Gastrin Releasing Peptide Antagonists with Improved Potency and Stability[1]

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Gastrin releasing peptide (GRP) is a 27 amino acid peptide hormone which is homologous to the amphibian peptide bombesin. Two series of novel GRP antagonists were developed by C-terminal modification of *N*-acetyl-GRP-20–27 amide. Peptide derivatives within each series resist enzymatic degradation in serum and exhibit strong affinity for the GRP receptor. The first series of compounds replaces the Leu[26]-Met[27] region of GRP with an alkyl ether moiety. One member of this series, *N*-acetyl-GRP-20–25-NH-[(*S*)-1-ethoxy-4-methyl-2-pentane], specifically blocked radiolabeled GRP binding with an IC_{50} of 6 nM. In the second series of antagonists the oxygen of the ether moiety is replaced with a methylene group, resulting in GRP antagonists which are equipotent to native GRP in receptor binding assays (IC_{50} = 2 nM) and are also resistant to proteolytic degradation in vitro. All of the C-terminally modified peptides tested blocked GRP-stimulated mitogenesis in Swiss 3T3 mouse fibroblasts. Representative compounds also blocked GRP-induced elevation of $[Ca^{2+}]_i$ in human SCLC cells, and inhibited GRP-independent release of gastrin in vivo.

Introduction

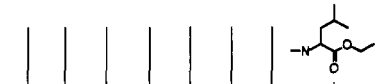
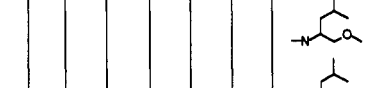
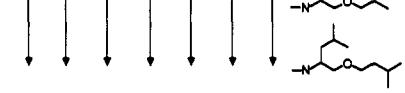
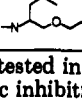
Gastrin releasing peptide (GRP) is a peptide hormone containing 27 amino acids which is structurally analogous to the amphibian peptide bombesin.[2] GRP stimulates a wide variety of biological responses in different tissues and cell lines, including mitogenesis in 3T3 mouse fibroblasts and the release of gastrin into the systemic circulation of rats and dogs.[3-6] GRP has also been proposed to play a central role in the pathophysiology of small cell lung cancer (SCLC) via an autocrine growth mechanism.[7-11] In addition, GRP may be involved in the formation of gastric ulcers,[12] pancreatic dysfunction,[13] and cystic fibrosis.[14] These observations suggest that GRP antagonists may have clinical utility as inhibitors of the pathophysiological response to GRP in human diseases.

A number of studies describing the preparation of peptide-based antagonists of GRP have been published.[15-20] One potent GRP antagonist is *N*-acetyl-GRP-20–26-OCH_2CH_3, with an IC_{50} ca. 2-fold higher than that of GRP itself in competitive binding inhibiition assays.[19] This derivative was shown to antagonize the biological activity of GRP in vitro in SCLC cells and in vivo in rats. Unfortunately, this peptide was found to rapidly degrade in human serum, which severely limits the peptide's potential clinical utility. The current report describes modifications of the C-terminal portion of this molecule, resulting in novel antagonists with improved receptor binding potency and improved stability in human serum.

Results

Alkyl Ether Derivatives. The GRP antagonist *N*-acetyl-GRP-20–26-OCH_2CH_3 (compound 2 in Table I), contains a C-terminal ester moiety which is essential to its activity.[19] When the stability of this antagonist in human serum in vitro was monitored by HPLC, the peptide was observed to degrade with a half-life of ca. 30 min (Figure 1). One degradation product expected to result from the action of serum esterases, *N*-acetyl-GRP-20–26-OH, was detected in the reaction mixture (data not shown). For this reason, it was anticipated that replacement of the ester functionality with a moiety less susceptible to hydrolysis might yield an antagonist with improved serum stability.

Table I. Activity of GRP Alkyl Ether Derivatives in Swiss 3T3 Fibroblasts[a]

#	Structures	Binding Inhibition (nM)	Mitogenic Stimulation (nM)	Mitogenic Inhibition (nM)
1	Ac-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	1.8	0.20	–
2		3.9	–	20
3		81	–	300
4		8.3	–	91
5		6.2	–	31
6		3.2	–	18
7		11	–	32
8	Ac-His-Trp-Ala-Val-Ala-His-Leu-Met-NH_2	180	20.0	–
9	D-Ala -Leu-Met-NH_2	3.2	0.20	–
10	Ala 	410	–	> 300
11	D-Ala 	5.0	–	35

[a] The peptide derivatives were tested in binding inhibition, mitogenic stimulation, and mitogenic inhibition assays in Swiss 3T3 fibroblasts as described in the Experimental Section. Numerical values represent the mean IC_{50} (binding inhibition and mitogenic inhibition) or EC_{50} (mitogenic stimulation) for at least two independent experiments.

Replacement of the C-terminal carbonyl of *N*-acetyl-GRP-20–26-OCH_2CH_3 with a methylene group yields an

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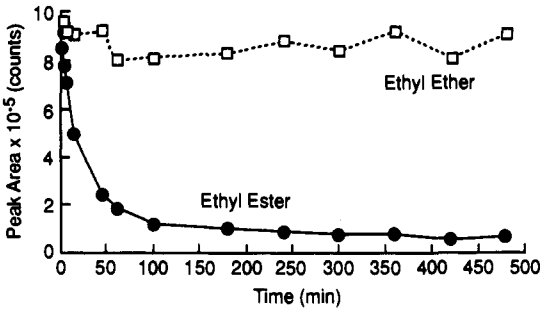

Figure 1. Degradation of compound 2 and compound 5 in human serum in vitro. Each peptide was incubated in human serum at 37 °C as described in the Experimental Section. Aliquots were withdrawn at various times and deproteinized, and the concentrations of compounds 2 (●) and 5 (□) were determined by HPLC.

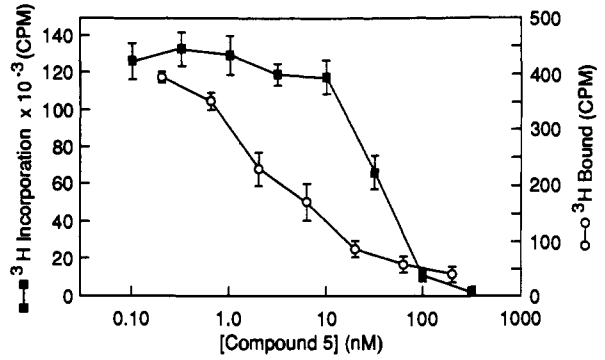

Figure 2. Inhibition of GRP binding and GRP-dependent mitogenesis in 3T3 mouse fibroblasts by compound 5. The peptide was titrated against 2 nM [^{3}H-Phe^{15}]GRP-15-27 amide (competitive binding inhibition, ○, right *Y* axis) or 3 nM *N*-acetyl-GRP-20-27 amide (mitogenic inhibition, ■, left *Y* axis). Error bars represent the standard error of the mean for triplicate samples in a single experiment. Independent binding inhibition titrations were performed at least twice for each compound.

ethyl ether GRP derivative, *N*-acetyl-GRP-20-25-NH-[(*S*)-1-ethoxy-4-methyl-2-pentane] (compound 5). This compound displays potency comparable to that of the ethyl ester derivative in competitive binding inhibition assays (IC_{50} = 6.2 ± 2.3 nM) (Figure 2). However, the ether derivative exhibits no detectable degradation in human serum in vitro (Figure 1), suggesting that a critical step in the degradation of *N*-acetyl-GRP-20-26-OCH_2CH_3 in human serum is the hydrolysis of the C-terminal ester functionality.

A series of ether derivatives were prepared to optimize the antagonist activity. The methyl (4), propyl (6), and isopentyl (7) ether derivatives all bind to the GRP receptor (Table I). As seen with the ester-containing antagonists,[19] the propyl derivative appeared to be the most potent member of this series. The corresponding alcohol, compound 3, was considerably less effective at blocking GRP binding to its receptor (binding inhibition IC_{50} = 81 ± 19 nM) than the ether derivatives examined. The specificity of these antagonists for the GRP receptor was verified by demonstrating that the propyl ether derivative does not inhibit the binding of radiolabeled epidermal growth factor (EGF) to its receptor at concentrations as high as 10 μM.[21]

Role of β-Turn Configuration. The conservation of glycine at position 24 in GRP and at the homologous residue in bombesin suggests that it may have a structural role in establishing a β-turn configuration.[4,17] If it exists, the β-turn configuration may contribute either to high-affinity binding of GRP to its receptor or to the initiation of a functional response by the receptor. To investigate the role of a possible β-turn structure in GRP, we prepared a number of GRP derivatives with amino acid substitutions at the critical Gly^{24} residue. Replacement of a glycine in a β-turn with L-alanine should destabilize this conformation, due to unfavorable steric interactions.[22] When this substitution was performed on *N*-acetyl-GRP-20-27 amide, the resulting peptide displayed dramatically reduced affinity for the GRP receptor (compound 8), but retained the functional agonist activity (mitogenic stimulation) of GRP (see Biological Activities). Substitution of the glycine with D-alanine, which should not interfere with formation of a β-turn, yields a peptide (compound 9) which is virtually equipotent to GRP in both receptor binding and mitogenic stimulation assays. These results imply that, as has been previously suggested,[4,17] adoption of a β-turn configuration may be an important factor in maintaining high-affinity binding of GRP to its receptor.

To investigate whether similar conformational factors contribute to receptor binding of the ethyl ether antagonist, analogous substitutions were made in compound 5. Similar to the results described above, the D-Ala^{24} derivative (compound 11) bound to the receptor with ca. 60-fold greater potency than the L-Ala^{24} peptide (compound 10). The D-Ala^{24} alkyl ether derivative also retained mitogenic antagonist activity. These data suggest that a β-turn structure may be important in high-affinity binding of both

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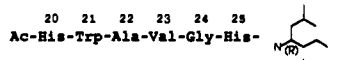
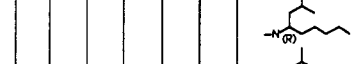
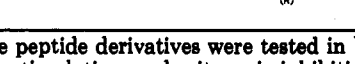
Table II. Activity of GRP Alkylamide Derivatives in Swiss 3T3 Fibroblasts[a]

#	Structures	Binding inhibition (nM)	Mitogenic stimulation (nM)	Mitogenic inhibition (nM)
12		2.6	–	10
13		4.9	–	35
14		1.4	–	3
15		2.4	–	6
16		2.0	–	4
17		11	–	22
18		2.8	–	7
19		16	–	80
20		1.2	–	8
21		3.3	–	11
22		4.1	0.20	–
23		1.8	–	0.6

[a] The peptide derivatives were tested in binding inhibition, mitogenic stimulation, and mitogenic inhibition assays in Swiss 3T3 fibroblasts as described in the Experimental Section. Numerical values represent the mean IC_{50} (binding inhibition and mitogenic inhibition) or EC_{50} (mitogenic stimulation) for at least two independent experiments.

GRP agonists and antagonists to GRP receptors on murine fibroblasts. However, the presence or absence of amino acids at position 24 conducive to β-turn formation does not appear to be critical to determining whether a GRP derivative is a functional agonist or an antagonist.

Alkylamide Derivatives. The alkylamide derivatives are structurally similar to the alkyl ether antagonists described above, except that the ether oxygen is replaced with a methylene group. This alteration is expected to change both the polarity and the bond angles of the C-terminal region of the modified GRP peptide. Since the sequence rule for specification of absolute configuration is highly dependent upon the presence of heteroatoms, the stereochemical nomenclature of the alkylamide derivatives is reversed compared to the nomenclature of the ether antagonists and Leu[26] of GRP itself. Therefore, the α-carbon configuration of the (*R*)-alkylamides is the same as that of (*S*)-leucine.

The (*R*)-alkylamide derivatives demonstrate even higher affinity for the GRP receptor than the corresponding ether derivatives (Table II). For instance, *N*-acetyl-GRP-20–25-NH-[(*R*)-2-methyl-4-octane] (compound **14**), which is an approximate isostere of compound **5**, exhibits an IC_{50} of 1.4 ± 0.3 nM in the competitive binding inhibition assay. This value is comparable to that of GRP itself and is ca. 4 times better than that of the corresponding ether antagonist, compound **5**. In addition, no degradation of this derivative was detected in human serum in vitro (data not shown). The effect of variation of the chain length of the alkylamide antagonists is less dramatic than with the ether and ester antagonists.[19] The (*R*)-alkylamide antagonist in this series exhibited IC_{50}s ranging from 1.4 to 2.9 nM in the binding inhibition assay (compounds **12**, **14**, **16**, **18**).

Effects of Stereochemical and N-Terminal Modification. The alkylamide antagonists outlined above are structurally homologous to the ether and ester-based GRP antagonists. The role of structural features in the C-terminal and N-terminal domains of these antagonists were investigated to better define the structure–function relationship. The effect of reversing the stereochemical configuration at the "α-carbon" of the modified Leu[26] residue was investigated by preparing a series of (*S*)-alkylamide derivatives. The (*S*)-2-methyl-4-heptyl (compound **13**) and (*S*)-2-methyl-4-nonyl (compound **17**) derivatives displayed 3–10-fold lower affinities for the GRP receptor than the corresponding (*R*)-alkylamides. Both of the *S* derivatives were mitogenic antagonists (see Biological Activities). This loss of potency is compatible with earlier reports indicating reduced biological activity of GRP-related [(*R*)-Leu[26]] peptides.[4] The specificity of this class of GRP antagonists is maintained irrespective of stereochemistry, however, since compound **15** did not inhibit binding of EGF to its receptor at concentrations up to 10 μM. Several symmetrical GRP-alkylamide peptides were also prepared (Table II). The 4-heptyl (compound **19**) and 5-nonyl (compound **21**) derivatives were all less potent in the competitive binding inhibition assay than compound **14**, while the 2,6-dimethyl-4-heptyl antagonist (compound **20**) displayed comparable activity.

The effect of modifying the N-terminal blocking group was evaluated with compounds **22** and **23**. Previous studies have demonstrated that the positive charge on the N-terminal amino group of GRP-20–27 amide must be suppressed by acetylation or a similar modification to obtain high-affinity binding to the GRP receptor.[19,23] Because of the potential lability of the N-acetyl group in vivo, the *N*-pivaloyl derivatives of GRP-20–27 amide and compound **16** were prepared. The results presented in Table II demonstrate that the peptides with alternative N-terminal blocking groups display affinity for the GRP receptor comparable to those of their N-acetylated counterparts.

Biological Activities. To determine whether the alkyl ether and alkylamide binding antagonists were also functional antagonists of GRP's biological activities, three types of assays were performed. All 23 compounds listed in Tables I and II were tested in mitogenesis or mitogenic inhibition assays on Swiss 3T3 murine fibroblasts in cell culture. Each of the alkyl ether (compounds **4–7**, **10**, **11**) and alkylamide (compounds **12–21**, **23**) derivatives behaved as antagonists of GRP-stimulated mitogenesis (Tables I and II). The relative potencies of these compounds as antagonists of GRP's biological activities were commensurate with their IC_{50}s as receptor binding antagonists. A representative study examining mitogenic inhibition is shown in Figure 2 for compound **5**.

To ensure that the alkyl ether and alkylamide compounds also behaved as functional antagonists of GRP activity in human cells, representative compounds from the alkyl ether and alkylamide series were selected for additional testing. Ethyl ether **5** and (*R*)-2-methyl-4-nonyl alkylamide **16** were assayed for their ability to block GRP-dependent elevation of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in H345 SCLC cells in vitro.[19,24,25] As previously reported, the addition of 100 nM GRP causes

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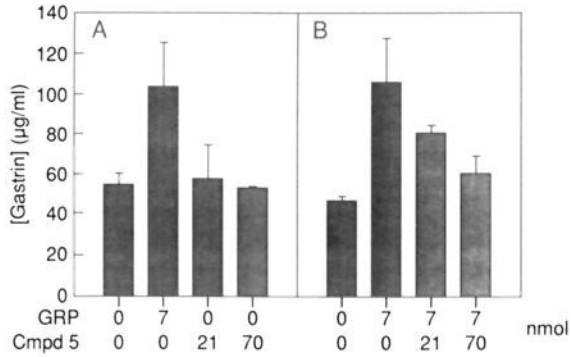

Figure 3. Inhibition of GRP-dependent elevation of serum gastrin concentrations in rats by compound **5**. Panel A: saline (bar 1), GRP (bar 2), or compound **5** (bars 3 and 4) were each administered to four rats at the doses indicated. Serum gastrin concentrations were measured in duplicate for each rat by radioimmunoassay. Bars represent the mean serum gastrin concentration for each group of four rats, and error bars represent the standard error of the mean. Panel B: saline (bar 1), GRP (bar 2), or GRP plus compound **5** (bars 3 and 4) were each administered to four rats at the doses indicated. Determination of serum gastrin levels and statistical analysis was the same as for panel A.

a transient increase in $[Ca^{2+}]_i$. Addition of compounds **5** or **16** at concentrations up to 1 μM ellicited no $[Ca^{2+}]_i$ response in H345 cells. Treatment of SCLC cells with these derivatives blocked the GRP-dependent elevation of $[Ca^{2+}]_i$. The IC_{50}s for inhibition of intracellular calcium elevation were 250 and 150 nM for compound **5** and compound **16**, respectively (data not shown). These results demonstrate that both the ether and alkylamide derivatives are functional GRP antagonists in human SCLC cells.

Finally, a single compound was examined to determine if these derivatives behaved as functional GRP antagonists in vivo. An ethyl ether derivative (compound **5**) was assayed for its ability to block GRP-stimulated elevation of serum gastrin concentrations in rats.[19] Intraperitoneal administration of 7 nmol of GRP to Sprague–Dawley rats elicits a 3-fold increase in serum gastrin. Coadministration of compound **5** with 7 nmol of GRP reduced the GRP-dependent elevation of serum gastrin in a dose-dependent fashion (Figure 3). These results demonstrate that compound **5** is a GRP antagonist in rats in vivo.

Discussion

Gastrin releasing peptide is a multifunctional hormone, eliciting a variety of responses in different tissue types. Several of these responses have been hypothesized to contribute to the pathophysiology of various human diseases. Monoclonal antibodies directed against the amphibian homologue of GRP, bombesin, have been reported to inhibit the growth of SCLC cells in vitro and in vivo.[10,11] In addition, GRP immunoreactivity has been detected in tracheobroncheal secretions of patients with cystic fibrosis, and GRP may serve as an inflammatory mediator in this disease.[13] Gastrin releasing peptide also acts as a secretagogue for several small peptide hormones including gastrin, cholecystokinin, and pancreatic polypeptide.[26] This activity may contribute to the formation or maintenance of gastric ulcers and pancreatitis. An effective GRP antagonist may be clinically useful in the treatment of one or more of these diseases, as well as in clarifying the specific contributions this peptide makes to various disease states.

Many of the peptide-based GRP antagonists developed to date have suffered from a lack of specificity, poor receptor binding potency, or physical instability in serum.[15-19] One potent antagonist is *N*-acetyl-GRP-20–26-OCH_2CH_3, which has an IC_{50} only 3-fold higher than GRP itself in competitive GRP binding inhibition assays.[19] In the current study, we found that the C-terminal ester moiety of this antagonist was vulnerable to serum esterase activity. Hydrolysis of this ester moiety was associated with a loss of receptor binding activity. Reduction of the carbonyl to a methylene group appears to eliminate serum degradation of the antagonist in vitro, with no loss of receptor binding potency. These data suggest that the ester carbonyl contributes relatively little to high affinity binding of the peptide to the GRP receptor. The ether oxygen can also be replaced by a methylene group, with an additional 3-fold increase in binding affinity. The optimal length of the C-terminal moieties attached to the histidine at position 25 are approximately the same for the ester, ether, and alkylamide antagonists[19] and may mimic the α-carbon backbone of the Leu[26]-Met[27] portion of native GRP.

Previous work has suggested that a β-turn configuration in the structure of GRP might be important for receptor binding activity. Our data indicate that substitutions for the glycine at position 24 which would reduce the tendency of the peptide to adopt a β-turn configuration cause a significant reduction in receptor binding affinity in both *N*-acetyl-GRP-20–27 amide and the ethyl ether antagonist (compound **5**). This substitution pattern in GRP agonists and antagonists is analogous to results observed with luteinizing hormone releasing hormone and suggests that a type II or type II′ β-turn configuration is important for high-affinity binding of GRP to its receptor.[27] Peptide–receptor interactions may play an important role in stabilizing the active conformation of GRP, since compound **5** lacks two C-terminal carbonyls which might normally stabilize a β-turn in GRP. However, the presence or absence of amino acids at position 24 conducive to β-turn formation does not appear to be critical to determining whether a GRP derivative is a functional agonist or an antagonist.

Receptor labeling studies suggest that the gross molecular weight and level of glycosylation of GRP receptors is similar in murine fibroblasts and human SCLC cells.[28,29] The relative affinities of our antagonists for GRP receptors on mouse 3T3 cells, rat gastrointestinal cells, and human SCLC cells are similar for the ester, ether, and alkylamide compounds.[19] While relatively few compounds have been tested in all three systems, these results suggest that the critical binding domains of GRP and the importance of the C-terminal portion for initiation of a biological response are conserved in each of these GRP receptor systems.

We have described the development of GRP antagonists which are equipotent to native GRP in competitive binding inhibition assays, stable in human serum in vitro, and block GRP-stimulated effects in human SCLC cells in vitro and in murine tissues in vivo. Previous reports suggest that these types of antagonists may be useful in slowing or arresting the growth of SCLC cells in vitro and in vivo.[7-11] In addition to the potential clinical utility of GRP antagonists, the compounds reported here provide significant new tools to unravel the structure–function–activity relationships between GRP and its receptor. The localization of the "biological trigger" (i.e. receptor activation) domain

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to the C-terminus of GRP, and the relatively minor structural modifications required to generate potent antagonists from the parent hormone, may provide a general starting point for the development of potent antagonists for other C-terminal amidated peptide hormones.[30]

Experimental Section

Preparation of Ligands. Peptide ligands were synthesized in an Applied Biosystems Model 430A peptide synthesizer by standard solid-state methodology on benzhydrylamine or (aminoacyl)(phenylacetamido)methyl resins using commercially available Boc-amino acids containing the normal side chain protecting groups.[31-33] Histidine was used in the form of *N*-α-Boc-*N*-π-[(benzyloxy)methyl]-L-histidine, and tryptophan was in the form of *N*-Boc-Trp. Dithioerythreitol was included in the trifluoroacetic acid deprotection reagent at a concentration of 0.5%. Cleavage and deprotection of the resin-bound peptide was achieved by treatment with liquid HF (20 mL/g of resin) containing anisole (2 mL/g of resin) as a scavenger. Purification of the crude material by reverse-phase HPLC on a Vydac C-18 support yielded a product whose purity was >98% by analytical HPLC.[34] Quantitative amino acid analysis gave the correct ratios for the given sequence. Molecular weight determination by fast-atom bombardment mass spectrometry provided confirmation of structure.

Alkylamide derivatives (compounds 12–21) were synthesized by coupling the appropriate His-alkylamide to *N*-acetyl-GRP-20–24-OH with dicyclohexylcarbodimide and 1-hydroxybenzotriazole in dimethylformamide. Since the glycine at position 24 of GRP is not optically active, this strategy ensures that racemization will not occur during the final coupling step. A typical synthesis is described in detail below.

***N*-Acetyl-GRP-20–25-(*R*)-2-methyl-4-nonanylamide (16).** A solution of *N*-Ac-GRP-20–25 (55.8 mg, 75 μmol), 1-hydroxybenzotriazole hydrate (16.1 mg, 105 μmol), (*R*)-2-methyl-4-nonanyl-(*S*)-histidinamide dihydrochloride (55 mg, 150 μmol), dicyclohexylcarbodiimide (31.1 mg, 150 μmol), and triethylamine (55 μL, 393 μmol) in DMF (15 mL) was stirred at room temperature under N_2 for 4 days. After concentrating under reduced pressure to remove DMF, the residue was triturated with distilled water three times. The aqueous extracts were combined, filtered, washed with diethyl ether, and refiltered, giving a total volume of 20 mL. Lyophilization gave a white solid which was purified by preparative HPLC to yield 12 mg of product. Amino acid analysis showed 61.3% peptide and the correct amino acid content. Mass spectral analysis yielded M + 1 = 887.7 *m/e* (calculated M = 886).

The histidinamide component was prepared by treatment of the corresponding bis-Boc derivative with dry HCl in ethyl acetate at 0 °C. The bis-Boc-His-alkylamide was obtained by activation of N_α,N_{im}-bis-Boc-histidine with equimolar amounts of isobutyl chloroformate and 4-methylmorpholine in ethyl acetate. After 15 min at 0 °C under nitrogen, an equimolar amount of (*R*)-2-methyl-4-aminononane hydrochloride was added, followed by an equivalent of 4-methylmorpholine. After 20 h, the product was extracteed and isolated by flash chromatography over silica gel.

Chiral amines were prepared by the method of Saari and Fisher.[35] Specifically, (*R*)-2-methyl-4-aminononane was prepared by catalytic hydrogenation of (*S*)-2-methyl-4-(Boc-amino)-5-nonene over a 5% palladium catalyst in ethanol followed by deprotection with HCl in ethyl acetate. (*S*)-2-Methyl-4-(Boc-amino)nonene was obtained by Wittig condensation of S-(2)-methyl-4-(Boc-amino)pentanal with *n*-butyltriphenylphosphonium bromide and potassium hexamethyldisilazane in tetrahydrofuran. The product was isolated by flash chromatography over silica gel.

The alkyl ether derivatives (compounds 2–7, 10, 11) were synthesized by coupling *N*-acetyl-GRP-20–24-OH with the appropriate C-terminal fragment as described for compound 16 above. The C-terminal fragment of the molecule was prepared by a procedure similar to that described below for the preparation of *N*-[(*S*)-1-ethoxy-4-methyl-2-pentyl]-(*S*)-histidinamide, which yields compound 5 upon coupling to *N*-acetyl-GRP-20–24-OH.

N-[(*S*)-1-ethoxy-4-methyl-2-pentyl]-(*S*)-histidinamide was prepared from the bis-Boc derivative as described above. *N*-[(*S*)-1-ethoxy-4-methyl-2-pentyl]-N_α,N_{im}-bis-Boc-(*S*)-histidinamide was obtained by condensation of N_α,N_{im}-bis-Boc-(*S*)-histidinamide with (*S*)-1-ethoxy-2-amino-4-methylpentane as described for the alkane analogue. (*S*)-1-ethoxy-2-amino-4-methylpentane was prepared by reaction of the sodium salt of (*S*)-leucinol with ethyl iodide.[36]

N-Pivaloyl peptides were prepared by deprotecting the appropriate *N*-Boc peptide with HCl in ethyl acetate, followed by reaction of the isolated peptide with pivaloyl anhydride. [^{3}H-Phe^{15}]GRP-15–27 amide was prepared as previously described.[37] All other chemicals were obtained from standard commercial sources.

Biological Assays. The degradation of GRP peptides in human serum was monitored by HPLC. Briefly, the peptide was added at a final concentration of 1 μM to human serum obtained from volunteers. The mixture was incubated at 37 °C, and aliquots withdrawn at various times. The serum was deproteinized by the addition of perchloric acid to a final concentration of 4%, chilled, and the precipitate removed by centrifugation. The peptide concentration was assayed by reverse-phase HPLC on a Vydac C-18 column.

Binding inhibition, mitogenic stimulation, and mitogenic inhibition assays were performed essentially as described.[19,37] Briefly, competitive binding inhibition data were measured on confluent monolayers of Swiss 3T3 fibroblasts maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine and 1% penicillin–streptomycin. The radioligand, [^{3}H-Phe^{15}]GRP-15–27 amide, was used at a final concentration of 2 nM. Binding inhibition experiments were performed at 15 °C. Maximal binding was observed 1–2 h after addition of the radioligand. Approximately 90–95% of the total counts bound were competed off by excess unlabeled GRP. Each experiment was performed in triplicate, in at least two separate trials. Mitogenic stimulation and mitogenic inhibition data were obtained on serum-starved 3T3 fibroblast monolayers. Confluent monolayers of Swiss 3T3 cells grown in 24-well plates were placed in serum-free DMEM for 48 h, at which time the peptides and 23 nM [^{3}H]thymidine were added. After an additional 48 h, cells were removed from the plate with 1 mL of 10× trypsin containing 5 mM EDTA. The cells were harvested with a Skatron filter apparatus (Skatron, Inc., Sterling VA). A 50-fold stimulation of [^{3}H]thymidine uptake was typically observed upon treatment of these cells with 3 nM GRP or 3 nM *N*-acetyl-GRP-20–27 amide. Mitogenesis inhibition studies were conducted by coadministration of the antagonist and 3 nM *N*-acetyl-GRP-20–27 amide.

Measurement of $[Ca^{2+}]_i$ flux in SCLC cells was performed essentially as described.[19,24,25] H345 human SCLC cells were obtained from E. Sausville (Georgetown University Medical Center, Washington, DC). Approximately 10^8 cells, maintained

(30) Since the completion of the current work, two other relevant studies have been published. The first (Wang, L. H., et al. *Biochemistry* **1990**, *29*, 616–622.) describes a series of potent [D-Phe^6]bombesin(6–13) alkylamide antagonists. The second (Wang, L. H.; et al. *J. Biol. Chem.* **1990**, *265*, 15695–15703) indicates that some [D-Phe^6]bombesin(6–13) alkylamide peptides exhibit partial agonist activity in some biological systems. The *N*-acetyl-GRP-20–25 alkyl ethers and alkylamides described in the current study did not exhibit significant agonist activity in the Swiss 3T3 cell mitogenesis assay at concentrations up to 316 nM.

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in suspension culture as described,[24] were harvested by gravity, washed once with RPMI-1640 (R_0), and resuspended in 2 mL of R_0. Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) was added at a final concentration of 1.2 nmol/10^6 cells from a fresh 10 mM stock in DMSO. After 15 min at 37 °C, the cells were diluted to 10 mL and incubated at 37 °C for 1 h. The cells were then centrifuged and resuspended in HEPES-saline (140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.4) at a density of 5×10^6 cells/mL. The cells were kept on ice until used. Fluorescence measurements were performed at 37 °C in an Aminco SPF-500 fluorimeter with a stirred cell attachment. The excitation wavelength was 340 nm and the emission wavelength was 510 nm.

The effect of GRP antagonists on gastrin release in vivo was measured in rats essentially as described.[19,38,39] Female Sprague-Dawley rats (≃150 g) were anesthesized by intramuscular injection of ketamine. One hundred microliters of sample was then injected intraperitoneally. After 5 min, blood samples were taken by cardiac puncture and immediately stored on ice. Gastrin concentration in serum was measured in duplicate by radioimmunoassay (Cambridge Medical Diagnostics, Cambridge, MA). In a pilot time-course experiment, the maximal gastrin response was observed 5 min after injection of GRP (data not shown). Each data point represents the mean value obtained from four separate rats.

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Determination of Dissociation Constants of High Affinity (pM) Human Renin Inhibitors: Application to Analogues of Ditekiren (U-71,038)

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A fluorescent human renin inhibitor, dansyl-Phe-His-LVA-Ile-Amp (3, U-80,825), was synthesized and utilized in a fluorescence energy transfer displacement assay to determine the dissociation constants (k_d's) of a series of ditekiren analogues. These studies have indicated that (1) both the parent ditekiren (2) and compounds 8a-3 are *up to 1 order of magnitude more potent* than revealed by their IC_{50}'s, (2) these dissociation constants are in good agreement with the independently determined K_i's for compounds **2**, **3**, and **8d**, and (3) the lower limit of the fluorescence energy transfer displacement assay has been extended beyond the picomolar range. It has therefore been suggested that many examples of underestimation of renin inhibitory activity may exist in the renin literature which could be discovered and rectified by using the methodology described herein.

The renin-angiotensin cascade continues to be a major focus in the search for new treatments for hypertension.[1] The therapeutic success of the angiotensin converting enzyme inhibitors has resulted in extensive efforts to synthesize and develop newer and potentially more selective drugs which act on this system,[2] primarily inhibitors of the aspartic proteinase renin. Indeed, numerous reports have appeared detailing the synthesis of potent (nanomolar) inhibitors of human renin.[3]

Experimental studies of protein inhibition by such high-affinity ligands are complicated by the difficulty in assaying such potent compounds. Specifically, inhibitors having dissociation constants (K_d's) in the nanomolar to subnanomolar range are particularly interesting to explore binding mechanisms and structure-activity relationships and to advance the investigation of the active-site chemistry of a proteins such as enzymes and receptors. This is especially true in research related to the potency assessment of peptide-based inhibitors of human renin (as well as for other enzymes) which are frequently expressed as IC_{50} values,[4] as determined by radioimmunoassay of angiotensin I (ANG I) generated in plasma as a result of angiotensinogen hydrolysis by plasma renin. These (IC_{50}) values are limited in sensitivity both by the accuracy with which the enzyme in the system can be determined and by the difficulty in working at sufficiently low levels of protein in order to be able to deal with very high affinity inhibitors. Thus, as is the case with renin, it is generally not possible to easily and accurately determine the IC_{50} values for large numbers of inhibitors whose activities fall below the 0.1 nM range. It has therefore been of interest to determine if the IC_{50}'s reported for this class of inhibitor are a true reflection of their inhibitory activity.

In an attempt to overcome these aforementioned difficulties, we developed a rapid and sensitive competitive displacement assay for the determination of renin inhibitor dissociation constants (K_d's) using fluorescence energy

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