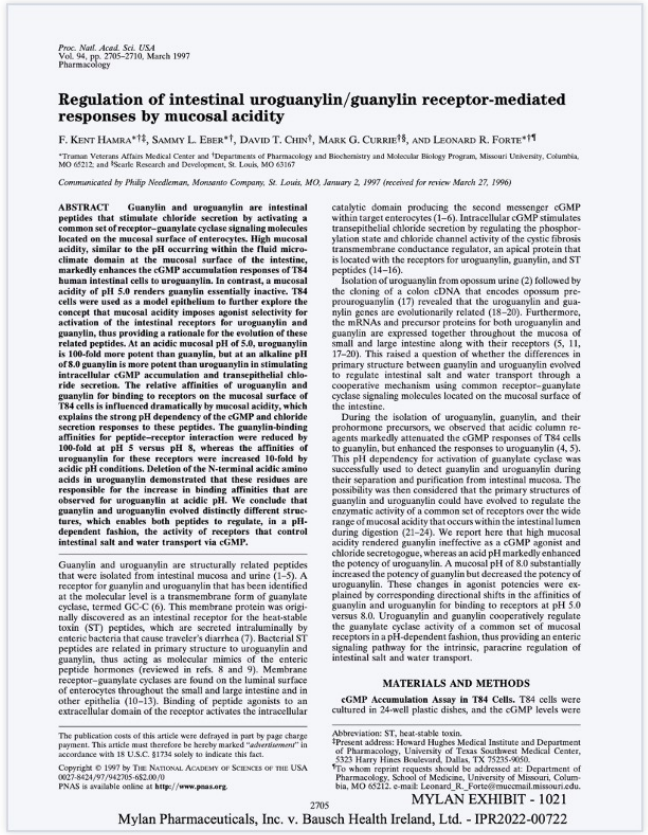
Q. In what ways does the formation of intrachain hydrogen bonds impact the modification of a lead compound for peptide drug development?

***

A. My answer is if an amino acid can form an intrachain amino acid, that could influence its pKa, and that might factor into drug development.

Ex. 2069 at 96:19-97:2, 98:12-22
Sur-Reply at 15-16; Ex. 2024 ¶¶ 173-182

Petitioner's Arguments re Increased pH Are Unfounded



Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 2705–2710, March 1997
Pharmacology

Regulation of intestinal uroguanylin/guanylin receptor-mediated responses by mucosal acidity

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Communicated by Philip Needleman, Monsanto Company, St. Louis, MO, January 2, 1997 (received for review March 27, 1996)

ABSTRACT **Guanylin and uroguanylin are intestinal peptides that stimulate chloride secretion by activating a common set of receptor–guanylate cyclase signaling molecules located on the mucosal surface of enterocytes. High mucosal acidity, similar to the pH occurring within the fluid microclimate domain at the mucosal surface of the intestine, markedly enhances the cGMP accumulation responses of T84 human intestinal cells to uroguanylin. In contrast, a mucosal acidity of pH 5.0 renders guanylin essentially inactive. T84 cells were used as a model epithelium to further explore the concept that mucosal acidity imposes agonist selectivity for activation of the intestinal receptors for uroguanylin and guanylin, thus providing a rationale for the evolution of these related peptides. At an acidic mucosal pH of 5.0, uroguanylin is 100-fold more potent than guanylin, but at an alkaline pH of 8.0 guanylin is more potent than uroguanylin in stimulating intracellular cGMP accumulation and transepithelial chloride secretion. The relative affinities of uroguanylin and guanylin for binding to receptors on the mucosal surface of T84 cells is influenced dramatically by mucosal acidity, which explains the strong pH dependency of the cGMP and chloride secretion responses to these peptides. The guanylin-binding affinities for peptide–receptor interaction were reduced by 100-fold at pH 5 versus pH 8, whereas the affinities of uroguanylin for these receptors were increased 10-fold by acidic pH conditions. Deletion of the N-terminal acidic amino acids in uroguanylin demonstrated that these residues are responsible for the increase in binding affinities that are observed for uroguanylin at acidic pH. We conclude that guanylin and uroguanylin evolved distinctly different structures, which enables both peptides to regulate, in a pH-dependent fashion, the activity of receptors that control intestinal salt and water transport via cGMP.**

Guanylin and uroguanylin are structurally related peptides that were isolated from intestinal mucosa and urine (1–5). A receptor for guanylin and uroguanylin that has been identified at the molecular level is a transmembrane form of guanylate cyclase, termed GC-C (6). This membrane protein was originally discovered as an intestinal receptor for the heat-stable toxin (ST) peptides, which are secreted intraluminally by enteric bacteria that cause traveler's diarrhea (7). Bacterial ST peptides are related in primary structure to uroguanylin and guanylin, thus acting as molecular mimics of the enteric peptide hormones (reviewed in refs. 8 and 9). Membrane receptor–guanylate cyclases are found on the luminal surface of enterocytes throughout the small and large intestine and in other epithelia (10–13). Binding of peptide agonists to an extracellular domain of the receptor activates the intracellular catalytic domain producing the second messenger cGMP within target enterocytes (1–6). Intracellular cGMP stimulates transepithelial chloride secretion by regulating the phosphorylation state and chloride channel activity of the cystic fibrosis transmembrane conductance regulator, an apical protein that is located with the receptors for uroguanylin, guanylin, and ST peptides (14–16).

Isolation of uroguanylin from opossum urine (2) followed by the cloning of a colon cDNA that encodes opossum prouroguanylin (17) revealed that the uroguanylin and guanylin genes are evolutionarily related (18–20). Furthermore, the mRNAs and precursor proteins for both uroguanylin and guanylin are expressed together throughout the mucosa of small and large intestine along with their receptors (5, 11, 17–20). This raised a question of whether the differences in primary structure between guanylin and uroguanylin evolved to regulate intestinal salt and water transport through a cooperative mechanism using common receptor–guanylate cyclase signaling molecules located on the mucosal surface of the intestine.

During the isolation of uroguanylin, guanylin, and their prohormone precursors, we observed that acidic column reagents markedly attenuated the cGMP responses of T84 cells to guanylin, but enhanced the responses to uroguanylin (4, 5). This pH dependency for activation of guanylate cyclase was successfully used to detect guanylin and uroguanylin during their separation and purification from intestinal mucosa. The possibility was then considered that the primary structures of guanylin and uroguanylin could have evolved to regulate the enzymatic activity of a common set of receptors over the wide range of mucosal acidity that occurs within the intestinal lumen during digestion (21–24). We report here that high mucosal acidity rendered guanylin ineffective as a cGMP agonist and chloride secretogogue, whereas an acid pH markedly enhanced the potency of uroguanylin. A mucosal pH of 8.0 substantially increased the potency of guanylin but decreased the potency of uroguanylin. These changes in agonist potencies were explained by corresponding directional shifts in the affinities of guanylin and uroguanylin for binding to receptors at pH 5.0 versus 8.0. Uroguanylin and guanylin cooperatively regulate the guanylate cyclase activity of a common set of mucosal receptors in a pH-dependent fashion, thus providing an enteric signaling pathway for the intrinsic, paracrine regulation of intestinal salt and water transport.

MATERIALS AND METHODS

cGMP Accumulation Assay in T84 Cells. T84 cells were cultured in 24-well plastic dishes, and the cGMP levels were

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0027-8424/97/942705-6$2.00/0
PNAS is available online at http://www.pnas.org.

Abbreviation: ST, heat-stable toxin.

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¶To whom reprint requests should be addressed at: Department of Pharmacology, School of Medicine, University of Missouri, Columbia, MO 65212. e-mail: Leonard_R_Forte@mucemail.missouri.edu.

secretion responses to these peptides. **The guanylin-binding affinities for peptide–receptor interaction were reduced by 100-fold at pH 5 versus pH 8, whereas the affinities of uroguanylin for these receptors were increased 10-fold by acidic pH conditions.** Deletion of the N-terminal acidic amino

Ex. 1021 at 2705 (Abstract)
Patent Owner's Resp. at 48; Ex. 2024 ¶ 44

Petitioner's Arguments re Increased pH Are Unfounded

MICHAEL SAMUEL EPSTEIN, M.D.

n colonic mucosa contains uroguanylin
nylin peptides

ENT HAMRA, WILLIAM J. KRAUSE, SAMMY L. EBER, RONALD H. FREEMAN.

Q. So are you aware of any pharmacologic therapies available as of January 17th, 2002 that acted only in the small intestine?

***

THE WITNESS: Not that I can answer for you.

Q. Is that a "no"?

***

THE WITNESS: I would say I don't know of any specifically on the top of my head.

monophosphate (cGMP) (7, 18). All species of mammals and birds examined express GC-C-like receptor activity on the apical surface of enterocytes throughout the intestine (21, 22). The opossum kidney also expresses high levels of GC-C-like receptors located in the apical membrane of proximal tubular cells (14). Guanylin was first isolated from rat jejunum as a heat-stable, 15-amino acid peptide that activated GC-C in human intestinal T84 cells (7). Guanylin cDNAs encoding 115- to 116-amino acid precursors have been isolated from rat, human, and mouse intestine (19). Uroguanylin was initially purified as 13- to 15-amino acid peptides from

has only been isolated from urine, and little is known regarding the tissues that may produce this peptide (10, 18, 20). The high levels of uroguanylin found in the urine of opossums, humans, and rats could be derived from the kidney and/or from other tissues via filtration of uroguanylin from the circulation. In the current study, we have isolated uroguanylin and guanylin peptides from the colonic mucosa of opossums. Several independent analytical techniques were used to identify the bioactive peptides. Thus intestinal mucosa is a potential tissue source for uroguanylin and guanylin found in urine (10, 18, 20).

G708 0193-1857/96 $5.00 Copyright © 1996 the American Physiological Society

MYLAN EXHIBIT - 1019 (Corrected)
Mylan Pharmaceuticals, Inc. v. Bausch Health Ireland, Ltd.

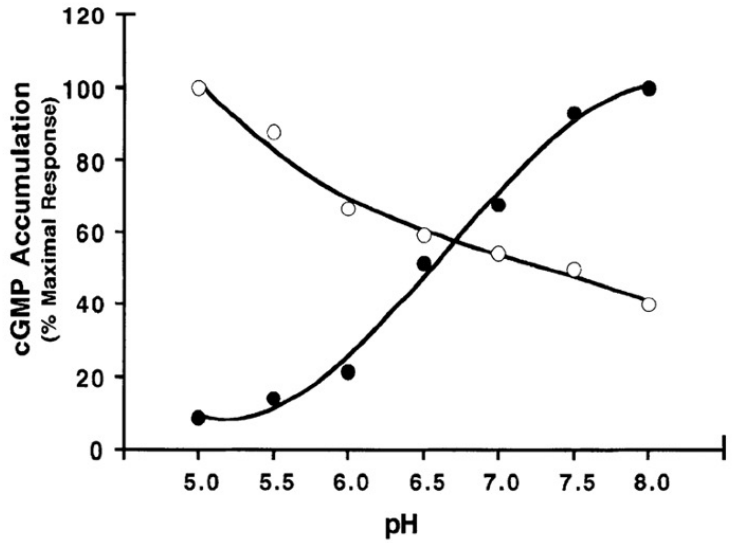

Fig. 1. Effects of medium pH on uroguanylin (○) and guanylin (●)-stimulated guanosine 3′,5′-cyclic monophosphate (cGMP) accumulation in T84 cells. Vehicle, 30 nM synthetic opossum uroguanylin, and 30 nM synthetic opossum guanylin were suspended in buffered assay medium previously adjusted to pH values indicated, as described in MATERIALS AND METHODS. Levels of T84 cell cGMP accumulation (pmol/well, average of 3 wells) elicited by vehicle and peptides in this experiment when tested at pH 5.0 and pH 8.0, respectively, were as follows: basal (vehicle control) = 0.45 and 0.78, uroguanylin = 43.9 and 17.5, and guanylin = 0.85 and 10.0. Data are representative of 4 experiments with similar results.

Ex. 1019 at G710, Fig. 1; Ex. 2070 at 52:6-16
Patent Owner's Resp. at 54; Sur-Reply at 9; Ex. 2024 ¶ 45; Ex. 2025 ¶ 38

Petitioner's Arguments re Increased pH Are Unfounded

PRP Pharmacology Research & Perspectives Open Access

ORIGINAL ARTICLE

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

Andrea Brancale[1], Kunwar Shailubhai[2], Salvatore Feria[1], Antonio Ricci[1], Marcella Bassetto[1] & Gary S Jacob[2]

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

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

doi: 10.1002/prp2.295

Abstract

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

Abbreviations

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

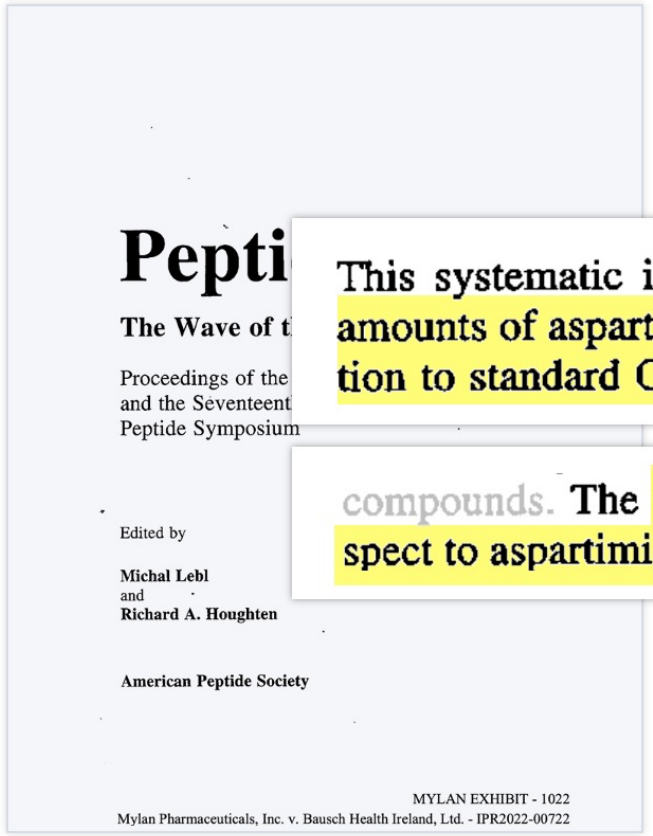
Introduction

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

As an analog of the pathological GC C agonist STh, linaclotide maintains many structural features of STh, including the presence of three disulfide bonds and an insensitivity to pH. MD simulations in this study show that the addition of a third intramolecular bond makes both STh and linaclotide insensitive to MD perturbations (Ozaki et al. 1991). The structural similarity of these two molecules is reflected by the low RMSD values of 1.28 Å for linaclotide. The amino acid substitutions that differen tiate linaclotide from STh further enhance the pharma cokinetic stability and proteolytic resistance of linaclotide, allowing it to remain active across a longer portion of the small intestine (Bharucha and Waldman 2010; Harris and Crowell 2007). The absence of pH sensing amino acid residues would additionally give these molecules maxi mum biological activity across the range of pH environ ments in the GI tract. This lack of focused areas of

Ex. 2019 at 7
Patent Owner's Resp. at 55

Aspartimide Formation Can Be Avoided Through Protecting Groups



Pepti

The Wave of t

Proceedings of the
and the Seventeent
Peptide Symposium

Edited by

Michal Lebl
and
Richard A. Houghten

American Peptide Society

MYLAN EXHIBIT - 1022
Mylan Pharmaceuticals, Inc. v. Bausch Health Ireland, Ltd. - IPR2022-00722

This systematic investigation clearly showed that in our test system, no detectable amounts of aspartimide were formed if Hmb-backbone protection was applied in addition to standard OtBu-protection of the Asp side chain. However, the synthesis of all

compounds. The OMpe-protecting group showed a significant improvement with respect to aspartimide formation when compared to regular OtBu-protection. Most inter-

Ex. 1022 at 64
Patent Owner's Resp. at 56-57; Ex. 2024 ¶¶ 198-199

Aspartimide Formation Can Be Avoided Through Protecting Groups

Letters in Peptide Science, **7**: 107–112, 2000.
KLUWER/ESCOM

Base-induced side reactions in Fmoc-solid phase peptide synthesis: Minimization by use of piperazine as N^{α}-deprotection reagent*

John D. Wade**, Marc N. Mathieu, Mary Macris & Geoffrey W. Tregear
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Received 1 November 1999; Accepted 24 November 1999

Key words: aspartimide formation, base-induced side reaction, Fmoc-solid phase peptide synthesis, N^{α}-deprotection reagent, piperazine

Summary

Base-induced aspartimide (cyclic imide) and subsequent base adduct formation in the Fmoc-solid phase synthesis of sensitive sequences are serious side reactions that are difficult to both anticipate and control. The effect of extended treatment of piperazine as N^{α}-Fmoc deprotection reagent on two sensitive peptide sequences was examined. For comparison, other bases were also investigated, including piperidine, 1-hydroxypiperidine, tetrabutylammonium fluoride, and 1,8-diazabicyclo[5.4.0]undec-7-ene. The results showed that all bases induced varying degrees of both aspartimide and, in some cases, base adduct formation, although piperazine caused the least side reaction. Use of *N*-(2-hydroxy-4-methoxybenzyl) peptide backbone amide protection was confirmed to confer complete protection against side reaction. In the absence of such protection, for all bases, the use of 1-hydroxybenzotriazole as additive had some, but not complete, beneficial effect in further reducing side reaction. Best results were obtained with piperazine containing 0.1M 1-hydroxybenzotriazole indicating that this reagent merits serious consideration for N^{α}-deprotection in the Fmoc-solid phase synthesis of base-sensitive sequences. A further advantage of this reagent is that it causes little racemisation of resin-bound C-terminal cysteine, an occasionally serious base-mediated problem in Fmoc-solid phase assembly.

Introduction

Aspartimide (cyclic imide) formation is a long-recognized side reaction that can occur both during solid phase peptide synthesis (SPPS) and storage of peptides, and may be either acid- or base-catalyzed [1]. Numerous studies on the mechanism of the reaction have shown it to be dependent on the nature of the acid or base, and the residue adjoining the carboxyl of the aspartate as well as the side chain protecting group used [1]. Imide formation was originally thought not to occur in Fmoc-SPPS. However, several recent studies have shown it to be a significant side reaction and one that is highly sequence and conformation dependent [2–4]. The problem is not confined exclusively to Asp-X sequences, for there has also been a report of Asn-X cyclization [5]. An additional side reaction now known to be associated with sensitive Asp-X sequences is subsequent modification of the imide by nucleophilic base to produce a base adduct (Figure 1). Several palliative measures for controlling imide and adduct formation have been recommended. These include addition to the N^{α}-Fmoc deprotection reagent of choice, piperidine, of agents such as 1-hydroxybenzotriazole (HOBt), but none completely suppress side reaction [2,6,7]. Aspartyl side chain protecting groups other than the commonly employed *tert*-butyl ester have also been reported to give improved yields of α-aspartyl peptides through increased steric hindrance. These include 1-adamantyl and β-3-methylpent-3-yl esters [2,8]. However, these are either not entirely compatible with Fmoc-SPS or commercially unavailable. The sole effective preventive measure to date is the use of Asp-X amide bond protec-

* A preliminary account of this work was presented at the 25th European Peptide Symposium, Budapest, Hungary, 1998.
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Conclusions

In the absence of prior information regarding the susceptibility of a new peptide sequence to modification during Fmoc-solid phase synthesis, it is recommended that – where feasible – Asp-X pairs be routinely protected with the Hmb moiety. Should this not be possible, then piperazine containing 0.1M HOBt is a practical and effective alternative.

Ex. 1023 at 111
Patent Owner's Resp. at 56-57; Ex. 2024 ¶¶ 198, 200

Aspartimide Formation Can Be Avoided Through Protecting Groups

Letters in Peptide Science, 1 (1994) 197–205 197
ESCOM

LIPS 026

Sequence dependence of aspartimide formation during 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis

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Received 31 January 1995
Accepted 23 February 1995

Key words: Aspartimide; Fmoc solid-phase peptide synthesis; Piperidides; Side reactions

SUMMARY

We have examined the sequence dependence of aspartimide formation during Fmoc-based solid-phase synthesis of the peptide Val-Lys-Asp-X-Tyr-Ile. The extent of aspartimide formation and subsequent conversion to the α- or β-piperidide was characterized and quantitated by analytical reversed-phase high-performance liquid chromatography and fast atom bombardment mass spectrometry. Aspartimide formation occurred for X = Arg(Pmc), Asn(Trt), Asp(*Ot*Bu), Cys(Acm), Gly, Ser, Thr and Thr(*t*Bu). No single approach was found that could inhibit this side reaction for all sequences. The most effective combinations, in general, for minimization of aspartimide formation were (i) *tert*-butyl side-chain protection of aspartate, piperidine for removal of the Fmoc group, and either 1-hydroxybenzotriazole or 2,4-dinitrophenol as an additive to the piperidine solution; or (ii) 1-adamantyl side-chain protection of aspartate and 1,8-diazabicyclo[5.4.0]undec-7-ene for removal of the Fmoc group.

INTRODUCTION

Successful solid-phase syntheses are dependent upon highly efficient coupling/deprotection cycles and minimization of deleterious side reactions. The cyclization of aspartate to form aspartimide has long been recognized as a substantial side reaction occurring during both synthesis and storage of peptides [1–3]. Aspartimide formation during peptide synthesis can be either acid or base catalyzed, with the kinetics of ring closure depending upon the nature and strength of the acid or base, the structure of the aspartate side-chain protecting group, and the aspartate carboxyl neighboring residue. Extensive studies have been published of aspartimide formation during *tert*-butyloxycarbonyl (Boc)-based peptide synthesis which have focused on protecting group strategies [4,5], sequence dependence [6], and the nature and strength of the acid or tertiary base [5,7]. These studies found that aspartimide formation can be adequately suppressed by additives such as 1-hydroxybenzotriazole (HOBt) or 2,4-dinitrophenol (Dnp) during base neutralization [8] or by using 2-adamantyl (2-Ada) or cyclohexyl side-chain protection of aspartate instead of benzyl (Bzl) protection [4,5].

It had been assumed that for 9-fluorenyl-

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alkyl amino acid prior to Fmoc-Asp(*Ot*Bu). Based on our study, the most effective combinations for minimization of aspartimide formation were (i) *t*Bu side-chain protection of aspartate, piperidine for removal of the Fmoc group, and either HOBt or Dnp as an additive to the piperidine solution; or (ii) 1-Ada side-chain protection of aspartate and DBU for removal of the Fmoc group.

Ex. 1024 at 204
Patent Owner's Resp. at 56-57; Ex. 2024 ¶¶ 198, 201-202

Aspartimide Formation Can Be Avoided Through Protecting Groups

BLAKE ROBERT PETERSON , Ph.D.

Q. How can aspartimide formation in peptides be reduced or avoided using protecting groups?

THE COURT REPORTER: I'm sorry, protecting?

THE WITNESS: Protecting groups.

THE COURT REPORTER: Thank you.

MR. HASFORD: Protecting groups.

THE WITNESS: One can reduce side reactions in chemical synthesis in general by using protecting groups in some cases. They -- they can limit the undesired pathways that lead to undesired products.

Ex. 2069 at 22:3-12
Sur-Reply at 17; Ex. 2024 ¶¶ 198-203

Objective Evidence Supports the Nonobviousness of the Claims

Federal Circuit: Objective Evidence of Nonobviousnses

Objective evidence . . . must be considered before a conclusion on obviousness is reached and is not merely "icing on the cake."

Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1380 (Fed. Cir. 1986)
Patent Owner's Resp. at 26-27, 57

Plecanatide's Unexpected Stabilization Against Interconversion

STUDIES ON SP-304 THERMOSTABILITY, pH DEPENDENCY AND TOPOISOMERIC STABILITY

Study Number:	SP-PH-004
Test Article:	SP-304
Author:	Kunwar Shailubhai Senior Vice President, Discovery Synergy Pharmaceuticals, Inc.
Study Dates:	November 24, 2001 to December 25, 2001
Testing Facility:	R&D Center, Synergy Pharmaceuticals, Inc., Norristown, PA
Final Report Date:	February 15, 2008
Source Data:	Synergy Pharmaceuticals Lab Notebook # 2; Pages: 170-185 Of Dr. Surendra Dheer

Synergy Pharmaceuticals, Inc.
420 Lexington Ave. - Suite 1609
New York, NY 10170

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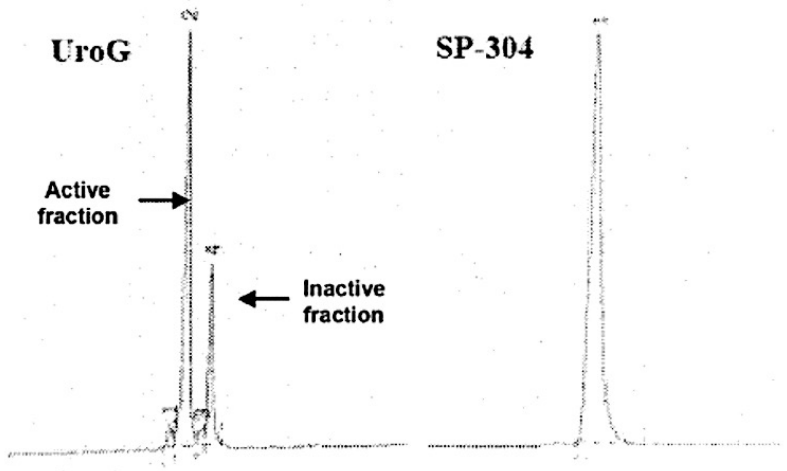
HIGHLY CONFIDENTIAL INFORMATION TRUL00018260

PROTECTIVE ORDER MATERIAL

Figure 1: HPLC Chromatographs of Uroguanylin and SP-304 Following 16-Hour Incubations at pH 3.0 in Aqueous Media at 37°C



Ex. 2028 at TRUL00018269 (Fig. 1)
Patent Owner's Resp. at 59; Ex. 2024 ¶¶ 206-215

Marx: Topoisomerism Not Affected by *N*-Terminal Region

U.C. Marx
J. Klodt
M. Meyer
H. Gerlach
P. Rösch
W.-G. Forssmann
K. Adermann

One peptide, two topologies: structure and interconversion dynamics of human uroguanylin isomers

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ISSN 1397-002X

Key words: guanylin; heat-stable enterotoxin; isomerization; solution structure; topological stereoisomer; uroguanylin

Abstract: The peptide hormone uroguanylin stimulates chloride secretion via activation of intestinal guanylyl cyclase C (GC-C). It is characterized by two disulfide bonds in a 1–3/2–4 pattern that causes the existence of two topological stereoisomers of which only one induces intracellular cGMP elevation. To obtain an unambiguous structure-function relationship of the isomers, we determined the solution structure of the separated uroguanylin isoforms using NMR spectroscopy. Both isomers adopt well-defined structures that correspond to those of the isomers of the related peptide guanylin. Furthermore, the structure of the GC-C-activating uroguanylin isomer A closely resembles the structure of the agonistic *Escherichia coli* heat-stable enterotoxin. Compared with guanylin isomers, the conformational interconversion of uroguanylin isomers is retarded significantly. As judged from chromatography and NMR spectroscopy, both uroguanylin isoforms are stable at low temperatures, but are subject to a slow pH-dependent mutual isomerization at 37°C with an equilibrium isomer ratio of approximately 1:1. The conformational exchange is most likely under the sterical control of the carboxy-terminal leucine. These results imply that GC-C is activated by ligands exhibiting the molecular framework corresponding to the structure of uroguanylin isomer A.

Abbreviations: cGMP, cyclic 3′,5′-guanosine monophosphate; Clean-TOCSY, TOCSY with suppression of NOESY-type cross peaks; DG, distance geometry; DQF-COSY, double-quantum filtered COSY; DSS, 2,2-dimethyl-silapentane-5-sulfonic acid; GC-C, guanylyl cyclase C; JR-NOESY, 2D NOESY spectrum acquired with a jump-return observe pulse; MD, molecular dynamics; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect, also used for NOESY cross peak; NOESY, NOE spectroscopy; RMSD, root-mean-

The slow development of the equilibrium between uroguanylin isomers at alkaline pH indicates that the ion ization state of the isomeric molecules strongly influences the kinetics of transition between the isomers of uroguanylin 16 and uroguanylin 24. Thus, the terminal carboxyl, ionizable side-chains of Asp2, Asp3 and Glu5, or those groups able to form intrachain hydrogen bonds, may be involved in the control of stabilization of the two isomers. After 3 days at alkaline pH, both isoforms decomposed because of disulfide exchange. Comparison of the conversion of uroguanylin 16 isomers with the isomers of the N-terminally extended uroguanylin-24 resulted in identical kinetics for isoforms A and B at a pH of 4.5 and 7.7 for both peptides (Fig. 6C). This result clearly demonstrates that the isomerization is not affected by the amino-terminal region of uroguanylin. Corresponding to the

Ex. 2010 at 236
Patent Owner's Resp. at 59; Sur-Reply at 26; *see also* Ex. 2024 ¶¶ 37, 147, 210-214

Fiser: Asp Preferred Over Glu for Stabilization

FEBS 17787

FEBS Letters 397 (1996) 225-229

Conservation of amino acids in multiple alignments: aspartic acid has unexpected conservation

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Received 18 July 1996; revised version received 25 September 1996

Abstract Analysis of the relationship between surface accessibility and amino acid conservation in multiple sequence alignments of homologous proteins confirms expected trends for hydrophobic amino acids, but reveals an unexpected difference between the conservation of Asp, Glu and Gln. Even when not in an active site, Asp is more highly conserved than Glu. There is a clear preference for conserved and buried Asp to be present in coil, but there is no tendency for Asp to conserve ϕ/ψ in the ++ region of the Ramachandran map. Glu does not show any preference to be conserved in a particular secondary structure. Analysis of recently derived substitution matrices (e.g. BLOSUM) confirms that Glu tends to substitute more frequently with other amino acids than does Asp. Analysis of relative accessibility versus relative conservation for individual amino acid positions in alignments shows a negative correlation for all amino acid types. With the exception of Arg, Lys, Gly, Glu, Asp and Tyr, a relative conservation of > 2 suggests the amino acid will have a relative accessibility of $< 50\%$. Observation of conserved Cys, Gly or Asp in a reliable multiple alignment suggests a position important for the structure of the protein. Furthermore, the Asp is likely to be involved in polar interactions through its side chain oxygen atoms. In contrast, Gln is the least conserved amino acid overall.

Key words: Conservation analysis; Multiple sequence alignment; Protein structure prediction

1. Introduction

Knowledge of protein sequences is growing much faster than knowledge of either three-dimensional structure or function. Accordingly, the interpretation of sequence data to identify structurally or functionally important residues is essential if the data are to be effective in furthering understanding of biological systems. Multiple sequence alignments of families of protein sequences are now used routinely to indicate residues of key importance to the function of the protein. A position in an alignment that has identical residues in all members of a protein family may have a key catalytic role. A position where similar physico-chemical properties (e.g. hydrophobicity) are shared may suggest importance in stabilising the native conformation of the protein [1,2]. Identification of such conserved features in multiple alignments has been used to good effect to improve the accuracy of prediction of secondary structure and buried residues (α-helix and β-strand) (e.g. [3–7]).

Here we report a systematic study of residue conservation in multiple alignments where at least one protein is of known tertiary structure. Our analysis complements that of Overington et al. [8] who considered only pairwise substitution frequencies for amino acids in structurally aligned families.

2. Materials and methods

2.1. Data base

A non-redundant set of 81 proteins was generated from the April 1993 release of the Brookhaven Protein Data Bank (PDB) [9]. The set was chosen in a two-step procedure. First, all pairs of chains (over 50 residues and resolution better than 2.5 Å) in the data bank were compared by calculating correlation coefficients between the dipeptide frequencies in each protein. A set of 101 protein chains was selected such that all pairs had a correlation of < 0.4. All pairs in this set were then compared by a rigorous sequence comparison method [10,11] followed by cluster analysis. This reduced the set to 81 protein chains that show no obvious sequence similarity (PDB code and chain identifiers: 1SSC 1ACX 1ALC 1BBP_A 1CC5 1ECA 1FKF 1FNR 1GCR 1GP1_A 1HDSB 1HIP 1HOE 1LRD_A 1PAZ 1PCY 1PHH 1PRC_C 1RBP 1RHD 1RNH 1SN3 1TGS 1TPK_A 1WSY_B 256B_A 2ALP 2AZA_A 2CAB 2CD4 2CDV 2CPP 2FXB 2GN5 2LH7 2LIV 2LTN_A 2ORI_L 2PAB_A 2RNT 2RSP_A 2SEC_I 2SNL_E 2SNS 2SOD_B 2SSI 2STV 2TSI 2UTG_A 3ADK 3BSC 3CLA 3FXC 3GAP_B 3LZM 3SGB_I 451C 4BP2 4FD1 4FXN 4HHB_A 4PEP 4PFK 4PTP 4TNC 5CTS 5CYT_R 5EBX 5RUB_A 5RXN 6LDH 6TMN_E 7PTI 8ADH 8ATC_B 8CAT_A 8DFR 9PAP 9RSA_A 9WGA_A).

Each protein in the set was compared by the Smith-Waterman algorithm [11,12] to the NBRF-PIR sequence data bank (Release 38) and all sequences that gave probability values of $< 10^{-5}$ by a length-dependent scoring scheme (program SCANPS ftp://geoff.biop.ox.ac.uk/programs/scanps) were multiply aligned with the query sequence by the algorithm of Barton and Sternberg [13]. This gave 81 alignments with between 3 and 499 sequences in each (median of 28 sequences).

2.2. Calculation of conservation and accessibility

Conservation scores based upon the physico-chemical properties of the amino acids were calculated for each position in each alignment according to Livingstone and Barton [2]. Conservation scores range from 0 to 10 and represent the number of the properties: Hydrophobic, Positive, Negative, Polar, Charged, Small, Tiny, Aliphatic, Aromatic, Proline and their negations (e.g. *not* positive) that are shared at a position. The program AMAS [2], which calculates conservation values from a multiple alignment, may be run over the World Wide Web (http://geoff.biop.ox.ac.uk/servers/amas-server.html).

Although conservation scores are absolute, the relative importance of a conservation score is dependent on the overall similarity between the sequences in the multiple alignment. For example, in an alignment of 20 sequences that all share $> 90\%$ pairwise identity conservation scores above 8 may be interesting. In contrast, if the pairwise identity is below 30% then lower conservation scores will be informative. Accordingly, in this study we normalised conservation scores by the average conservation for each alignment to give relative conservation scores C_r. We refer to a position as 'conserved' if $C_r > 1$.

Accessible surface areas were calculated by the program DSSP [14] and converted to relative accessibilities by dividing by the accessibility of the residue in a Gly-X-Gly tripeptide [15]. Two relative accessibility classes were considered $0 \leq A \leq 0.25$ (buried) and $0.25 < A \leq max(A)$ (exposed).

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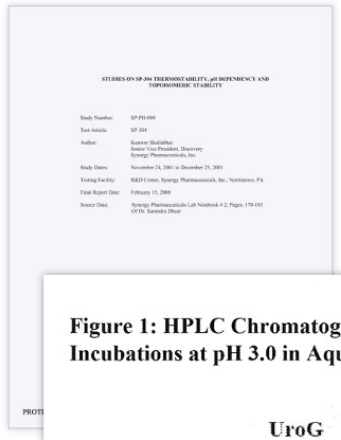
PII S0014-5793(96)01181-7

Since our data do not suggest a significant preference for ++ ϕ/ψ, the preferred conservation of Asp is likely to be due to differing side-chain interactions. The most obvious hypothesis is that since Glu has a higher proportion of non-polar atoms than Asp it can make more non-specific interactions and so there are fewer constraints on its environment. In

Why, then, is Asp most highly conserved when buried in coil? The short Asp side chain is restricted in mobility yet able to make strong polar interactions. It is possible that Asp may form a 'pin' that stabilises non-regular structures in loops.

Ex. 2036 at 227, 228
Patent Owner's Resp. at 59; *see also* Ex. 2024 ¶¶ 105, 147, 153

Patent Owner Did Not Use Wild-Type Human Uroguanylin



STUDIES ON SP-304 THERMOSTABILITY, pH DEPENDENCY AND TOPOISOMERIC STABILITY

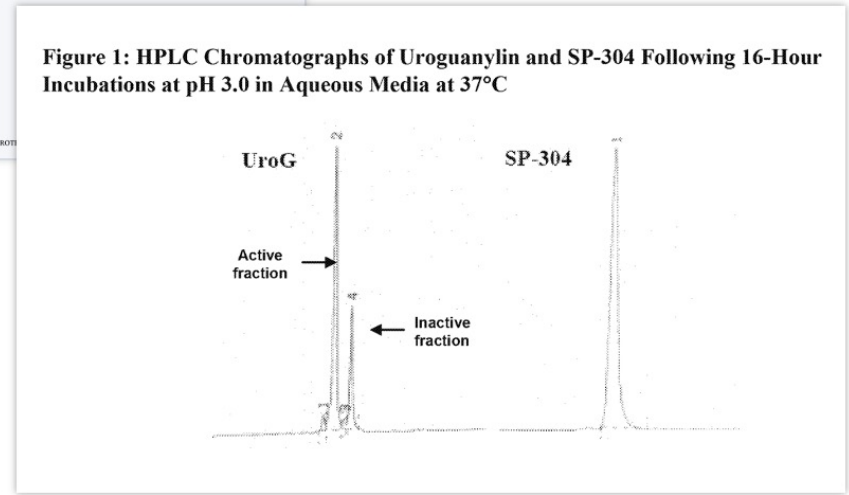
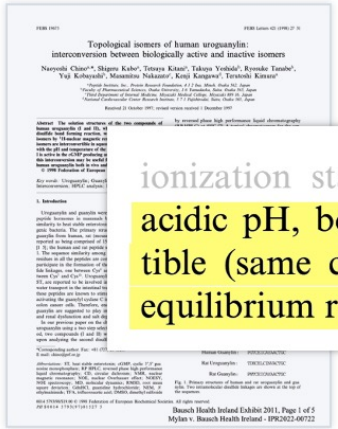


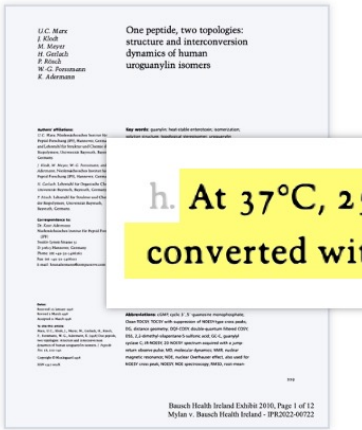
Figure 1: HPLC Chromatographs of Uroguanylin and SP-304 Following 16-Hour Incubations at pH 3.0 in Aqueous Media at 37°C



Topological isomers of human uroguanylin: interconversion between biologically active and inactive isomers

Bausch Health Ireland Exhibit 2011, Page 1 of 5
Mylan v. Bausch Health Ireland - IPR2022-00722

ionization state of functional group(s) in the molecule. At acidic pH, both compounds are, in one sense, freely convertible (same conversion rates) and eventually come to a 1:1 equilibrium ratio. In contrast, conversions of both compounds



One peptide, two topologies: structure and interconversion dynamics of human uroguanylin isomers

Bausch Health Ireland Exhibit 2010, Page 1 of 12
Mylan v. Bausch Health Ireland - IPR2022-00722

h. At 37°C, 25% of both uroguanylin-16 isomers are interconverted within 24 h. This and conversion experiments

Ex. 2028 at TRUL00018269 (Fig 1); Ex. 2010 at 236, Ex. 2011 at 30
Sur-Reply at 19; *see also* Patent Owner's Resp. at 59; Ex. 2024 ¶¶ 70, 131, 206-215

Petitioner's Uncorroborated Data Do Not Include Human Uroguanylin as a Comparator

Beschwerdekammern
Boards of Appeal
Chambres de recours

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Datum/Date
11.06.13

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°
I17839 OPPO01	02721604.3 / 1379224
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
Synergy Pharmaceuticals, Inc.	

Appeal number: T1366/12-3.3.04

Please find enclosed a copy of the decision dated 04.06.13.

The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Annex(es): ☑ Acknowledgement of receipt - EPO Form 2936
☐

Registered letter with advice of delivery

EPO Form 3032 01/11

MYLAN EXHIBIT - 1067 Pg. 001
Mylan Pharmaceuticals, Inc. v. Bausch Health Ireland, Ltd.
IPR2022-00722

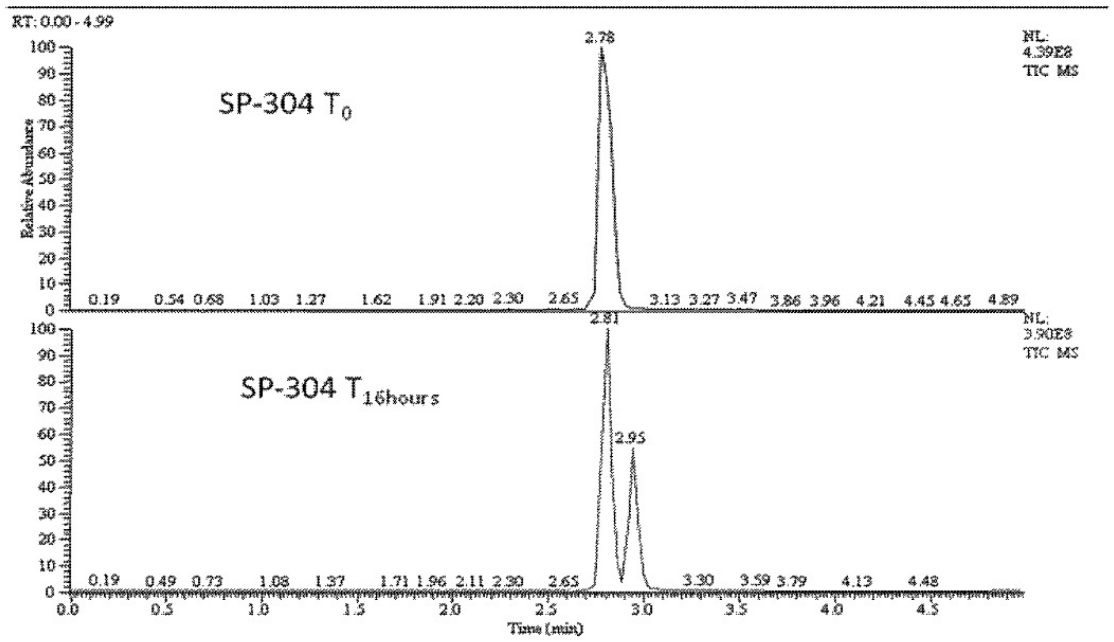
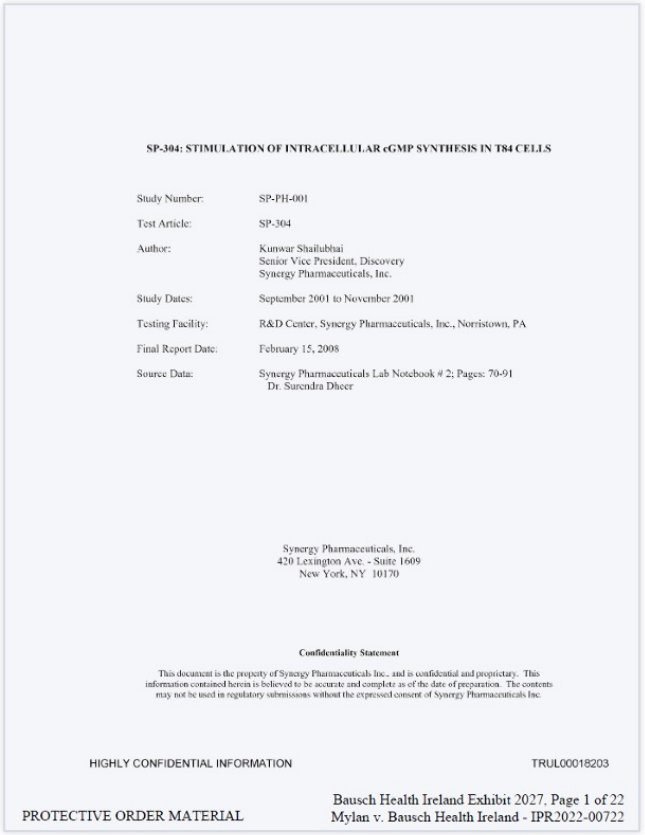


Figure 1

Ex. 1067 at 91
Sur-Reply at 25-26; *see also* Patent Owner's Resp. at 59; Ex. 2024 ¶¶ 206-215

Plecanatide's Significantly and Unexpectedly Superior Potency



SP-304: STIMULATION OF INTRACELLULAR cGMP SYNTHESIS IN T84 CELLS

Study Number: SP-PH-001

Test Article: SP-304

Author: Kunwar Shailubhai
Senior Vice President, Discovery
Synergy Pharmaceuticals, Inc.

Study Dates: September 2001 to November 2001

Testing Facility: R&D Center, Synergy Pharmaceuticals, Inc., Norristown, PA

Final Report Date: February 15, 2008

Source Data: Synergy Pharmaceuticals Lab Notebook # 2; Pages: 70-91
Dr. Surendra Dheer

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PROTECTIVE ORDER MATERIAL

Bausch Health Ireland Exhibit 2027, Page 1 of 22
Mylan v. Bausch Health Ireland - IPR2022-00722

Table 1. Effects of SP-304, Uroguanylin, and Other Test Peptides in the T84 cGMP Stimulation Bioassay

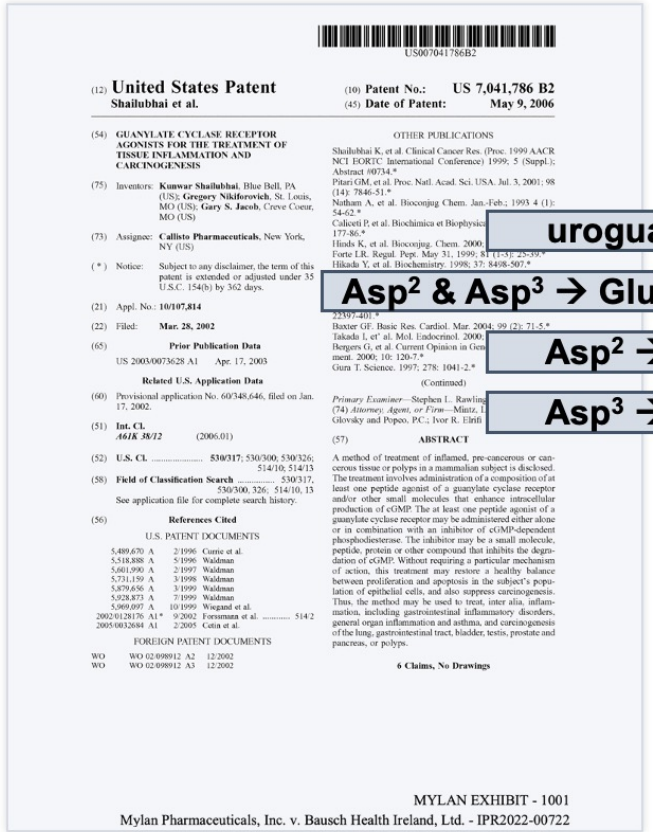
Test Material	Concentration		cGMP Levels (pmol/well) *	EC_{50} **	
	Molar	ng/mL		Molar	ng/mL
SP-301 (uroguanylin)	0	0	0	~~10^{-6} M~~ 2.3×10^{-7}M	~~1668~~ 383.6
	10^{-9} M	1.668	0		
	10^{-8} M	16.668	12		
	10^{-7} M	166.8	82		
	10^{-6} M	1668	205		
	10^{-5} M	16680	254		
SP-302	0	0	0	~~10^{-6} M~~ 3.5×10^{-7}M	~~1696~~ 593.6
	10^{-9} M	1.696	0		
	10^{-8} M	16.96	8		
	10^{-7} M	169.6	62		
	10^{-6} M	1696	185		
	10^{-5} M	16960	248		
SP-303	0	0	0	~~10^{-6} M~~ 2.4×10^{-7}M	~~1682~~ 403.7
	10^{-9} M	1.682	0		
	10^{-8} M	16.82	12		
	10^{-7} M	168.2	82		
	10^{-6} M	1682	195		
	10^{-5} M	16820	254		
SP-304	0	0	0	~~10^{-7} M~~ 1.1×10^{-7}M	~~168.2~~ 185.0
	10^{-9} M	1.682	0		
	10^{-8} M	16.82	17		
	10^{-7} M	168.2	149		
	10^{-6} M	1682	320		
	10^{-5} M	16820	315		

* cGMP levels in T84 cells after a 30-minute incubation.
** EC_{50}: median effective concentration (required to induce a 50% effect)

Reason for Amendment: To update the scientific notation for the EC50 molar value for each peptide to include the coefficient as well as the exponent and to update the corresponding ng/mL values. The new values were calculated using Prism 6 (Version 6.05) using a nonlinear regression curve fit (log[peptide concentration] versus cGMP level) using a least squares fit with no constraints)

Ex. 2027 at TRUL00018211-212, TRUL00018222
Sur-Reply at 26; *see also* Patent Owner's Resp. at 60-61; EX. 2024 ¶¶ 216-220; Ex. 2025 ¶¶ 97-99

Significantly Superior Potency Would Have Been Unexpected



US007041786B2

(12) **United States Patent**
Shailubhai et al.

(10) **Patent No.: US 7,041,786 B2**
(45) **Date of Patent: May 9, 2006**

(54) **GUANYLATE CYCLASE RECEPTOR AGONISTS FOR THE TREATMENT OF TISSUE INFLAMMATION AND CARCINOGENESIS**

(75) Inventors: **Kunwar Shailubhai**, Blue Bell, PA (US); **Gregory Nikiforovich**, St. Louis, MO (US); **Gary S. Jacob**, Creve Coeur, MO (US)

(73) Assignee: **Callisto Pharmaceuticals**, New York, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 362 days.

(21) Appl. No.: **10/107,814**

(22) Filed: **Mar. 28, 2002**

(65) **Prior Publication Data**

US 2003/0073628 A1 Apr. 17, 2003

Related U.S. Application Data

(60) Provisional application No. 60/348,646, filed on Jan. 17, 2002.

(51) **Int. Cl.**
A61K 38/12 (2006.01)

(52) **U.S. Cl.** **530/317**; 530/300, 530/326; 514/10, 514/13

(58) **Field of Classification Search** 530/317, 530/300, 326; 514/10, 13
See application file for complete search history.

(56) **References Cited**

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(Continued)

Primary Examiner—Stephen L. Rawlins
(74) *Attorney, Agent, or Firm*—Mintz, L
Glovsky and Popeo, P.C.; Ivor R. Elrifi

(57) **ABSTRACT**

A method of treatment of inflamed, pre-cancerous or cancerous tissue or polyps in a mammalian subject is disclosed. The treatment involves administration of a composition of at least one peptide agonist of a guanylate cyclase receptor and/or other small molecules that enhance intracellular production of cGMP. The at least one peptide agonist of a guanylate cyclase receptor may be administered either alone or in combination with an inhibitor of cGMP-dependent phosphodiesterase. The inhibitor may be a small molecule, peptide, protein or other compound that inhibits the degradation of cGMP. Without requiring a particular mechanism of action, this treatment may restore a healthy balance between proliferation and apoptosis in the subject's population of epithelial cells, and also suppress carcinogenesis. Thus, the method may be used to treat, inter alia, inflammation, including gastrointestinal inflammatory disorders, general organ inflammation and asthma, and carcinogenesis of the lung, gastrointestinal tract, bladder, testis, prostate and pancreas, or polyps.

6 Claims, No Drawings

MYLAN EXHIBIT - 1001
Mylan Pharmaceuticals, Inc. v. Bausch Health Ireland, Ltd. - IPR2022-00722

uroguanylin

Asp² & Asp³ → Glu² & Glu³

Asp² → Glu²

Asp³ → Glu³

TABLE 4

Peptide agonists evaluated for biological activity in the T84 cell bioassay.

SEQ ID NO.*	Compound Code	cGMP Level** (pmol/well)
1	SP301	205
6	SP302	225
7	SP303	195
20	SP304	315
14	SP306	0
4	SP310	0
21	SP316	275

*SEQ ID's for SP301, SP304 and SP316 are the precise amino acid sequences for these analogs as given in the text.
**Intracellular cGMP level observed in T84 cells following treatment with 1 micromolar solution of the respective peptide agonist for 30 minutes. The value observed for SP304 was statistically significant with a $p > 0.5$.

Peptides shown in Table 4 were custom synthesized and purified (>95% purity) using a published procedure (38).

Ex. 1001 at 15:53-54, 16 (Table 4)
Patent Owner's Resp. at 63-64; Sur-Reply at 18-19; EX. 2024 ¶¶ 221-222; Ex. 2025 ¶¶ 100-102

Currie's Data Are Internally Inconsistent

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Boards of Appeal
Chambres de recours

European Patent Office
80298 MUNICH
GERMANY

Adams, Harvey Vaughan John
Mathys & Squire LLP
120 Holborn
London
EC1N 2SQ
ROYAUME UNI

Datum/Date
11.06.13

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°
I17839 OPPO01	02721604.3 / 1379224

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
Synergy Pharmaceuticals, Inc.

Appeal number: T1366/12-3.3.04

Please find enclosed a copy of the decision dated 04.06.13.

The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Annex(es): ☑ Acknowledgement of receipt - EPO Form 2936
☐

Registered letter with advice of delivery

EPO Form 3032 01/11

MYLAN EXHIBIT - 1067 Pg. 001
Mylan Pharmaceuticals, Inc. v. Bausch Health Ireland, Ltd.
IPR2022-00722

~70 pmol/well

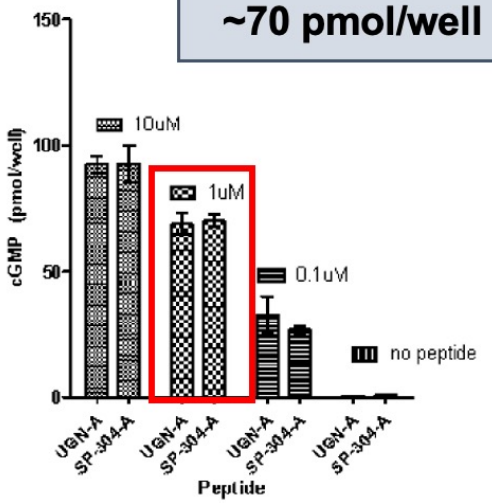


Figure 1

Concentration	p-value	Significance
10 µM	0.9889	ns
1 µM	0.8190	ns
0.1 µM	0.5402	ns

Table 1

~30-35 pmol/well

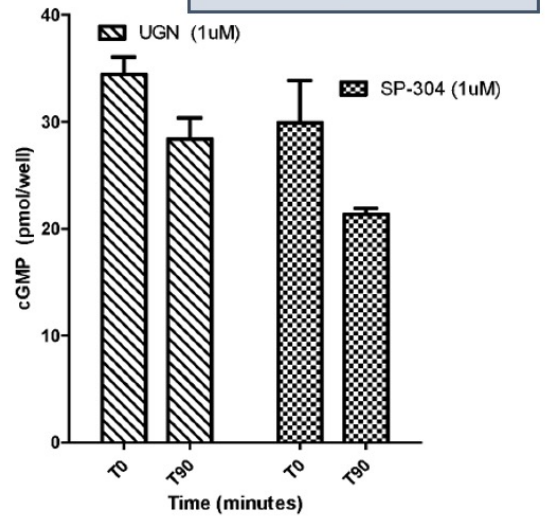


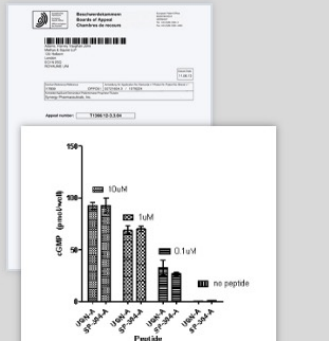
Figure 2

Time point	p-value	Significance
T0	0.2522	ns
T90	0.1008	ns

Table 2

Ex. 1067 at 121, 122
Sur-Reply at 21

Currie's Data Are Inconsistent with the Literature



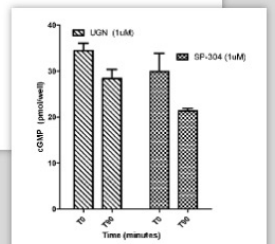
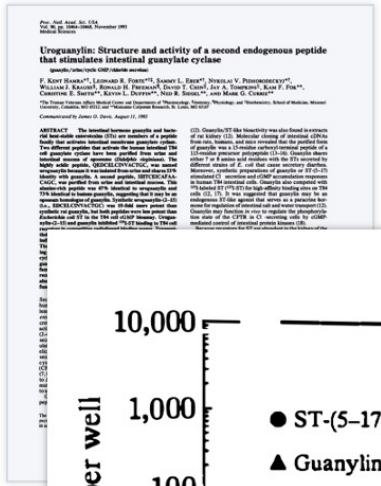


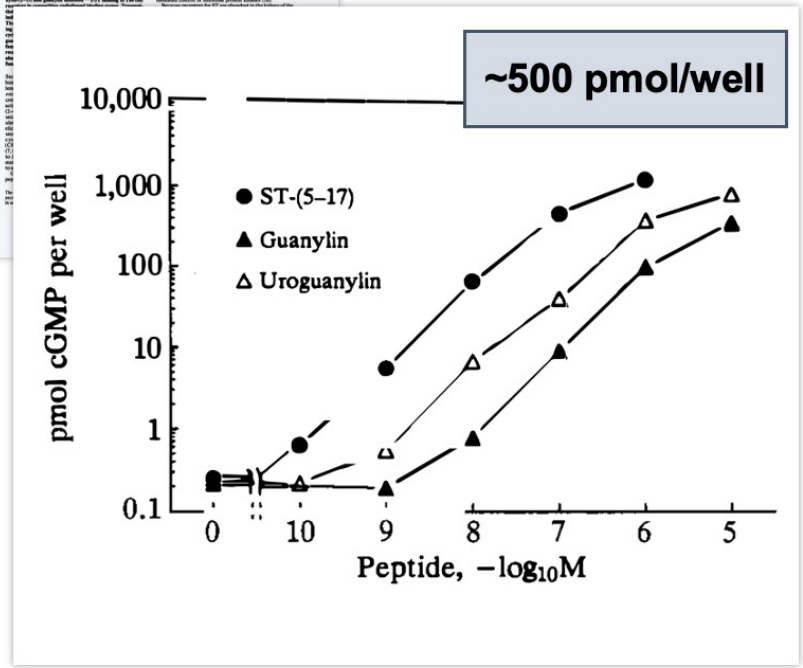
Figure 2

Time point	p-value	Significance
T0	0.2522	ns
T90	0.1068	ns

Table 2

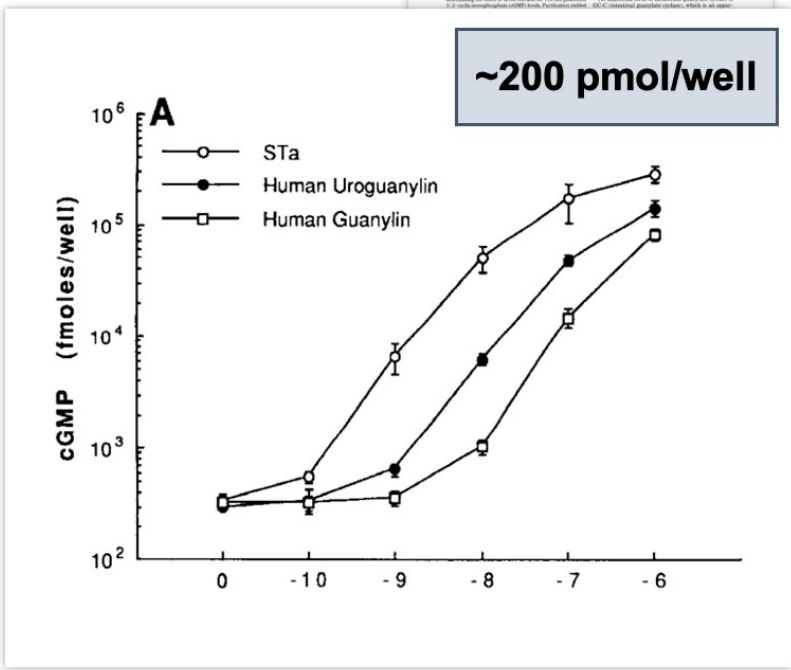


Uroguanylin: Structure and activity of a second endogenous peptide that stimulates intestinal guanylate cyclase



rapid communication

Characterization of human uroguanylin: a member of the guanylin peptide family



Ex. 2012 at 10467 (Fig. 5); Ex. 2065 at 346 (Fig. 4A)
Sur-Reply at 21-22

Plecanatide's Unexpected pH Sensitivity

STUDIES ON SP-304 THERMOSTABILITY, pH DEPENDENCY AND TOPOISOMERIC STABILITY

Study Number: SP-PH-004

Test Article: SP-304

Author: Kunwar Shailubhai
Senior Vice President, Discovery
Synergy Pharmaceuticals, Inc.

Study Dates: November 24, 2001 to December 25, 2001

Testing Facility: R&D Center, Synergy Pharmaceuticals, Inc., Norristown, PA

Final Report Date: February 15, 2008

Source Data: Synergy Pharmaceuticals Lab Notebook # 2; Pages: 170-185
Of Dr. Surendra Dheer

Synergy Pharmaceuticals, Inc.
420 Lexington Ave. - Suite 1609
New York, NY 10170

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This document is the property of Synergy Pharmaceuticals Inc., and is confidential and proprietary. This information contained herein is believed to be accurate and complete as of the date of preparation. The contents may not be used in regulatory submissions without the expressed consent of Synergy Pharmaceuticals Inc.

HIGHLY CONFIDENTIAL INFORMATION TRUL00018260

PROTECTIVE ORDER MATERIAL

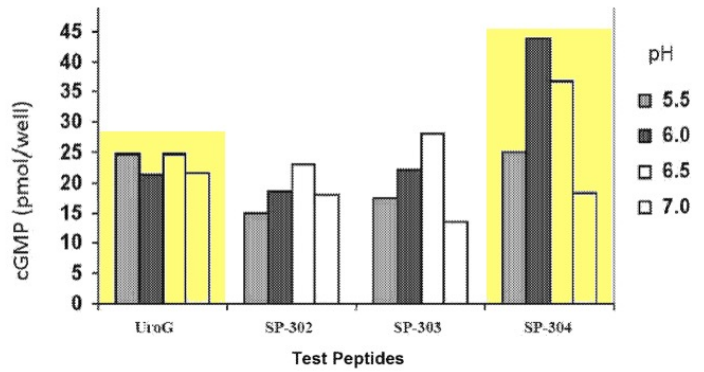
Bausch Health Ireland Exhibit 2028, Page 1 of 18
Mylan v. Bausch Health Ireland - IPR2022-00722

Table 2: Raw Data (pH sensitivity assays)

Test Peptide	Concentration	cGMP Levels (pmol/well) *			
		pH 5.5	pH 6.0	pH 6.5	pH. 7.0
Uroguanylin	0.1 µM (1.67 µg/mL)	24.6	21.36	24.6	21.72
SP-304	0.1 µM (1.68 µg/mL)	24.9	43.8	36.68	18.18
SP-302	0.1 µM (1.67 µg/mL)	14.80	18.6	22.98	17.94
SP-303	0.1 µM (1.68 µg/mL)	17.46	22.26	28.14	13.62

* cGMP levels in T84 cells after a 30-minute incubation at 37°C.

Figure 2: Effect of 30 Minute Incubations of 0.1 µM Concentrations of SP-304, Uroguanylin, SP-302 and SP-303 on cGMP Production at Various Physiological pH Values in T84 Cells



Ex.2028 at TRUL00018270, TRUL00018273 (Table 2)
Patent Owner's Resp. at 61-63; Sur-Reply at 24; EX. 2024 ¶¶ 223-228; Ex. 2025 ¶¶ 105-108

Plecanatide's Unexpected pH Sensitivity

SCOTT WALDMAN, M.D., Ph.D.

D STATES PATENT AND TRADEMARK OFFICE

RE THE PATENT TRIAL AND APPEAL BOARD

MYLAN PHARMACEUTICALS INC.,

Petitioner,

v.

BAUSCH HEALTH IRELAND LIMITED,

Patent Owner.

Case IPR2022-00722
U.S. Patent No. 7,041,786

DECLARATION OF SCOTT A. WALDMAN, M.D., PHD, FDP, FAHA, FNAI, FASPET

PROTECTIVE ORDER MATERIAL

Bausch Health Ireland Exhibit 2025, Page 1 of 64
Mylan v. Bausch Health Ireland - IPR2022-00722

110. In my opinion, these results are clinically significant and would have been unexpected to a person of ordinary skill in the art. Nothing in the prior art taught that the one amino acid substitution of plecanatide would produce this significant improvement. This increase in cGMP production is surprising, but additionally, plecanatide's increased pH sensitivity would have been entirely unexpected. It is also clinically meaningful. As discussed above, this unexpected pH sensitivity in the acidic areas of the intestines allows plecanatide's unexpectedly superior selective activity in the small intestine to provide fluid into the intestine to treat constipation, rather than in the colon, where its activity drops (pH 7) and is thus less likely to cause adverse effects, like diarrhea. In other words, plecanatide's targeted activity in the small intestine (not in the colon) advantageously reduces the diarrhea rate in patient populations.

Ex. 2025 ¶ 110
Patent Owner's Resp. at 61-63; Sur-Reply at 24; Ex. 2025 ¶ 110.

Plecanatide's Significantly and Unexpectedly Superior Heat Stability

STUDIES ON SP-304 THERMOSTABILITY, pH DEPENDENCY AND TOPOISOMERIC STABILITY

Study Number:	SP-PH-004
Test Article:	SP-304
Author:	Kunwar Shailubhai Senior Vice President, Discovery Synergy Pharmaceuticals, Inc.
Study Dates:	November 24, 2001 to December 25, 2001
Testing Facility:	R&D Center, Synergy Pharmaceuticals, Inc., Norristown, PA
Final Report Date:	February 15, 2008
Source Data:	Synergy Pharmaceuticals Lab Notebook # 2; Pages: 170-185 Of Dr. Surendra Dheer

Synergy Pharmaceuticals, Inc.
420 Lexington Ave. - Suite 1609
New York, NY 10170

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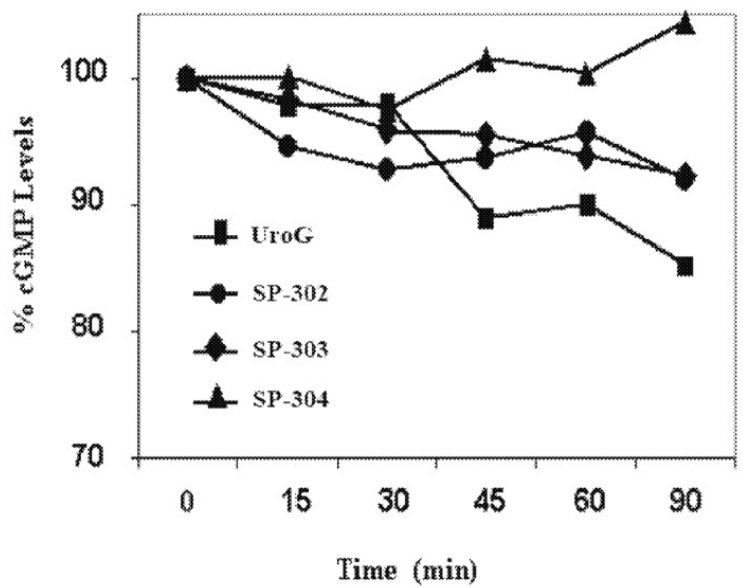
HIGHLY CONFIDENTIAL INFORMATION TRUL00018260

PROTECTIVE ORDER MATERIAL

Bausch Health Ireland Exhibit 2028, Page 1 of 18
Mylan v. Bausch Health Ireland - IPR2022-00722

Figure 3: Thermostability of SP-304, Uroguanylin, SP-302 and SP-303 Following Heat Treatment at 95°C in T84 Cells as a Function of Time



Ex. 2028 at TRUL00018268, TRUL00018270-271
Patent Owner's Resp. at 64-65; Sur-Reply at 25; Ex. 2024 ¶¶ 231-238

Plecanatide's Significantly and Unexpectedly Superior Binding Affinity

ANTICANCER RESEARCH 29: 3777-3784 (2009)

In Vivo Imaging of Human Colorectal Cancer Using Radiolabeled Analogs of the Uroguanylin Peptide Hormone

DIJIE LIU[1,5], DOUGLAS OVERBEY[1], LISA D. WATKINSON[1,5], SAID DAIBES-FIGUEROA[1,5], TIMOTHY J. HOFFMAN[1,4,5], LEONARD R. FORTE[1,3,5], WYNN A. VOLKERT[1,2,5] and MICHAEL F. GIBLIN[1,2,5,*]

[1]*Research Service, Harry S. Truman Memorial Veterans' Administration Hospital, Columbia, MO 65201; Departments of* [2]*Radiology,* [3]*Medical Pharmacology and Physiology, and* [4]*Internal Medicine, and* [5]*The Radiopharmaceutical Sciences Institute, University of Missouri-Columbia, Columbia, MO 65211, U.S.A.*

Abstract. *Background: Uroguanylin is an endogenous peptide agonist that binds to the guanylate cyclase C receptor (GC-C). GC-C is overexpressed in human colorectal cancer (CRC), and exposure of GC-C-expressing cells to GC-C agonists results in cell cycle arrest and/or apoptosis, highlighting the therapeutic potential of such compounds. This study describes the first use of radiolabeled uroguanylin analogs for in vivo detection of CRC. Materials and Methods: The peptides uroguanylin and E[3]-uroguanylin were N-terminally labeled with the DOTA chelating group via NHS ester activation and characterized by RP-HPLC, ESI-MS, and GC-C receptor binding assays. The purified conjugates were radiolabeled with In-111 and used for in vivo biodistribution and SPECT imaging studies. In vivo experiments were carried out using SCID mice bearing T84 human colorectal cancer tumor xenografts. Results: Alteration of the position 3 aspartate residue to glutamate resulted in increased affinity for GC-C, with IC_{50} values of 5.0±0.3 and 9.6±2.9 nM for E[3]-uroguanylin and DOTA-E[3]-uroguanylin, respectively. In vivo, [111]In-DOTA-E[3]-uroguanylin demonstrated tumor uptake of 1.17±0.23 and 0.61±0.07%ID/g at 1 and 4 h post injection, respectively. The specificity of tumor localization was demonstrated by co-injection of 3 mg/kg unlabeled E[3]-uroguanylin, which reduced tumor uptake by 69%. Uptake in kidney, however, was dramatically higher for the uroguanylin peptides than for previously characterized radiolabeled E. coli heat-stable enterotoxin (STh) analogs targeting GC-C, and was also inhibited by coinjection of unlabeled peptide in a fashion not previously observed. Conclusion: Use of uroguanylin-targeting vectors for in vivo imaging of colorectal cancers expressing GC-C resulted in tumor uptake that paralleled that of higher affinity heat-stable enterotoxin peptides, but also resulted in increased kidney uptake in vivo.*

Guanylate cyclase C (GC-C) is a type 1 transmembrane glycoprotein expressed on brush border membranes of intestinal epithelial cells, as well as on transformed human colon cancer cell lines such as the T-84 cell line (1, 2). In the normal intestinal mucosa, GC-C receptors are expressed within the apical (luminal) face of epithelial cell membranes, and are therefore isolated from the bloodstream by cell-cell tight junctions (3-6). GC-C expression is maintained in transformed cells throughout the process of colorectal carcinogenesis, while expression of the endogenous GC-C ligands guanylin and uroguanylin is typically lost (7-9). Normally expressed at high levels within the lumen of the gut, GC-C is expressed on virtually all histologically confirmed primary and metastatic colorectal tumors examined in human patients, while normal tissues and other types of cancer express minimal or no GC-C receptors (4-6). GC-C receptors on colorectal tumors retain their ligand-binding capacity, and expression of GC-C receptors does not vary as a function of metastatic site or grade of these tumors (5). The unique expression of GC-C by metastatic cells of colorectal origin within lymph nodes of patients undergoing staging for colorectal cancer (CRC) forms the basis for a PCR-based diagnostic test that is currently undergoing clinical trials (10). GC-C expression has also formed the basis of development of ligand-based molecular agents for *in vivo* detection and therapy of colorectal cancer (9, 11-20).

Uroguanylin is a 16 amino acid peptide with two disulfide bonds, and has nanomolar affinity for the GC-C receptor (21, 22). Secretion of the endogenous peptides guanylin and uroguanylin into the lumen of the gut by enterochromaffin cells plays a role in regulation of ion and fluid homeostasis

Correspondence to: Dr. Michael F. Giblin, Harry S. Truman Memorial VA Hospital, Research Service Room A204, 800 Hospital Drive, Columbia, MO 65201, U.S.A. Tel: +1 5738146000 ext. 53669, Fax: +1 5738821663, e-mail: giblinm@health.missouri.edu

Key Words: Uroguanylin, *E. coli* heat-stable enterotoxin, guanylyl cyclase C, single photon-emitting computed tomography (SPECT), colorectal cancer, *in vivo* imaging.

0250-7005/2009 $2.00+.40

Table I. *Calculated and observed $(M+H)^+$ values and IC_{50} values (± SD) for characterized peptides.*

Peptide	$(M+H)^+$ Calc.	$(M+H)^+$ Obs.	IC_{50} (nM)
Uroguanylin	1667.6	1667.7	39.8±14.9
DOTA-uroguanylin	2053.6	2053.9	34.5±3.3
E^3-uroguanylin	1681.6	1681.6	5.0±0.3
DOTA-E^3-uroguanylin	2067.6	2067.9	9.6±2.9

Ex. 2046 at 3779
Patent Owner's Resp. at 66; Sur-Reply at 19-20, 25; Ex. 2024 ¶¶ 241-242

Liu Did Not Use Wild-Type Human Uroguanylin

ANTICANCER RESEARCH 29: 3777-3784 (2009)

***In Vivo* Imaging of Human Colorectal Cancer Using Radiolabeled Analogs of the Uroguanylin Peptide Hormone**

DIJIE LIU[1,5], DOUGLAS OVERBEY[1], LISA D. WATKINSON[1,5], SAID DAIBES-FIGUEROA[1,5], TIMOTHY J. HOFFMAN[1,4,5], LEONARD R. FORTE[1,3,5], WYNN A. VOLKERT[1,2,5] and MICHAEL F. GIBLIN[1,2,5,*]

[1]Research Service, Harry S. Truman Memorial Veterans' Administration Hospital, Columbia, MO 65201;

as a 0.05 N HCl solution. Wild-type human uroguanylin was obtained from the American Peptide Company, and E^3-uroguanylin was kindly provided by Dr. Kunwar Shailubhai at Callisto Pharmaceuticals. All other reagents were purchased from Aldrich

Peptides were purificd by RP-HPLC and characterized by MALDI-TOF MS and by a competitive displacement receptor binding assay utilizing T84 human colorectal cancer cells and ^{125}I-labeled F^{19}-STh(1-19) (Table I, Figure 2).

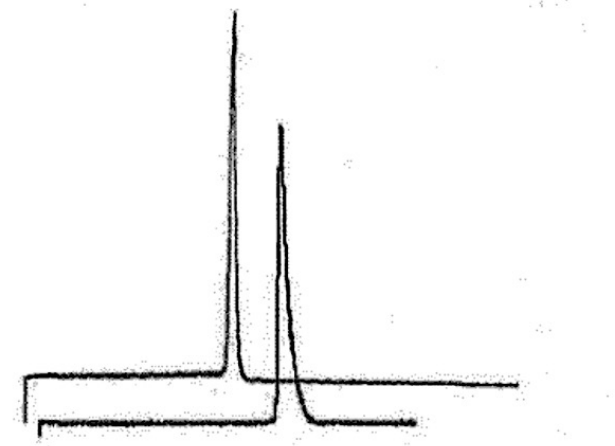
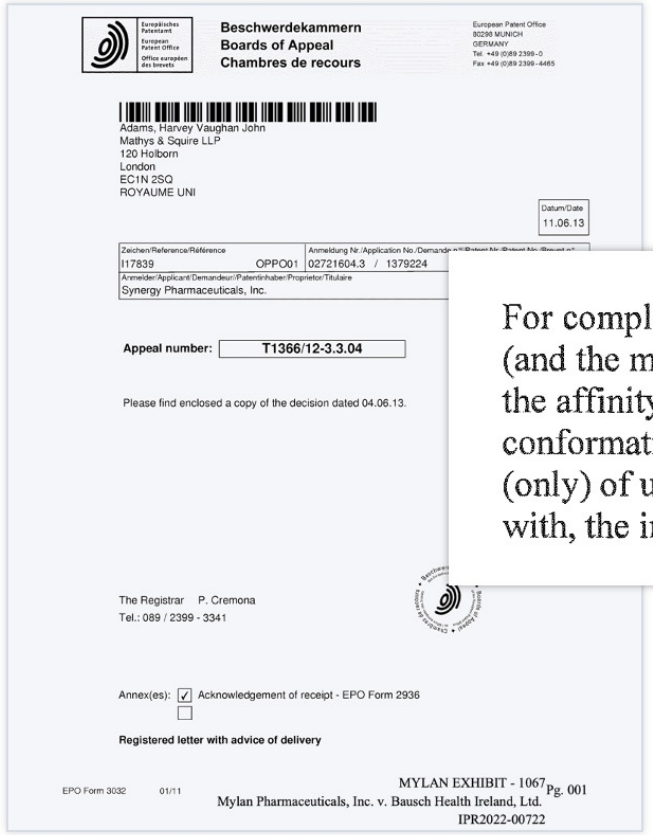


Figure 3. *RP-HPLC chromatograms of purified ^{111}In-DOTA-E^3-uroguanylin (top), ^{111}In-DOTA- uroguanylin (bottom).*

Ex. 2046 at 3778-3780, Fig. 3
Sur-Reply at 19-20, 25; *see also* Patent Owner's Resp. at 66; Ex. 2024 ¶¶ 241-242

Patent Owner Did Not Use Wild-Type Human Uroguanylin



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Datum/Date
11.06.13

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°
I17839 OPPO01	02721604.3 / 1379224

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
Synergy Pharmaceuticals, Inc.

Appeal number: T1366/12-3.3.04

Please find enclosed a copy of the decision dated 04.06.13.

The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Annex(es): ☑ Acknowledgement of receipt - EPO Form 2936
☐

Registered letter with advice of delivery

EPO Form 3032 01/11

MYLAN EXHIBIT - 1067 Pg. 001
Mylan Pharmaceuticals, Inc. v. Bausch Health Ireland, Ltd.
IPR2022-00722

For completeness, we note that, because of the rapid isomeric interconversion of uroguanylin (and the much slower interconversion of E^3-uroguanylin (SP-304)), it is impossible to compare the affinity of the active conformation (only) of uroguanylin to the affinity of the active conformation of E^3-uroguanylin (SP-304). Testing of the activity or affinity of the active form (only) of uroguanylin is not possible, as it rapids converts to, and eventually reaches equilibrium with, the inactive conformation.

Ex. 1067 at 140
Sur-Reply at 20; Ex. 2024 ¶ 56

The European Patent Office Did Not Credit Currie's Data

BLAKE ROBERT PETERSON , Ph.D

Q. Okay. Are you aware that the European Patent Office did not credit Dr. Currie's results and declined to invalidate the plecanatide patent?

***

THE WITNESS: I believe that to be true, yes.

Ex. 2069 at 104:15-19
Sur-Reply at 23

Federal Circuit: A Compound and All of Its Properties Are Inseparable

"From the standpoint of patent law, a compound and all of its properties are inseparable; they are one and the same thing."

Application of Papesch, 315 F.2d 381, 391 (C.C.P.A. 1963)
Sur-Reply at 1

Federal Circuit: Permitting Reliance on Non-Prior Art

Permitting reliance on non-prior art as "evidence of the motivation of a POSITA to explore less frequent dosing regimens as of the priority date."

Yeda Rsch. v. Mylan Pharms. Inc., 906 F.3d 1031, 1041-42 (Fed. Cir. 2018)
Patent Owner's Resp. at 37

Drs. Peterson and Epstein Are Not GCC Experts

BLAKE ROBERT PETERSON , Ph.D.

Q. You have never been qualified by any court or by the U.S. Patent and Trademark Office as an expert in the biochemistry of GC-C receptors, correct?

A. That's correct.

Q. In connection with your work in this case, have you had any communications with Dr. Mark Currie?

A. No, I have not.

Q. Aside from your work on this case, have you ever had any communications with Dr. Mark Currie?

A. No, I have not.

MICHAEL SAMUEL EPSTEIN, M.D.

Q. Do you consider yourself an expert in guanylin cyclase C receptors?

A. An expert, no.

Q. But you personally have never developed a GCC receptor agonist?

A. That is correct.

Ex. 2069 at 10:6-9, 15:22-16:2; Ex. 2070 at 25:14-16, 26:2-4
Sur-Reply at 23

Dr. Peterson Has Never Conducted a T84 Cell Bioassay

BLAKE ROBERT PETERSON , Ph.D.

Q. Have you yourself ever conducted a T84 cell bioassay?

***

THE WITNESS: I have conducted countless different whole cell assays. That particular specific assay I have not conducted in my laboratory, no.

Ex. 2069 at 10:15-20
Sur-Reply at 23

Dr. Epstein's Lack of Expertise

MICHAEL SAMUEL EPSTEIN, M.D.

Q. Do you have a Ph.D. in chemistry?
A. No, I do not.
Q. Do you have a master's in chemistry?
A. No, I do not.
Q. Do you have a B.S. in chemistry?
A. No.
Q. Do you have a Ph.D. in protein engineering?
A. No, I do not.
Q. Do you have a master's in protein engineering?
A. No, I do not.
Q. Do you have a B.S. in protein engineering?
A. No, I do not.
Q. Do you have any degrees in pharmaceutical chemistry?
A. No, I do not.
Q. Do you have any degrees in pharmacy?
A. No, I do not.
Q. Do you have any degrees in clinical pharmacology?
A. No, I do not.

Ex. 2070 at 22:21-24:9
Sur-Reply at 23

Dr. Epstein's Lack of Expertise in Statistics and Biostatistics

BLAKE ROBERT PETERSON , Ph.D.

Q. You have never been qualified by any court or by the U.S. Patent and Trademark Office as an expert in statistics or biostatistics, correct?

***

THE WITNESS: I use statistics regularly in my research, but I'm not qualified by a court in an official certified sense, no.

Ex. 2069 at 114:13-19
Sur-Reply at 23

Petitioner's Topoisomer "Reservoir" Argument Is Unavailing

BLAKE ROBERT PETERSON , Ph.D.

Q. There is no reservoir of inactive topoisomer for a drug that does not exhibit topoisomeric interconversion, correct?

THE WITNESS: I mean if there's no interconversion, there is no reservoir. If that answers your question.

Ex. 2069 at 50:18-24, 106:9-19
Sur-Reply at 5

Petitioner's Reliance on Currie's Diarrhea Statements Are Misplaced

BLAKE ROBERT PETERSON , Ph.D.

Q. Trulance is not approved for use in infants, correct?

***

THE WITNESS: I believe that's correct.

Q. Linzess is not approved for use in infants, correct?

***

THE WITNESS: I believe that's correct.

Q. Trulance is not approved for use in domestic animals, correct?

***

THE WITNESS: I'm not sure about the veterinary implications or indications of Trulance.

Q. Linzess is not approved for use in domestic animals, correct?

***

THE WITNESS: Again, I'm not sure if it's been approved for veterinary use.

Ex. 2069 at 28:14-29:11
Sur-Reply at 7

HPLC Is Not Suitable for Manufacturing Scale

BLAKE ROBERT PETERSON , Ph.D.

Q. High-performance liquid chromatography, or HPLC, is an analytical chemistry technique used to separate compounds in a chemical mixture, correct?

A. That is correct.

Q. HPLC can be used for research purposes, correct?

A. That is correct.

Q. HPLC can be used for manufacturing purposes, correct?

A. That is correct.

Q. What are some of the different considerations for using HPLC on a research scale versus using HPLC on a manufacturing scale?

***

THE WITNESS: HPLC is better suited to smaller scales, and so on a manufacturing scale, it might not be a cost-effective method.

Ex. 2069 at 22:14- 23:4, 28:5-12
Sur-Reply at 4

Any Compound Is Toxic at High Levels

MICHAEL SAMUEL EPSTEIN, M.D.

Q. Insulin is a peptide that the human body naturally produces; is that correct?

***

THE WITNESS: Yes.

Q. And it's possible to overdose on insulin; is that correct?

***

THE WITNESS: You may overdose on insulin.

Q. And an insulin overdose can lead to death; is that correct?

***

THE WITNESS: It may lead to hypoglycemia which, if left untreated, could be fatal.

Ex. 2070 at 35:19-36:13
Sur-Reply at 4

Certain Drugs Can Slow Colonic Transit Times

MICHAEL SAMUEL EPSTEIN, M.D.

THE WITNESS: I'm not sure exactly what you're asking. I'm a little bit lost in that. What are you asking? You mean something like that would cause the colon to slow down or something like that or...

Q. Correct.

A. Well, it could be longer if you take an antidiarrheal agent like Imodium or Lomotil that would slow the colon down. It's meant to do that. So it might slow down the transit through the colon.

Q. And opioids are another example of that would delay the transit time?

A. Those are actually opioids. So yes --

Q. Perfect.

A. -- opioids would do that.

Ex. 2070 at 45:18-47:7
Sur-Reply at 3

“Medicine Is an Art, Not a Science”

MICHAEL SAMUEL EPSTEIN, M.D.

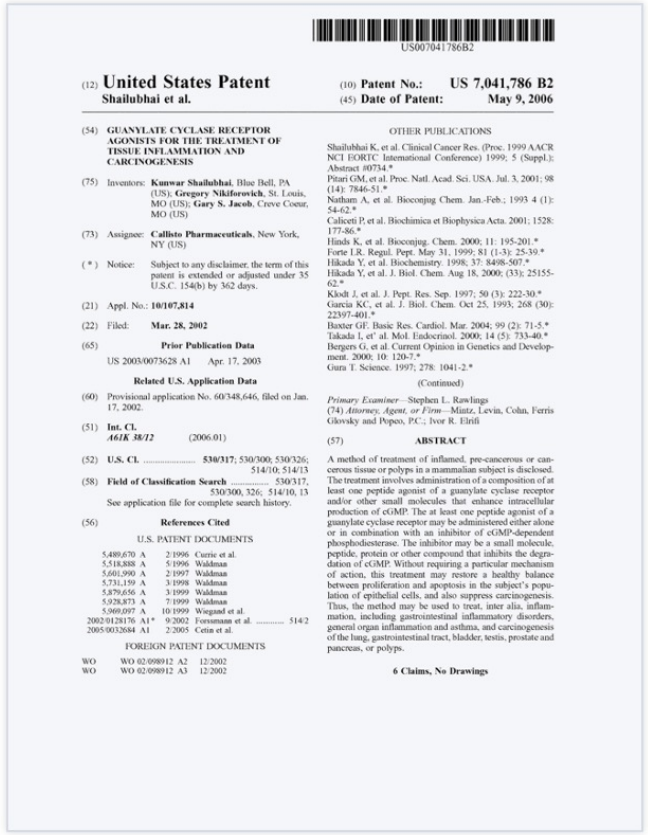
Q. And how frequently do constipated patients have bowel movements?

A. That's actually an interesting question. I have patients who have a bowel movement every day and say they're constipated, and I have patients that have a bowel movement less than once a week and say they're constipated.

There's so many factors that goes into that, whether they're obstipated, whether they have tenesmus or, you know, pressure in the rectum, urgency or straining or incomplete evacuation or crampy discomfort. It really is very variable. And sometimes what we doctors think is not what the patient thinks. So it’s more a matter of what does the patient feel, not so much what we think. That's why medicine is an art, not a science.

Ex. 2070 at 38:6-22
Ex. 2025 ¶¶ 23-24

Claim 1 of the ’786 Patent



US007041786B2

(12) **United States Patent**
Shailubhai et al.

(10) **Patent No.: US 7,041,786 B2**
(45) **Date of Patent: May 9, 2006**

(54) **GUANYLATE CYCLASE RECEPTOR AGONISTS FOR THE TREATMENT OF TISSUE INFLAMMATION AND CARCINOGENESIS**

(75) Inventors: **Kunwar Shailubhai**, Blue Bell, PA (US); **Gregory Nikiforovich**, St. Louis, MO (US); **Gary S. Jacob**, Creve Coeur, MO (US)

(73) Assignee: **Callisto Pharmaceuticals**, New York, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 362 days.

(21) Appl. No.: **10/107,814**

(22) Filed: **Mar. 28, 2002**

(65) **Prior Publication Data**

US 2003/0073628 A1 Apr. 17, 2003

Related U.S. Application Data

(60) Provisional application No. 60/348,646, filed on Jan. 17, 2002.

(51) **Int. Cl.**
A61K 38/12 (2006.01)

(52) **U.S. Cl.** **530/317**; 530/300; 530/326; 514/10; 514/13

(58) **Field of Classification Search** 530/317, 530/300, 326; 514/10, 13
See application file for complete search history.

(56) **References Cited**

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Primary Examiner—Stephen L. Rawlings
(74) *Attorney, Agent, or Firm*—Mintz, Levin, Cohn, Ferris Glovsky and Popeo, P.C.; Ivor R. Elrifi

(57) **ABSTRACT**

A method of treatment of inflamed, pre-cancerous or cancerous tissue or polyps in a mammalian subject is disclosed. The treatment involves administration of a composition of at least one peptide agonist of a guanylate cyclase receptor and/or other small molecules that enhance intracellular production of cGMP. The at least one peptide agonist of a guanylate cyclase receptor may be administered either alone or in combination with an inhibitor of cGMP-dependent phosphodiesterase. The inhibitor may be a small molecule, peptide, protein or other compound that inhibits the degradation of cGMP. Without requiring a particular mechanism of action, this treatment may restore a healthy balance between proliferation and apoptosis in the subject's population of epithelial cells, and also suppress carcinogenesis. Thus, the method may be used to treat, inter alia, inflammation, including gastrointestinal inflammatory disorders, general organ inflammation and asthma, and carcinogenesis of the lung, gastrointestinal tract, bladder, testis, prostate and pancreas, or polyps.

6 Claims, No Drawings

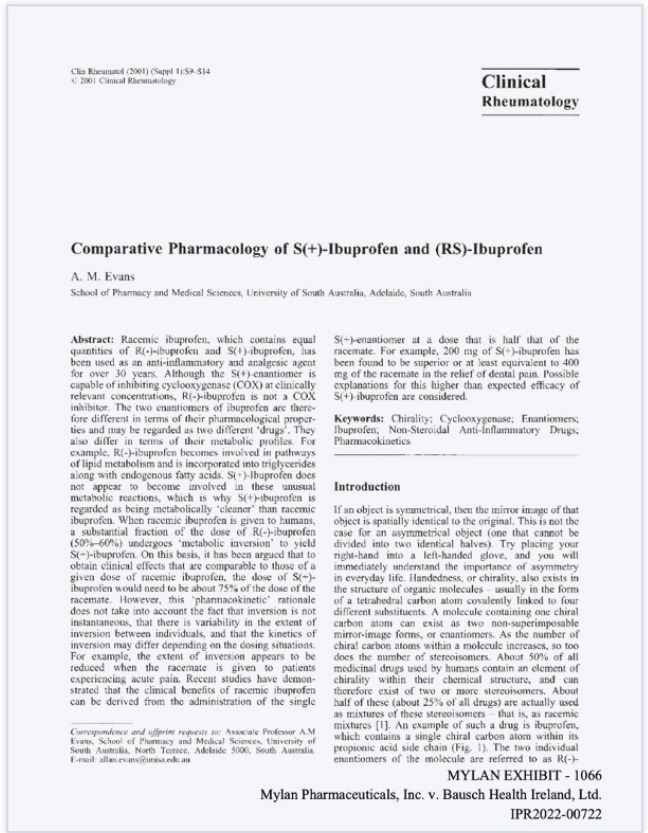
1. A peptide consisting of the amino acid sequence of SEQ ID NO:20.

<400> SEQUENCE: 20

```
Asn Asp Glu Cys Glu Leu Cys Val Asn Val Ala Cys Thr Gly Cys Leu
  1               5                  10                  15
```

Ex. 1001 at claim 1; 35-36.
Patent Owner’s Resp. at 1

Evans: Isomer Activity Is Unpredictable



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Clinical Rheumatology

Comparative Pharmacology of S(+)-Ibuprofen and (RS)-Ibuprofen

A. M. Evans
School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, South Australia

Abstract: Racemic ibuprofen, which contains equal quantities of R(-)-ibuprofen and S(+)-ibuprofen, has been used as an anti-inflammatory and analgesic agent for over 30 years. Although the S(+)-enantiomer is capable of inhibiting cyclooxygenase (COX) at clinically relevant concentrations, R(-)-ibuprofen is not a COX inhibitor. The two enantiomers of ibuprofen are therefore different in terms of their pharmacological properties and may be regarded as two different "drugs". They also differ in terms of their metabolic profiles. For example, R(-)-ibuprofen becomes involved in pathways of lipid metabolism and is incorporated into triglycerides along with endogenous fatty acids. S(+)-Ibuprofen does not appear to become involved in these unusual metabolic reactions, which is why S(+)-ibuprofen is regarded as being metabolically 'cleaner' than racemic ibuprofen. When racemic ibuprofen is given to humans, a substantial fraction of the dose of R(-)-ibuprofen (50%–60%) undergoes 'metabolic inversion' to yield S(+)-ibuprofen. On this basis, it has been argued that to obtain clinical effects that are comparable to those of a given dose of racemic ibuprofen, the dose of S(+)-ibuprofen would need to be about 75% of the dose of the racemate. However, this 'pharmacokinetic' rationale does not take into account the fact that inversion is not instantaneous, that there is variability in the extent of inversion between individuals, and that the kinetics of inversion may differ depending on the dosing situations. For example, the extent of inversion appears to be reduced when the racemate is given to patients experiencing acute pain. Recent studies have demonstrated that the clinical benefits of racemic ibuprofen can be derived from the administration of the single S(+)-enantiomer at a dose that is half that of the racemate. For example, 200 mg of S(+)-ibuprofen has been found to be superior or at least equivalent to 400 mg of the racemate in the relief of dental pain. Possible explanations for this higher than expected efficacy of S(+)-ibuprofen are considered.

Keywords: Chirality; Cyclooxygenase; Enantiomers; Ibuprofen; Non-Steroidal Anti-Inflammatory Drugs; Pharmacokinetics

Introduction

If an object is symmetrical, then the mirror image of that object is spatially identical to the original. This is not the case for an asymmetrical object (one that cannot be divided into two identical halves). Try placing your right-hand into a left-handed glove, and you will immediately understand the importance of asymmetry in everyday life. Handedness, or chirality, also exists in the structure of organic molecules – usually in the form of a tetrahedral carbon atom covalently linked to four different substituents. A molecule containing one chiral carbon atom can exist as two non-superimposable mirror-image forms, or enantiomers. As the number of chiral carbon atoms within a molecule increases, so too does the number of stereoisomers. About 50% of all medicinal drugs used by humans contain an element of chirality within their chemical structure, and can therefore exist of two or more stereoisomers. About half of these (about 25% of all drugs) are actually used as mixtures of these stereoisomers – that is, as racemic mixtures [1]. An example of such a drug is ibuprofen, which contains a single chiral carbon atom within its propionic acid side chain (Fig. 1). The two individual enantiomers of the molecule are referred to as R(-)-

Correspondence and offprint requests to: Associate Professor A.M Evans, School of Pharmacy and Medical Sciences, University of South Australia, North Terrace, Adelaide 5000, South Australia. E-mail: allan.evans@unisa.edu.au

[12–15]. However, the extent of chiral inversion varies between individuals – for example, in osteoarthritis patients being treated with racemic ibuprofen, the fractional inversion of R(-)-ibuprofen varied between 35% and 85% [13].

The second important feature that distinguishes R(-)-ibuprofen from S(+)-ibuprofen is its ability to interfere with normal lipid metabolism. In rats, the formation of

placebo. These results were not unexpected. However, 200 mg of S(+)-ibuprofen provided a more rapid onset of analgesic action than 400 mg of the racemate and provided better pain relief over the first 3 h after dosing.

Ex. 1066 at 11, 12
Sur-Reply at 5-6

HPLC Is Not Suitable for Manufacturing Scale

BLAKE ROBERT PETERSON , Ph.D.

Q. High-performance liquid chromatography, or HPLC, is an analytical chemistry technique used to separate compounds in a chemical mixture, correct?

A. That is correct.

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A. That is correct.

Q. HPLC can be used for manufacturing purposes, correct?

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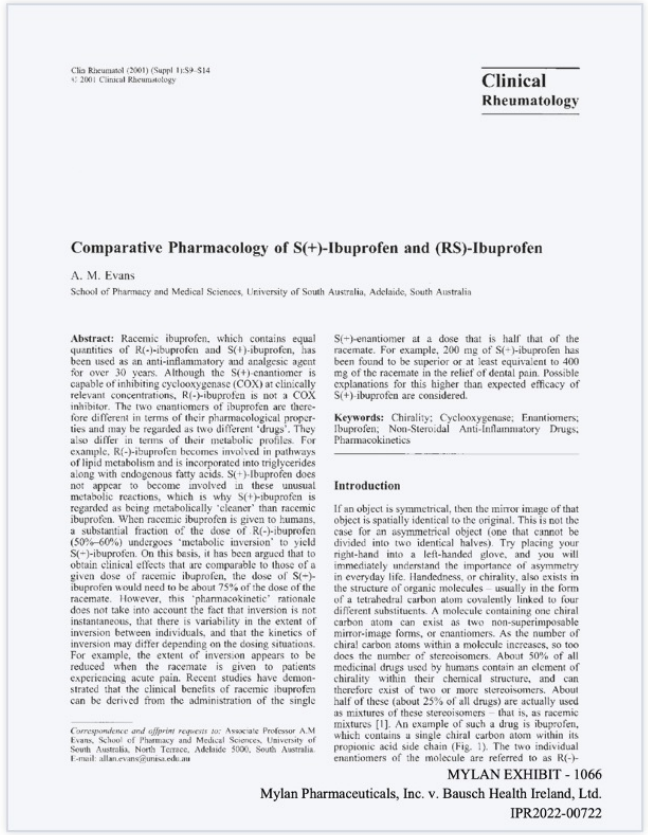
Q. What are some of the different considerations for using HPLC on a research scale versus using HPLC on a manufacturing scale?

***

THE WITNESS: HPLC is better suited to smaller scales, and so on a manufacturing scale, it might not be a cost-effective method.

Ex. 2069 at 22:14- 23:4, 28:5-12
Sur-Reply at 4; *see also* Ex. 2024 ¶¶ 65, 130

Evans: Pure Preparations Are Preferred



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In 1992, and again in 1996, I identified a range of potential advantages of administering profens, including ibuprofen, as enantiomerically pure preparations of the S-enantiomers [4,6]. These advantages included: reduced metabolic load, reduced chance of pharmacokinetic interactions with other drugs, avoidance of involvement in lipid metabolism and of the pharmacokinetic variability that arises from the metabolic inversion of R(-)-ibuprofen, and prevention of adverse events that may arise from the COX-independent actions of R(-)-ibuprofen. In addition, it was suggested that patient acceptability could be improved through the use of smaller doses. At that time, potential interactions

Ex. 1066 at 13
Sur-Reply at 6

Hamra 1996: Opossum Uroguanylin Is More Potent than Human Uroguanylin

Opossum colonic mucosa contains uroguanylin and guanylin peptides

F. KENT HAMRA, WILLIAM J. KRAUSE, SAMMY L. EBER, RONALD H. FREEMAN, CHRISTINE E. SMITH, MARK G. CURRIE, AND LEONARD R. FORTE
The Truman Veterans Affairs Medical Center and Departments of Pharmacology, Anatomy, and Physiology, School of Medicine, Missouri University, Columbia 65212; and Searle Research and Development, St. Louis, Missouri 63167

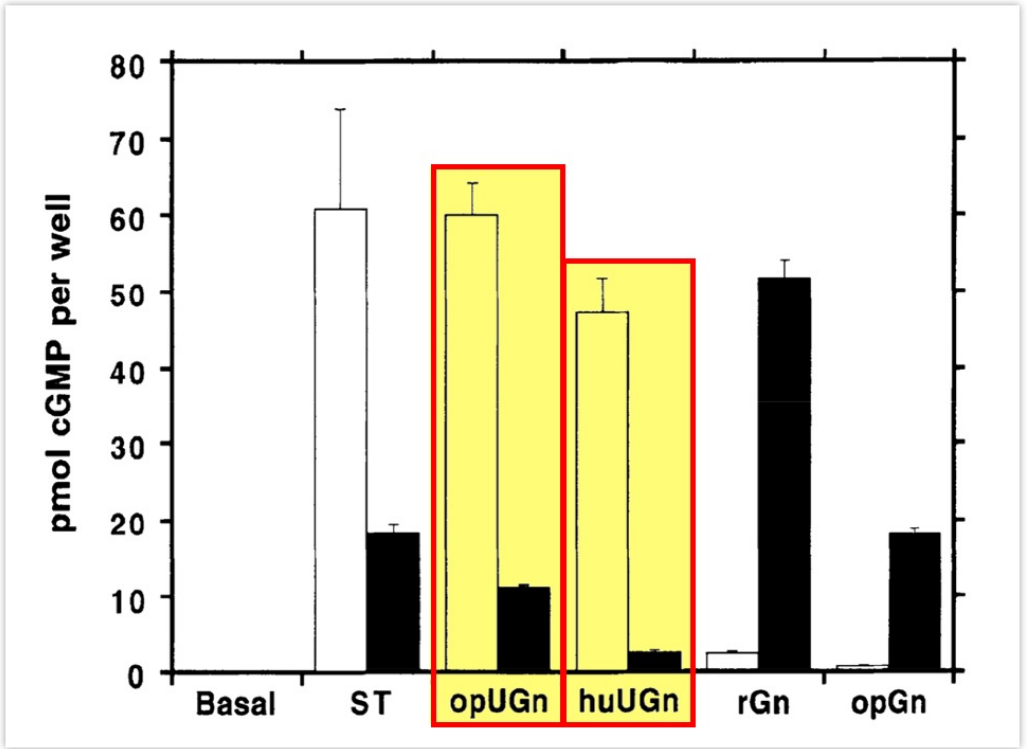
Hamra, F. Kent, William J. Krause, Sammy L. Eber, Ronald H. Freeman, Christine E. Smith, Mark G. Currie, and Leonard R. Forte. Opossum colonic mucosa contains uroguanylin and guanylin peptides. *Am. J. Physiol.* 270 (*Gastrointest. Liver Physiol.* 33): G708–G716, 1996.—Uroguanylin and guanylin are structurally related peptides that opossum urine (18) and was named on the basis of its structural and functional similarities to guanylin and its isolation from urine. Uroguanylin was confirmed as a second member in the guanylin peptide family by the subsequent isolation of opossum guanylin (18) and the

Fig. 2. Agonist-stimulated cGMP accumulation in T84 cells at pH 5.5 (open bars) and pH 8.0 (solid bars). Peptides and vehicle were suspended in HEPES and Dulbecco's modified Eagle's medium (DMEM) containing 50 mM sodium bicarbonate (pH 8.0), or 2-(*N*-morpholino)ethanesulfonic acid (MES) and DMEM at pH 5.5 (pH 5.5) for analysis in the T84 cell cGMP accumulation bioassay. Basal, vehicle control; ST, synthetic *E. coli* ST-(5—17); opGn, synthetic opossum guanylin; opUGn, synthetic opossum uroguanylin; huUGn, synthetic human uroguanylin; rGn, synthetic rat guanylin-(101—115). All peptides were tested at 30 nM except for *E. coli* ST-(5—17), which was tested at 3 nM. Error bars indicate standard error of the mean for 3 experiments.

intestine (21, 22). The opossum kidney also expresses high levels of GC-C-like receptors located in the apical membranes of proximal tubular cells (14). Guanylin was first isolated from rat jejunum as a heat-stable, 15-amino acid peptide that activated GC-C in human intestinal T84 cells (7). Guanylin cDNAs encoding 115- to 116-amino acid precursors have been isolated from rat, human, and mouse intestine (19). Uroguanylin was initially purified as 13- to 15-amino acid peptides from

urine of opossums, humans, and rats could be derived from the kidney and/or from other tissues via filtration of uroguanylin from the circulation. In the current study, we have isolated uroguanylin and guanylin peptides from the colonic mucosa of opossums. Several independent analytical techniques were used to identify the bioactive peptides. Thus intestinal mucosa is a potential tissue source for uroguanylin and guanylin found in urine (10, 18, 20).

0193-1857/96 $5.00



Ex. 1019 at G710 (Fig. 2)
Patent Owner's Resp. at 48