Desmethionine Alkylamide Bombesin Analogues: A New Class of Bombesin Receptor Antagonists with Potent Antisecretory Activity in Pancreatic Acini and Antimitotic Activity in Swiss 3T3 Cells†

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ABSTRACT: Bombesin-related peptides have a large number of physiological functions as well as having an autocrine growth mechanism for the regulation of small cell lung cancer cells. In the present study we have synthesized 21 des-Met amide or alkylamide analogues of bombesin and compared their abilities to function as bombesin receptor antagonists in guinea pig pancreatic acini and Swiss 3T3 cells with those of the previously most potent antagonist described, [Leu$^{13}\psi$(CH$_2$NH)Leu^{14}]bombesin (analogue I). All des-Met analogues functioned as antagonists. Bn(1–13)NH$_2$ was approximately equipotent to I (K_i = 60–80 nM) whereas Bn(6–13)NH$_2$ was 30-fold less potent (K_i = 1800 nM). Formation of an ethylamide, Bn(6–13)ethylamide, increased the potency 30-fold such that this octapeptide was equipotent to I. The addition of a D-Phe^6 moiety to I did not change potency but caused a 30-fold increase in potency of Bn(6–13)NH$_2$ and a 8-fold increase in the potency of Bn(6–13)ethylamide (K_i = 16 nM). Additional studies of both NH$_2$- and COOH-terminal alterations in Bn(6–13)NH$_2$ demonstrated that the most potent antagonist was [D-Phe^6]Bn(6–13)propylamide (PA), having IC$_{50}$'s of 1.6 nM and 0.8 nM for bombesin-stimulated amylase release and Swiss 3T3 cell growth, respectively. Detailed studies of the most potent amide analogue, [D-Phe^6]Bn(6–13)NH$_2$, and alkylamide analogue, [D-Phe^6]Bn(6–13)PA, demonstrated that these analogues functioned as competitive antagonists and that their action was selective for the bombesin receptor. These results demonstrate that, as with CCK- and gastrin-related peptides, the C-terminal amino acid is important for initiating a biologic response but not essential for determining receptor affinity. Furthermore, the most potent des-Met analogue, [D-Phe^6]Bn(6–13)PA, is 30-fold more potent than any previously described bombesin receptor antagonist. This member of this new class of antagonists can be easily synthesized, offers fewer proteolytic degradation sites, and should be useful for in vivo studies.

Bombesin and structurally related naturally occurring peptides [gastrin-releasing peptide (GRP), neuromedin B, neuromedin C [GRP(18–27)]] have a wide range of biological responses. These include release of numerous gastrointestinal hormones (Ghatei et al., 1982), stimulation of pancreatic enzyme secretion (Jensen et al., 1988a), regulation of central nervous functions such as thermoregulation (Marki et al., 1981), and the ability to function as a growth factor in 3T3 mouse fibroblasts and small cell lung cancer cells (SCLC) (Cuttitta et al., 1985; Corps et al., 1985). Bombesin-related peptides have been proposed to have an autocrine growth mechanism in regulating growth of SCLC cells because both anti-bombesin antibodies and bombesin receptor antagonists have inhibited growth in vivo and in vitro (Cuttitta et al., 1985; Woll et al., 1988; Trepel et al., 1988; Mahoud et al., 1989). These observations suggest that bombesin receptor antagonists would have not only widespread utility in resolving the role of bombesin-related peptides in physiologic processes but also perhaps a clinical role as inhibitors of the pathophysiologic effect of bombesin in human disorders.

Previous studies have described a number of classes of bombesin receptor antagonists (Woll et al., 1988; Coy et al., 1988a; Heinz-Erian et al., 1987; Jensen et al., 1984, 1988b; Heimbrook et al., 1989a). The pseudotetradecapeptide [Leu$^{13}\psi$(CH$_2$NH)Leu^{14}]bombesin (Coy et al., 1988a) (analogue I) was the first bombesin receptor antagonist with sufficient potency (K_i = 50 nM) to be generally useful. This peptide has been shown to inhibit in vivo bombesin-stimulated gastric acid secretion in rats (Rossowski et al., 1989), autocrine signals in 3T3 cells (Woll et al., 1988), growth of certain strains of human SCLC (Trepel et al., 1988), basal growth of strains of human SCLC, and basal growth of NCI-N592 SCLC (Mahmoud et al., 1989). However, in various test systems bombesin has an affinity 15–50 times higher than analogue I; thus a more potent antagonist is preferable for possible in vivo studies.

Gastrin and CCK have a Phe amide at their COOH terminus, and in previous studies des-Phe amidated analogues of gastrin and CCK have been shown to function as potent antagonists or partial agonists (Spanarkel et al., 1983; Martinez et al., 1984). In the present study we have undertaken a similar strategy. We have coupled COOH alterations with D-amino acid substitutions for Asn^6 of bombesin because in a recent study (Saeed et al., 1987), we have found that substitutions in this position increase affinity of [D-Phe^{12}]bombesin receptor antagonists for the receptor. Our results demonstrate

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that [D-Phe^6]Bn(6–13)alkylamides are specific bombesin receptor antagonists with up to 30-fold greater activity than any known antagonists.

Experimental Procedures

Materials

Protected amino acids and other synthetic reagents were obtained from Advanced ChemTech, Louisville, KY. NIH-Hartley strain guinea pigs (150–200 g) were obtained from the Small Animals Section, Veterinary Resources Branch, NIH. HEPES was from Boehringer Mannheim Biochemicals, Indianapolis, IN; purified collagenase (type CLSPA, 440 units/mg) was from Worthington Biochemicals, Freehold, NJ; soybean trypsin inhibitor, carbamylcholine, theophylline, and bacitracin were from Sigma Chemical Co., St. Louis, MO; essential vitamin mixture (100× concentrated) was from Microbiological Associates, Bethesda, MD; glutamine and gastrin I(2–17) were from Research Plus Laboratories, Bayonne, NJ; Na^{125}I was from Amersham Searle, Arlington Heights, IL; [^{3}H]thymidine was from New England Nuclear, Boston, MA; Phadebas amylase test reagent was from Pharmacia Diagnostics, Piscataway, NJ; bovine plasma albumin (Fraction V) was from Miles Laboratories, Elkhart, IN; A23187 was from Calbiochem-Behring, La Jolla, CA; and vasoactive intestinal polypeptide (VIP), secretin, calcitonin gene related peptide (CGRP), gastrin-releasing peptide (GRP), C-terminal octapeptide of cholecystokinin (CCK-8), [Tyr^4]-bombesin, bombesin, and substance P were from Peninsula Laboratories, Belmont CA.

The standard incubation solution used in experiments involving pancreatic acini contained 24.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM theophylline, 1% (w/v) albumin, 0.01% (w/v) trypsin inhibitor, 1% (v/v) amino acid mixture, and 1% (v/v) essential vitamin mixture. The incubation solution was equilibrated with 100% O_2, and all incubations were carried out with O_2 as the gas phase.

Methods

Preparation of Peptides. Solid-phase syntheses of peptide amides, including introduction of the reduced peptide bond, were carried out by the standard methods described recently (Sasaki & Coy, 1987; Coy et al., 1988b) on methylbenzhydrylamine resin (Advanced ChemTech, Louisville, KY). Alkylamide analogues were synthesized on a standard Leu-*O*-polystyrene resin by using tosyl group protection for the imidazole group of His. Free peptides were then cleaved in good yield by treatment overnight with the appropriate amine. Analogue XXII (Table I), which has a free C-terminal COOH group, was also synthesized on the Leu-*O*-polystyrene resin followed by HF cleavage. The crude hydrogen fluoride cleaved peptides or the amine-cleaved alkylamides were then purified on a column (2.5 × 90 cm) of Sephadex G-25 and eluted with linear gradients of acetonitrile in 0.1% trifluoroacetic acid using an Eldex Chromatrol gradient controller (flow rate ca. 1 mL/min). Peptides were further purified by rechromatography on the same column with slight modifications to the gradient conditions when necessary. Homogeneity of the peptides was assessed by thin-layer chromatography and analytical reverse-phase high-pressure liquid chromatography and for each peptide purity was at least 97%. Amino acid analysis gave the expected amino acid ratios. The presence of the reduced peptide bond or C-terminal alkyl groups was demonstrated by fast atom bombardment mass spectrometry. Each of the 22 analogues gave good recovery of the molecular ions corresponding to the calculated molecular weights.

Tissue Preparation. Dispersed acini from guinea pig pancreas were prepared by using the modification (Jensen et al., 1982) of the method described previously (Peikin et al., 1978).

Amylase Release. Dispersed acini from one guinea pig pancreas were suspended in 150 mL of standard incubation solution, samples (250 mL) were incubated for 30 min at 37 °C, and amylase release was measured as described previously (Peikin et al., 1978; Gardner & Jackson, 1979). Amylase activity was determined by the method of Ceska et al. (1969a,b), using the Phadebas reagent. Amylase release was calculated as the percentage of amylase activity in the acini at the beginning of the incubation that was released into the extracellular medium during the incubation.

Effect of Peptides on Bombesin-Stimulated Amylase Release. Antagonist activity was determined as described previously (Jensen et al., 1988b). Various concentrations of peptides were incubated alone or with 0.3 nM bombesin, a concentration that causes half-maximal stimulation, which in the present studies was a 10-fold increase over basal secretion.

Binding of ^{125}I-Labeled [Tyr^4]Bombesin to Acini or *3T3 Fibroblasts.* ^{125}I-[Tyr^4]bombesin (2000 Ci/mmol) was prepared by using the modification (von Schrenck et al., 1989) of the method described previously (Jensen et al., 1988b). ^{125}I-[Tyr^4]bombesin was separated from ^{125}I by using a SepPak and from unlabeled peptide by reverse-phase high-pressure liquid chromatography on a column (0.46 × 25 cm) of μBondapak C_{18}. The column was eluted isocratically with acetonitrile (22.5%) and triethylammonium phosphate (0.25 M, pH 3.5) (77.5%) at a flow rate of 1 mL/min. Binding of ^{125}I-[Tyr^4]bombesin to 3T3 cells or pancreatic acini was performed as reported previously (Jensen et al., 1978, 1988b). Incubations contained 0.05 nM ^{125}I-[Tyr^4]bombesin and were for 60 min at 37 °C for pancreatic acini and for 30 min at 22 °C for 3T3 cells. Nonsaturable binding of ^{125}I-[Tyr^4]-bombesin was the amount of radioactivity associated with the acini or 3T3 cells when containing 0.05 nM ^{125}I-[Tyr^4]bombesin plus 1 μM bombesin. Nonsaturable binding was <10% of total binding in all experiments. All values in this paper are for saturable binding, i.e., total binding minus nonsaturable binding.

Growth of Swiss 3T3 Fibroblasts. Stock cultures of Swiss 3T3 cells (kindly provided by Dr. E. Rozengurt, Imperial Cancer Research Fund) were grown and assayed for [^{3}H]-thymidine incorporation as described previously (Coy et al., 1988a; Dicker & Rozengurt, 1980; Rozengurt & Sinnet-Smith, 1983) in the presence of bombesin (3 nM) and several concentrations of selected antagonist analogues.

Results

To determine whether the 22 bombesin analogues synthesized functioned as antagonists, the initial screening was for their effects on basal and bombesin-stimulated amylase release from guinea pig pancreatic acini which have been shown to exhibit a well-characterized bombesin receptor mediating stimulation of enzyme secretion (Jensen et al., 1978, 1988a). Each peptide when present at concentrations up to 10 μM produced no enzyme stimulation, whereas 10 nM bombesin caused a 10-fold increase over basal. However, each bombesin analogue inhibited 0.3 nM bombesin stimulated amylase release. The dose–response curves for selected analogues are shown in Figure 1 and the calculated IC_{50} values for all analogues in Table I. Dose–inhibition curves for each selected analogue on binding of ^{125}I-[Tyr^4]bombesin to pan-

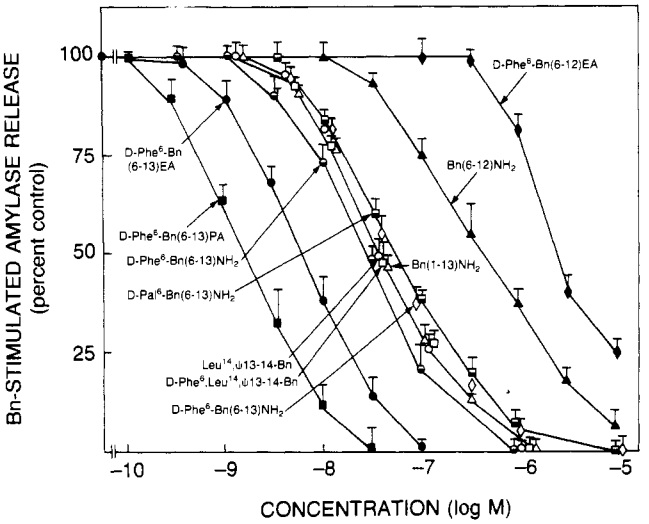

FIGURE 1: Ability of various bombesin analogues to inhibit bombesin-stimulated amylase release from pancreatic acini. Pancreatic acini were incubated with 0.3 nM bombesin either alone or with the indicated concentrations of the analogues. Amylase release is expressed as the percentage of the stimulated release caused by 0.3 nM bombesin with no analogue present. The control and 0.3 nM bombesin stimulated release were 2.4 ± 0.1% and 22.7 ± 3.5% of the total cellular amylase (n = 4), respectively. Each value is the mean ± SEM of four separate experiments.

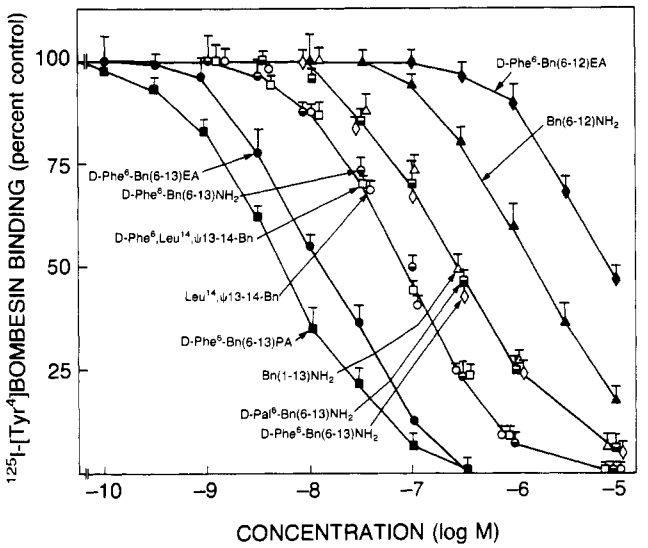

FIGURE 2: Ability of various bombesin analogues to inhibit binding of ^{125}I-[Tyr^4]bombesin to pancreatic acini. Acini were incubated with 50 pM ^{125}I-[Tyr^4]bombesin either alone or with 1 μM bombesin or the indicated concentrations of the bombesin analogues as described under Methods. Results are expressed as the percentage of saturable binding with no analogue present (i.e., percent control). Values are means ± SEM of five separate experiments.

creatic acini are shown in Figure 2 and the calculated K_i values for all analogues in Table I. Analogues in Table I were divided into three main groups, containing analogues representing full-sequence analogues based on bombesin (I–V), short-sequence des-Met amides of bombesin with or without D-amino acid substitutions in position 5 or 6 (VI–XVI), and short bombesin des-Met analogues which are either alkylamides or substitutions for the amide and which are primiarly with D-amino acid substitutions in position 6 (XVII–XXII).

In general, there was a close relative correlation between the abilities of the various bombesin analogues to inhibit bombesin-stimulated amylase release (Figure 1, Table I) and binding of ^{125}I-[Tyr^4]bombesin (Figure 2, Table I). The des-Met amide analogue of bombesin itself was an antagonist

Table I: Comparison of the Ability of Bombesin Analogues To Inhibit Bombesin-Stimulated Amylase Release from Pancreatic Acini and Inhibition of Binding of ^{125}I-[Tyr^4]bombesin[a]

		IC_{50} or K_i (nM)	
no.	analogue	inhibition of 0.3 nM Bn stimulated amylase release	binding of ^{125}I-[Tyr^4]-bombesin
I	[Leu^{14}, ψ13–14]Bn	31 ± 4	60 ± 6
II	[D-Phe^6,Leu^{14},ψ13–14]Bn	31 ± 8	70 ± 6
III	Bn(1–13)NH$_2$	33 ± 10	216 ± 30
IV	[D-Phe^6]Bn(1–13)NH$_2$	41 ± 23	239 ± 21
V	[D-Ala^5]Bn(1–13)NH$_2$	170 ± 25	412 ± 87
VI	Bn(6–13)NH$_2$	610 ± 230	1796 ± 310
VII	[D-Phe^6]Bn(6–13)NH$_2$	24 ± 11	96 ± 21
VIII	[N-Ac-D-Phe^6]Bn(6–13)NH$_2$	86 ± 8	154 ± 45
IX	[D-Pal^6]Bn(6–13)NH$_2$	101 ± 17	307 ± 39
X	[D-Leu^6]Bn(6–13)NH$_2$	71 ± 3	168 ± 18
XI	[D-p-Cl-Phe^6]Bn(6–13)NH$_2$	54 ± 1	102 ± 15
XII	[D-Tyr^6]Bn(6–13)NH$_2$	72 ± 13	115 ± 21
XIII	[D-Trp^6]Bn(6–13)NH$_2$	64 ± 13	88 ± 11
XIV	[D-Nal^6]Bn(6–13)NH$_2$	34 ± 6	95 ± 12
XV	[D-Nal^6,Ala^7]Bn(6–13)NH$_2$	500 ± 170	1002 ± 127
XVI	[D-Phe^6]Bn(6–12)NH$_2$	632 ± 195	>1000
XVII	Bn(6–13)EA	54 ± 1	95 ± 5
XVIII	[D-Phe^6]Bn(6–13)EA	7 ± 1	16 ± 4
XIX	[D-Phe^6]Bn(6–13)PA	1.6 ± 0.3	4.4 ± 0.9
XX	[D-Phe^6]Bn(6–12)PA	3370 ± 580	6568 ± 749
XXI	N-Ac-Bn(7–13)EA	29 ± 10	50 ± 9
XXII	[D-Phe^6]Bn(6–13)OH	1026 ± 622	7931 ± 1428

[a] K_i values for binding of the analogues were calculated as described previously (Cheng & Prusoff, 1980). IC_{50} values are from data shown in Figure 1 and represent the concentrations of the analogues causing half-maximal inhibition of 0.3 nM Bn stimulated amylase release. Each value is the mean ± 1 SEM from at least five experiments, and in each experiment each point was determined in duplicate.

equal in potency to the most potent antagonist described previously, [Leu^{14},ψ13–14]Bn (compare III and I) (Coy et al., 1988a). Particularly apparent in Figure 1, Figure 2, and Table I is that reducing the length of the des-Met amide bombesin analogue from 13 to 8 COOH-terminal amino acids (compare III to VI) resulted in a 20-fold decrease in potency; however, there is 30-fold increase in potency of the octapeptide amide created by the presence of D-Phe in position 6 (compare VII and VI). In contrast, substitution of a D-Phe in position 6 of the original bombesin des-Met amide did not alter the inhibitory potency (compare III and IV) similar to that seen with such a substitution in the original pseudotetradecapeptide analogue (compare I and II). A previous study has reported that a D-amino acid substitution in position 5 or 6 of [D-Phe^{12}]bombesin antagonists increased potency (Saeed et al., 1989) but a D-Ala^5 substitution in the present des-Met amide decreased potency 4-fold (compare IV and V).

To investigate the importance of the side-chain characteristics of the substitutions in position 6 of the Bn(6–13)NH$_2$ analogue in determining increased antagonist activity, various Bn(6–13)NH$_2$ analogues with substitution with differing properties were synthesized (VII, IX–XIV, Table I). Replacement of D-Phe^6 with D-β-naphthylalanine^6, D-Trp^6, D-Tyr^6, D-p-chloro-Phe^6, or D-Leu^6 resulted in only a slight decrease in potency (<3-fold) (compare VII, X–XIV, Table I) whereas replacement with D-pyridylalanine instead of D-Phe^6 resulted in a 5-fold decrease in potency (compare VII and IX, Table I).

Adding an N-acetyl to the N-terminus of [D-Phe^6]Bn(6–13)NH$_2$ resulted in only a slight decrease in potency (compare VII and VIII, Table I). The replacement of Gln^7 in [D-Phe^6]Bn(6–13)NH$_2$ by Ala^7 decreased antagonist potency 20-fold (compare VII and XV, Table I). Further deletion of

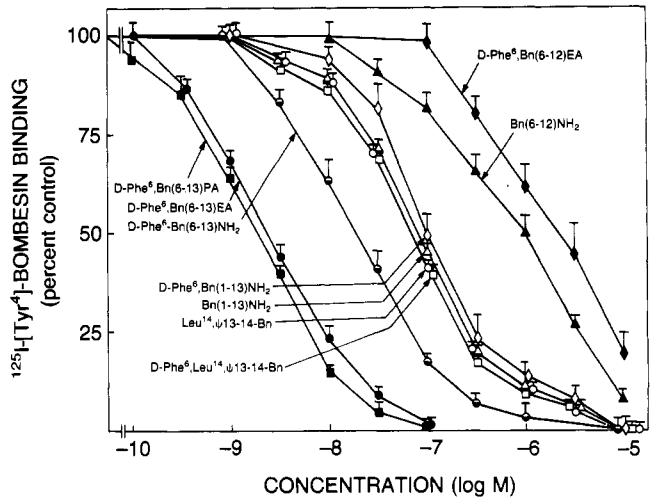

FIGURE 3: Ability of various bombesin analogues to inhibit binding of ^{125}I-[Tyr^4]bombesin to murine Swiss 3T3 cells. 3T3 cells were incubated with 50 pM ^{125}I-[Tyr^4]bombesin alone or with 1 μM bombesin or the indicated concentrations of bombesin analogues. Results are expressed as the percentage of saturable binding with no bombesin analogue present (i.e., percent control). In each experiment each value was determined in duplicate, and each point is the mean ± SEM of at least four experiments.

COOH-terminal amino acids had a significant effect on potency because [D-Phe^6]Bn(6–12)NH$_2$ was 30-fold less potent than [D-Phe^6]Bn(6–13)NH$_2$ (compare VII and XVI).

Adding an alkyl group to the amide had a marked effect on potency. Bn(6–13)ethylamide was 15 times more potent than Bn(6–13)NH$_2$ (compare XVII and VI), whereas [D-Phe^6]Bn(6–13)ethylamide and [D-Phe^6]Bn(6–13)propylamide were 3 and 15 times more potent than [D-Phe^6]Bn(6–13)NH$_2$ and 100 and 400 times more potent than Bn(6–13)NH$_2$, respectively. [D-Phe^6]Bn(6–13)propylamide (XIX) was an especially potent antagonist, being 30-fold more potent than the most potent bombesin antagonist described previously (Coy et al., 1988a) (compare I and XIX, Table I). As with the des-Met amide analogues the chain length of the bombesin alkyl amide analogue was important for determining potency because the [D-Phe^6]Bn(6–12)propylamide analogue was 2500 times less potent than the [D-Phe^6]Bn(6–13)propylamide analogue (compare XIX and XX). However, the Bn(7–13)-ethylamide analogue was only 2-fold more potent than Bn-(6–13)ethylamide analogue (compare XXI and VII). A free carboxy terminus in [D-Phe^6]Bn(6–13)OH reduced inhibitory potency almost 40-fold from that of the amide analogue (compare XXII and VII).

Selected bombesin analogues were also tested for their abilities to interact with bombesin receptors on murine 3T3 cells and inhibit binding of ^{125}I-[Tyr^4]bombesin (Figure 3) as well as bombesin-stimulated growth of the same cells (Table II). The relative abilities of the different bombesin analogues to inhibit binding to 3T3 cells agreed closely with those for inhibition of binding to pancreatic acini or bombesin-stimulated amylase release (compare Figure 3 to Figures 1 and 2). As shown for pancreatic acini, [D-Phe^6]Bn(6–13)propylamide (XIX) was the most potent antagonist (K_i = 1.7 nM, Table II, Figure 3). It was 2 times as potent as the next most potent analogue [[D-Phe^6]Bn(6–13)ethylamide (XVIII)], 40 times more potent than [Leu^{14},ψ13–14]Bn(I), which was the previous most potent antagonist described in these cells, 60 times more potent than Bn(1–13)NH$_2$ (III), and 700 times more potent than the unsubstituted Bn(6–13)NH$_2$ (VI) (Figure 3). The ability of various bombesin analogues to occupy the bombesin receptor on 3T3 cells and inhibit bombesin-stimulated growth

Table II: Comparison of the Ability of Bombesin-Related Analogues To Inhibit Bombesin-Stimulated Incorporation of [^{3}H]Thymidine into Murine 3T3 Cells and Inhibit Binding of ^{125}I-[Tyr^4]bombesin[a]

		IC_{50} or K_i (nM)	
no.	analogue	growth	binding
I	[Leu^{14},ψ13–14]Bn	18 ± 12	65 ± 8
VI	Bn(6–13)NH$_2$	1627 ± 756	1216 ± 173
VII	[D-Phe^6]Bn(6–13)NH$_2$	29 ± 16	23 ± 1
VIII	[*N*-Ac-D-Phe^6]Bn(6–13)NH$_2$	92 ± 45	113 ± 18
XIV	[D-Nal^6]Bn(6–13)NH$_2$	55 ± 20	28 ± 3
XVIII	[D-Phe^6]Bn(6–13)EA	0.7 ± 0.3	2.9 ± 0.4
XIX	[D-Phe^6]Bn(6–13)PA	0.8 ± 0.1	1.7 ± 0.2
XX	[D-Phe^6]Bn(6–12)EA	>1000	5006 ± 906

[a] K_i values for binding ofthe analogues as described previously (Cheng & Prusoff, 1980). IC_{50} values represent concentrations of peptides causing half-maximal inhibition of 3.0 nM bombesin stimulated growth. Each value for thymidine incorporation or 3T3 binding is the mean ± SEM of a minimum of three or five experiments, respectively.

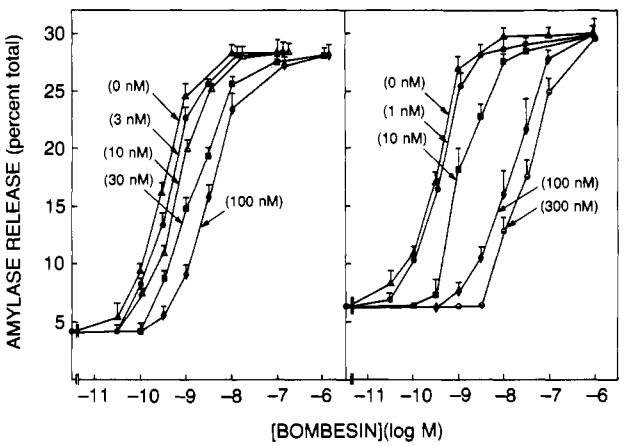

FIGURE 4: Effect of [D-Phe^6]Bn(6–13)NH$_2$ (VII, Table I) (left panel) or [D-Phe^6]Bn(6–13)PA (XIX, Table I) (right panel) on bombesin-stimulated amylase release from pancreatic acini. Acini were incubated with or without the indicated concentration of bombesin or the concentrations of the two bombesin analogues shown in parenthesis. Amylase release is expressed as the percentage of the total cellular amylase released into the extracellular medium during the incubation. Each value is the mean ± SEM of five experiments.

of 3T3 cells was compared in Table II. There was generally a close agreement, with [D-Phe^6]Bn(6–13)propylamide and -ethylamide (XIX and XVIII) analogues being extremely potent and inhibiting growth at 0.7 nM. These analogues were 25 times more potent than [Leu^{14},ψ13–14]Bn(I), the most potent antagonist previously described, and 40 times more potent than [D-Phe^6]Bn(6–13)NH$_2$ (VII) (Table II).

The nature of inhibition caused by these analogues was investigated further by testing the ability of the most potent amide analogue, [D-Phe^6]Bn(6–13)NH$_2$ (VII), and alkylamide analogue, [D-Phe^6]Bn(6–13)PA (XIX), to inhibit bombesin-stimulated amylase release (Figure 4) and the ability of the antagonist [D-Phe^6]Bn(6–13)PA to interact with bombesin receptors on Swiss 3T3 cells (Figure 5). Bombesin caused detectible amylase release from pancreatic acini at 0.03 nM, half-maximal release at 0.3 nM, and maximal release at 1 nM (Figure 4). Increasing concentrations of [D-Phe^6]Bn(6–13)-NH$_2$ (VII) (Figure 4, left) or [D-Phe^6]Bn(6–13)PA (XIX) (Figure 4, right) caused a parallel rightward shift in the dose–response curve for bombesin-stimulated amylase secretion with no change in maximal secretion. When these data were plotted in the form of Schild (1949), the data for [D-Phe^6]-Bn(6–13)NH$_2$ (VII) were best fit by the equation y = 1.0(±0.21)x + 7.9 and for [D-Phe^6]Bn(6–13)PA (XIX) by y = 1.1(±0.09)x + 9.31. For each analogue the slope was not

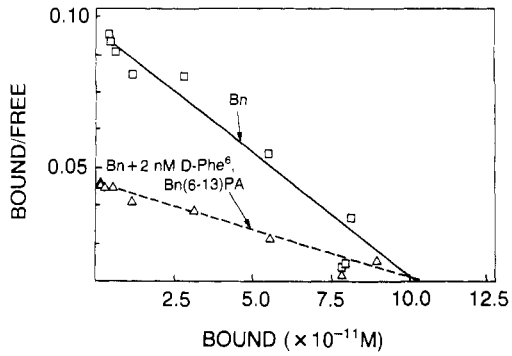

FIGURE 5: Effect of 2 nM [D-Phe^6]Bn(6–13)PA (XIX, Table I) on the bombesin dose–inhibition curve for binding of ^{125}I-[Tyr^4]bombesin to murine 3T3 cells. 3T3 cells were incubated with 50 pM ^{125}I-[Tyr^4]bombesin alone or with 2 nM [D-Phe^6]Bn(6–13)PA plus increasing concentrations (0.01 nM–1 μM) of bombesin. Results were analyzed by using a nonlinear curve-fitting program (LIGAND) (Munson & Rodbard, 1980), and Scatchard plots were shown. Data from four separate experiments are given, and in each experiment each value was determined in duplicate.

significantly different from unity, and the calculated affinities were K_i = 12.5 ± 2.6 and K_i = 3.4 ± 0.4 nM for [D-Phe^6]-Bn(6–13)NH_2 and [D-Phe^6]Bn(6–13)PA, respectively. When the ability of 2 nM [D-Phe^6]Bn(6–13)PA (XVIV) to affect binding of ^{125}I-[Tyr^4]bombesin to Swiss 3T3 cells (Figure 5) was analyzed by a nonlinear least-squares program (Munson & Rodbard, 1980), the [D-Phe^6]Bn(6–13)PA caused a significant decrease in the affinity (2.6 ± 0.2 versus 0.92 ± 0.06 nM, $p < 0.01$, $n = 4$) but caused no change in the total number of binding sites (66 400 ± 5300 versus 62 700 ± 3800 sites/cell, $n = 4$), compatible with competitive inhibition.

The specificity of the inhibitory action was tested by examining the ability of the most potent amide analogue (VII) and alkylamide analogue (XIX) to inhibit amylase release produced by secretagogues that do not stimulate secretion through the bombesin receptor. Analogues VII (10 μM) or XIX (0.1 μM) completely inhibited stimulation by bombesin, GRP, and neuromedin C, each of which stimulates secretion by interacting with bombesin receptors (Table III). However, each analogue did not inhibit secretion stimulated by CCK-8, carbamylcholine, substance P, vasoactive intestinal peptide, secretin, or CGRP, each of which interacts with its receptor on pancreatic acini, or by TPA or A23187 which have postreceptor mechanisms of stimulating secretion.

Table III: Effect of [D-Phe^6]Bn(6–13)NH_2 (VII, Table I) and [D-Phe^6]Bn(6–13)PA (XVIII, Table I) on Amylase Release Stimulated by Various Pancreatic Secretagogues[a]

	amylase release (% of total)		
secretagogue	alone	plus [D-Phe^6]Bn-(6–13)NH_2 (10 μM)	plus [D-Phe^6]Bn-(6–13)PA (0.1 μM)
none	3.6 ± 0.2	3.7 ± 0.2	3.8 ± 0.3
bombesin (0.3 nM)	13.4 ± 0.8	3.5 ± 0.3[b]	2.6 ± 1.0[b]
GRP (1 nM)	18.2 ± 2.9	3.9 ± 1.4[b]	5.0 ± 1.0[b]
neuromedin C (1 nM)	16.4 ± 2.4	3.6 ± 0.3[b]	3.7 ± 0.2[b]
carbachol (10 μM)	24.0 ± 1.5	22.6 ± 1.1	23.8 ± 2.2
substance P (3 nM)	7.6 ± 0.6	7.8 ± 0.4	7.6 ± 0.5
CCK-8 (0.1 nM)	17.0 ± 1.4	15.9 ± 0.8	17.9 ± 1.4
CGRP (0.1 μM)	8.5 ± 0.9	8.5 ± 0.3	8.5 ± 1.1
VIP (0.3 nM)	15.6 ± 1.7	16.2 ± 0.5	17.0 ± 0.4
secretin (0.1 μM)	15.0 ± 0.8	16.5 ± 1.2	16.4 ± 0.3
A23187 (1 μM)	6.9 ± 0.8	7.0 ± 0.4	6.6 ± 0.2
TPA (0.1 μM)	25.7 ± 3.2	28.5 ± 1.1	29.6 ± 1.1

[a] Dispersed acini from guinea pig were incubated for 30 min at 37 °C with the secretagogues shown alone or with the indicated concentration of either [D-Phe^6]Bn(6–13)NH_2 (VII, Table I) or [D-Phe^6]Bn-(6–13)PA (XIX, Table I). Results are expressed as the percentage of total cellular amylase release into the incubation medium. The results are mean ± 1 SEM from at least four separate experiments, and in each experiment each point was determined in duplicate. Abbreviations are described in the text. [b] Significantly different ($p < 0.01$) from corresponding value obtained with secretagogue alone.

Discussion

This study demonstrates that des-Met alkylamide analogues of bombesin are extremely potent, specific bombesin receptor antagonists. The potency of these analogues is shown by the fact that the most potent peptide, [D-Phe^6]Bn(6–13)propylamide, inhibits bombesin-stimulated growth of murine Swiss 3T3 cells, bombesin-stimulated amylase release from pancreatic acini, and ^{125}I-[Tyr^4]bombesin binding to bombesin receptors on both cell types with IC_{50}'s and K_d's from 1 to 4 nM. Therefore, this analogue is 20–30 times more potent than the most potent antagonist described previously ([Leu$^{13}\psi$-(CH_2NH)Leu^{14}]bombesin) (Coy et al., 1988a). It is 1000 times more potent than the most potent substance P antagonist which also functions as a bombesin receptor antagonist, [D-Arg^1,D-Trp^{7,9},Leu^{11}]substance P (Jensen et al., 1984, 1988b), and 5000 times more potent than the bombesin receptor antagonist [D-Phe^{12}]bombesin (Heinz-Erian et al., 1987). That these analogues are functioning as specific inhibitors of the action of bombesin is supported by the specificity studies with the most potent des-Met amide analogue, [D-Phe^6]Bn(6–13)NH_2, and the most potent des-Met alkylamide analogue, [D-Phe^6]Bn(6–13)propylamide. Each of these analogues, at a concentration that completely inhibited the ability of GRP, bombesin, or neuromedin C [GRP(18–27)] to interact with the bombesin receptor, had no effect on agents that interact with receptors for cholecystokinin, muscarinic cholinergic agents, substance P, calcitonin gene related peptide, or vasoactive intestinal peptide (VIP, secretin), nor did they inhibit the action of agents such as TPA or A23187 that have a postreceptor mechanism of action (Jensen & Gardner, 1981; Gardner & Jensen, 1987). The ability of these analogues to distinguish the bombesin from substance P receptors is particularly important because previous studies have demonstrated that a number of the other classes of bombesin receptor antagonists can also function as substance P receptor antagonists (Jensen et al., 1988a,b; Merali et al., 1988; Mizrahi et al., 1985). The fact that these analogues were functioning as bombesin receptor antagonists is supported by a number of results. There was a close correlation between the ability of each analogue to inhibit ^{125}I-[Tyr^4]bombesin binding to receptors on either cell type and their abilities to inhibit bombesin-stimulated enzyme secretion or growth. In pancreatic acini both [D-Phe^6]Bn(6–13)NH_2 and [D-Phe^6]Bn(6–13)-propylamide caused a parallel rightward shift of the bombesin dose–response curve for amylase release with no change in maximal response, and the slope of the Schild plot was not significant from unity for each analogue, demonstrating competitive inhibition. Lastly, in Swiss 3T3 cells, analysis of the effect of 2 nM [D-Phe^6]Bn(6–13)propylamide on the dose–inhibition curve of bombesin for binding of ^{125}I-[Tyr^4]bombesin demonstrated a change only in affinity, not in the number of sites, demonstrating competitive inhibition.

The results from this study demonstrate that the method originally described by Martinez et al. (Spanarkel et al., 1983; Martinez et al., 1984) of forming potent antagonists for gastrin/CCK peptides by making des-Phe amidated analogues can also work with bombesin-related peptides if the proper additional substitutions are made. Similar to a previous report describing GRP(20–26)amide as a weak bombesin receptor

antagonist (K_i = 1570 nM) (Heimbrook et al., 1989a), we found that Bn(6–13)NH_2 was a very weak antagonist (K_i = 1800 nM). One of the strategies that has proved useful in numerous peptides for improving potency is the substitution of a D-amino acid in various points in the chain. [D-Trp^6]-LHRH (Coy et al., 1976), [D-Trp^8]somatostatin (Rivier et al., 1975), [D-Phe^7]MSH(4–10) (Sawyer et al., 1980), [D-Phe^4]glucagon (Sueiras-Diaz et al., 1984), and [D-Ala^2]-GHRF(1–29) (Lance et al., 1984) are all more potent than the corresponding peptide. In a previous study (Saeed et al., 1989) when we applied a similar strategy to [D-Phe^{12}]bombesin analogues which function as antagonists, we found that a D-Phe substitution in position 5 or 6 increased inhibitory affinity 3- and 7-fold, respectively. When similar changes were made in native bombesin analogues functioning as antagonists such as Bn(1–13)NH_2 or [Leu^{14},ψ13–14]bombesin, no change in affinity occurred; however, D-Phe^6 substitutions in all Bn(6–13)amide analogues markedly increase inhibitory potency (10–30-fold). Therefore, the substitution of a D-Phe^6 compensated for the shortening of the peptide in that [D-Phe^6]-Bn(6–13)NH_2 was equipotent to Bn(1–13)NH_2, each of which were 30-fold more potent than Bn(6–13)NH_2. Our results demonstrate that the chemical characteristics of the D-amino acid substituted in the 6-position of Bn(6–13)NH_2 to increase potency are not narrow. Steric requirements for the aromatic amino acid substituted in the 6-position of Bn(6–13)NH_2 did not appear to be too exacting, because there were only small differences between D-Trp^6, D-Tyr^6, and D-Phe^6 derivatives. Furthermore, the hydrophobicity or size of the D-amino acid substituted in this position was not particularly important because substitution of D-β-naphthylalanine^6, containing a larger and more hydrophobic side chain than D-Phe^6, or a more hydrophilic group such as D-pyridylalanine or D-Tyr^6 had only a minimal effect on inhibitory potency. In contrast to recent studies with glutaramic acid analogues which function as CCK receptor antagonists (Makovec et al., 1986; Jensen et al., 1986) the addition of an electron-withdrawing group to the aromatic moiety (D-*p*-chlorophenylalanine^6) also had only a minimal effect on potency. Lastly, an aromatic group in position 6 of Bn(6–13)NH_2 was not required to increase potency because the D-Leu^6 analogue was 6-fold more potent than Bn(6–13)-NH_2 itself.

Another important finding in the present study in addition to the D-amino acid substitution in position 6 for increasing inhibitory potency of shorter des-Met bombesin amides was the addition of alkyl groups to the amide moiety. All alkylamide analogues of Bn(6–13)NH_2 were 5–11 times more potent than the amide analogue. The chain length of the alkyl group was also important in determining the increase in affinity because the propylamide analogue was 2- to 4-fold more potent than the ethylamide derivative. These results coupled with other recent results (Coy et al., 1988a; Saeed et al., 1989; Heimbrook et al., 1988) provide insights into the importance of the COOH-terminal amino acid of bombesin in determining the ability of bombesin to interact with receptors or to initiate a biologic response and therefore into possible future alterations that may lead to the development of even more potent agonists or antagonists. C-Terminal structural modifications which have now been determined to produce either full agonists or antagonists are summarized in Figure 6. Recent studies have demonstrated that a C-terminal Met side chain per se in bombesin is not essential for agonist activity since other diverse amino acid substitutions in position 14 also yield agonists (Coy et al., 1988a; Saeed et al., 1989; Heimbrook et al., 1988). However, it is now apparent that the position 14 carboxamide

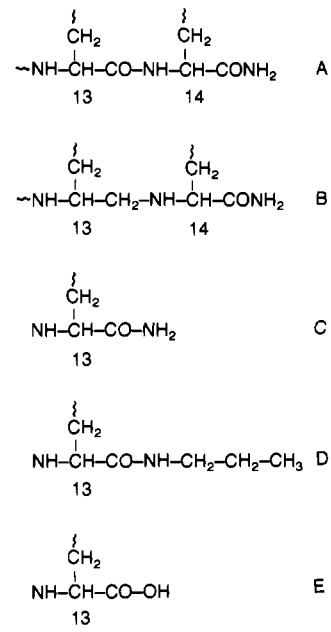

FIGURE 6: C-Terminal structural modifications to bombesin that are responsible for either receptor agonist or antagonist activity. (A) Bombesin with Leu^{13} and Met^{14} (agonist); (B) [Leu$^{13}\psi$(CH_2NH)-Leu^{14}]Bn with the position 13 CO replaced by CH_2 (antagonist); (C) des-Met^{14}-amidated analogues (antagonist); (D) des-Met^{14} alkylamide analogues (more potent antagonists); (E) des-Met^{14} free acid analogue (very weak antagonist).

group is of prime importance in bombesin expression of biological activity since its removal always produces pure antagonists in the present series of analogues. Our previous work has shown (Coy et al., 1988a) that reduction of the position 13 carbonyl group also completely converts agonist to antagonist activity despite the continued presence of the position 14 carboxamide group. This might best be explained, as was originally proposed (Coy et al., 1988a), by a conformational shift in the position 14 carboxamide group in the receptor-bound peptide promoted by the increased rotational freedom and flexibility of the C-terminal region of the reduced peptide bond analogue. The present data also reveal that alkyl substituents on the position 13 NH_2 group dramatically improve binding affinity and antagonist potency. This perhaps implies that the position 13 CO group, at least in C-terminally deleted analogues, is involved in binding to the bombesin/GRP receptor via hydrogen bonding since such an interaction would be enhanced by electron-releasing alkyl substituents. This is also supported by the greater potency of the propylamide XIX compared to the ethylamide analogue XVIII and the much reduced antagonist potency of the position 13 free carboxy analogue XXII in which the CO electrons are distributed over two CO groups.

In conclusion, structural alterations in bombesin analogues that function as antagonists have been developed that allow chain length to be reduced from 13 to 8 amino acids while increasing inhibitory potency and retention of specificity. The most potent analogue, [D-Phe^6]Bn(6–13)propylamide, is 30 times more potent than any other known antagonist, has approximately equal affinity to bombesin (Jensen et al., 1988b) for the bombesin receptor, and it is hoped, because of its increased affinity and fewer proteolytic degradation sites, will prove generally useful for in vivo studies.

Added in Proof

Since submission of this paper, two additional studies have been published, demonstrating various des-Met bombesin or GRP analogues function as receptor antagonists. One study

(Camble et al., 1989) demonstrates that (2-methylpropionyl)[D-Ala^{24}]GRP(20–26) methyl ester and pivaloyl-[D-Ala^{24}]GRP(20–26)methylamide were potent antagonists (K_i = 2–4 nM in 3T3 cells). Another study (Heimbrook et al., 1989b) demonstrates various des-Met GRP esters function as potent Bn receptor antagonists with *N*-Ac-GRP(20–26) ethyl ester (K_i = 4 nM, 3T3 cells) being the most potent. In this study Ac-GRP(20–26)ethylamide was also shown to be an antagonist (K_i = 53 nM, 3T3 cells).

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Registry No. I, 117016-31-6; II, 124199-84-4; III, 124176-03-0; IV, 124176-04-1; V, 124176-05-2; VI, 124176-06-3; VII, 124176-07-4; VIII, 124176-08-5; IX, 124199-85-5; X, 124176-09-6; XI, 124199-86-6; XII, 124176-10-9; XIII, 124176-11-0; XIV, 124199-87-7; XV, 124199-88-8; XVI, 124176-12-1; XVII, 124199-89-9; XVIII, 124199-90-2; XIX, 124199-91-3; XX, 124199-92-4; XXI, 124199-93-5; XXII, 124176-13-2; amylase, 9000-92-4; bombesin, 31362-50-2; gastrin-releasing peptide, 80043-53-4; neuromedin C, 81608-30-2.

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