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Potent bombesin antagonists with C-terminal Leu-ψ(CH_2-N)-Tac-NH_2 or its derivatives

(peptide synthesis/pseudononapeptide analogs/tumor growth inhibition/peptide receptors)

R.-Z. CAI*†, H. REILE*†, P. ARMATIS*, AND A. V. SCHALLY*†‡

*Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center, New Orleans, LA 70146; and †Section of Experimental Medicine, Tulane University School of Medicine, New Orleans, LA 70112

Contributed by A. V. Schally, August 25, 1994

ABSTRACT **Various pseudononapeptide bombesin (BN)-(6–14) antagonists with a reduced peptide bond (CH_2-NH) between positions 13 and 14 can suppress the mitogenic activity of BN or gastrin-releasing peptide in 3T3 fibroblast cells and small cell lung carcinoma. In the search for more potent BN antagonists, 10 modified nonapeptide BN antagonists containing N-terminal D-Phe, D-Cpa, and D- or L-Tpi and C-terminal Leu-ψ(CH_2-N)-Tac-NH_2, Leu-ψ(CH_2-N)-MeTac-NH_2, or Leu-ψ(CH_2-N)-Me_2Tac-NH_2 have been synthesized by incubating [$^{13}\psi^{14}$,CH_2-NH,Cys^{14}]BN-(6–14) or [$^{13}\psi^{14}$-CH_2-NH,Pen^{14}]BN-(6–14) with formaldehyde or acetaldehyde (Cpa = 4-chlorophenylalanine, Tac = thiazolidine-4-carboxylic acid, Tpi = 2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indol-3-carboxylic acid, and Pen = penicillamine). The biological activities of these compounds were then evaluated. [D-Phe^6,$^{13}\psi^{14}$,CH_2-N,Tac^{14}]BN-(6–14) (RC-3950-II) and [D-Phe^6,$^{13}\psi^{14}$,CH_2-N,Me_2Tac^{14}]BN-(6–14) (RC-3985-II) exhibited greater potency in inhibition of ^{125}I-labeled [Tyr^4]BN binding to Swiss 3T3 cells than their parent compounds [D-Phe^6,$^{13}\psi^{14}$,CH_2-NH,Cys^{14}]BN-(6–14) (RC-3950-I) and [D-Phe^6,$^{13}\psi^{14}$,CH_2-NH,Pen^{14}]BN-(6–14) (RC-3985-I). The order of binding affinities of these compounds was as follows: [$^{13}\psi^{14}$,CH_2-N,Tac^{14}]BN-(6–14) > [$^{13}\psi^{14}$,CH_2-N,Me_2Tac^{14}]BN-(6–14) > [$^{13}\psi^{14}$,CH_2-N,$MeTac^{14}$]BN-(6–14). In most cases, the analogs with C-terminal Leu-ψ(CH_2-N)-Tac-NH_2 were also more potent growth inhibitors of 3T3 cells than compounds containing C-terminal Leu-ψ(CH_2-N)-Me_2Tac-NH_2 or Leu-ψ(CH_2-N)-MeTac-NH_2. The best BN antagonists of this series, RC-3950-II and [D-Cpa^6,$^{13}\psi^{14}$,CH_2-N,Tac^{14}]BN-(6–14) (RC-3925-II), inhibited gastrin-releasing peptide-stimulated growth of Swiss 3T3 cells with IC_{50} values of 1 nM and 6 nM, respectively. Since antagonists of this class inhibit growth of various tumors in animal cancer models, some of them may have clinical applications.**

Bombesin (BN) and its mammalian counterpart, gastrin-releasing peptide (GRP), possess a variety of physiological and pharmacological functions (1, 2). It is also thought that BN-like peptides act as autocrine growth factors in human small cell lung carcinoma and other cancers (3–7). The development of BN/GRP antagonists might lead to hormonal approaches for the treatment of small cell lung carcinoma and other malignancies.

BN and GRP have similar amino acid sequences in their C-terminal regions that are responsible for the biological activities, including mitogenic effects in Swiss 3T3 cells. Coy *et al.* (8) reported that the modification of the C-terminal peptide bond (between residues 13 and 14, $^{13}\psi^{14}$) to a reduced form (CH_2-NH) yields potent BN antagonists. Incorporation of a D-amino acid at the N terminus in short-chain [$^{13}\psi^{14}$,CH_2-NH]BN-(6–14) antagonists results in an additional increase in inhibitory potency (9). Other types of BN antagonists contain a C-terminal alkyl ester or an alkyl amide instead of Met-NH_2 (10, 11). We have reported the synthesis of [$^{13}\psi^{14}$,CH_2-N,Tpi^{14}]BN-(6–14) antagonists (12), which showed greater growth inhibitory potencies in 3T3 cells than the [$^{13}\psi^{14}$,CH_2-NH,Trp^{14}]BN-(6–14) reduced bond compounds. Tpi (2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indol-3-carboxylic acid) is a constrained Trp analog containing a secondary amine. The reduced bond Tpi compound cannot be formed by the alkylation reaction. To form the Leu-ψ(CH_2-N)-Tpi derivative, we allowed Leu-ψ(CH_2-NH)-Trp to react on benzhydrylamine resin with formaldehyde.

In this study, we report the synthesis of a series of BN analogs containing C-terminal Leu-ψ(CH_2-N)-Tac-NH_2, Leu-ψ(CH_2-N)-MeTac-NH_2, or Leu-ψ(CH_2-N)-Me_2Tac-NH_2 by incubating C-terminal Leu-ψ(CH_2-NH)-Cys-NH_2 or Leu-ψ(CH_2-NH)-Pen-NH_2 compounds with formaldehyde or acetaldehyde. These modified reduced bond BN analogs were then investigated for their abilities to inhibit both the binding of ^{125}I-labeled [Tyr^4]BN to 3T3 cells and GRP-(14–27)-stimulated growth of these cells *in vitro*. Tac (thiazolidine-4-carboxylic acid) and its 2-methyl (MeTac) and 5,5-dimethyl (Me_2Tac) derivatives are constrained analogs of cystine containing a secondary amine; Pen is penicillamine.

MATERIALS AND METHODS

Synthesis of Peptides. *Amino acid derivatives. tert*-Butoxycarbonyl (Boc) amino acids and Boc-Leu-aldehyde were purchased from Advanced ChemTech with the exception of Boc-D-Tpi and Boc-L-Tpi, which were prepared in our laboratory (12). Functional side groups were protected by 4-methylbenzyl in the case of Cys, benzyl for Pen, and benzyloxycarbonyl for the nitrogen of the imidazole group of His. The positions of amino acid residues are numbered relative to BN.

(*i*) [$^{13}\psi^{14}$,CH_2-NH,Cys^{14}]BN-(6–14) and [$^{13}\psi^{14}$,CH_2-NH,Pen^{14}]BN-(6–14). These two derivatives were prepared by standard solid-phase synthesis on benzhydrylamine resin (Advanced ChemTech). Introduction of the reduced bond $^{13}\psi^{14}$(CH_2-NH) was carried out using Boc-Leu-aldehyde and $NaBH_3CN$ in acidified dimethylformamide as described by Sasaki and Coy (13). After the formation of a reduced peptide bond, all of the coupling reactions were performed by the active ester method using 1,3-diisopropylcarbodiimide and 1-hydroxybenzotriazole. The peptide was cleaved by treatment with liquid HF containing anisole and dithiothreitol as scavenger. The crude peptide was purified by HPLC as described in the *Purification and analytical HPLC* section.

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Abbreviations: BN, bombesin; GRP, gastrin-releasing peptide; Cpa, 4-chlorophenylalanine; Pen, penicillamine; Tac, thiazolidine-4-carboxylic acid; MeTac, 2-methylthiazolidine-4-carboxylic acid; Me_2Tac, 5,5-dimethylthiazolidine-4-carboxylic acid; Tpi, 2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indol-3-carboxylic acid; Boc, *tert*-butoxycarbonyl.

‡To whom reprint requests should be addressed at: Veterans Affairs Medical Center, 1601 Perdido Street, New Orleans, LA 70146.

(*ii*) [$^{13}\psi^{14}$,CH_2-N,Tac^{14}]BN-(6–14). Twenty to 30 mg of crude [$^{13}\psi^{14}$,CH_2-NH,Cys^{14}]BN-(6–14) in 0.8 ml of glacial acetic acid was incubated with 100 μl of 1% formaldehyde (wt/vol in water) at room temperature for 30 sec, followed by addition of 100 μl of 3% (wt/vol) cystine solution in water. This reaction mixture was immediately subjected to purification by HPLC to yield the desired peptide.

(*iii*) [$^{13}\psi^{14}$,CH_2-N,MeTac^{14}]BN-(6–14). Twenty to 30 mg of [$^{13}\psi^{14}$,CH_2-NH,Cys^{14}]BN-(6–14) in 0.8 ml of glacial acetic acid was mixed with 100 μl of 50% (vol/vol) acetaldehyde at room temperature for 5 min. This reaction mixture was purified by HPLC to yield [$^{13}\psi^{14}$,CH_2-N,MeTac^{14}]BN-(6–14).

(*iv*) [$^{13}\psi^{14}$,CH_2-N,Me_2Tac^{14}]BN-(6–14). Twenty to 30 mg of [$^{13}\psi^{14}$,CH_2-NH,Pen^{14}]BN-(6–14) in 0.8 ml of glacial acetic acid was combined with 100 μl of 10% formaldehyde and stirred at 0°C for 30 min. The reaction mixture was purified by HPLC to yield [$^{13}\psi^{14}$,CH_2-N,Me_2Tac]BN-(6–14).

Purification and analytical HPLC. Purification was carried out by a Beckman HPLC system using a Vydac column (10 × 250 mm) packed with C_{18} silica gel (300-Å pore size, 5-μm particle size) developed at a flow rate of 10 ml/min and utilizing a solvent system consisting of components A and B as follows: A, 0.1% trifluoroacetic acid; B, 0.1% trifluoroacetic acid in 70% aqueous acetonitrile. The fractions were eluted with a linear gradient of 20–60% B in 40 min and were then examined by analytical HPLC (Hewlett–Packard model HP-1090) with a 4.6 × 250 mm W-Porex 5-μm C_{18} column (Phenomenex, Torrance, CA) using the solvent system described above at a flow rate of 1.2 ml/min. The fractions showing purity >95% were combined and lyophilized. The homogeneity of peptides was assessed by analytical HPLC, and retention times of these peptides on analytical HPLC are listed in Table 1.

Biological Activities. *Cell cultures.* Swiss 3T3 fibroblasts, obtained from the American Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, penicillin G (sodium salt; 100 units/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (0.25 μg/ml) (all from GIBCO) in a humidified atmosphere containing 5% CO_2/95% air at 37°C. The cells were passaged twice per week and were judged free of mycoplasma by a mycoplasma detection kit (Boehringer Mannheim). All studies were performed with density-arrested, quiescent cells.

Proliferation assay. Inhibition of [*methyl*-^{3}H]thymidine incorporation into DNA in Swiss 3T3 cells was used to determine the antagonistic activity of BN analogs. The methods were similar to those described previously (12, 14), except that the incubation time with the antagonists was reduced to 24 hr and the labeling with [^{3}H]thymidine was reduced to 4 hr. Cells were seeded into 96-well microplates (Falcon) at 5 × 10^3 cells per well in culture medium containing 10% newborn calf serum. After 3 days, the confluent cultures were washed with DMEM and maintained in DMEM supplemented with 0.5% newborn calf serum for 2 more days. The BN analogs were added alone or in combination with 1 nM GRP-(14–27) in DMEM containing insulin (Sigma) at 1 μg/ml. Control cultures received 1 nM GRP-(14–27) or medium without additions. After an incubation period of 20 hr, the cells were pulsed with 0.25 μCi (1 Ci = 37 GBq) of [*methyl*-^{3}H]thymidine (specific activity, 25 Ci/mmol; Amersham) per well in a total volume of 175 μl per well for 3–4 hr. The cells were fixed with ice-cold 10% (wt/vol) trichloroacetic acid, washed twice with cold trichloroacetic acid, and solubilized overnight in 0.2 M NaOH at 37°C. The lysate was mixed with scintillation cocktail (Cyto Scint; ICN), and radioactivity was determined by liquid scintillation counting (Searle Analytic Model 6880). Since the extent of stimulation with GRP-(14–27) varied depending on the number of cell passages, the agonistic activity was expressed as the percent stimulation, where 100% is the value obtained for 1 nM GRP-(14–27). Antagonistic activity was defined as the percent inhibition of the stimulation caused by 1 nM GRP-(14–27).

Radioiodination of [Tyr^4]BN. [Tyr^4]BN was synthesized in our laboratory. The radioiodination of [Tyr^4]BN using a Bio-Rad enzymobead radioiodination kit (Bio-Rad) has been described (15). [^{125}I-Tyr^4]BN was separated from ^{125}I and unlabeled [Tyr^4]BN by HPLC using an automated gradient controller (Waters, Milford, MA) and a C_{18} column (SynChropak RP-8, 250 × 4.1 mm i.d.; SynChrom, Lafayette, IN), which was eluted with a linear gradient at a flow rate of 1.2 ml/min. The radioactivity in the effluent was continuously monitored with a system based on an SML-2 radiation detector (Technical Associates, Canoga Park, CA) and a UV detector at 280 nm. The elution gradient was 35–55% B, and the peak fraction of monoiodinated [^{125}I-Tyr^4]BN appeared at 41% solvent B. The fractions were collected at a rate of 0.96 ml per fraction with an ISCO model 1850 fraction collector (ISCO), and the radioactivity of each fraction was measured. The peak fraction of the monoiodinated peptide was diluted with 0.25 M phosphate buffer (pH 7.4) containing 1% bovine serum albumin to 60–100 μCi/ml and stored at −70°C. Specific activity of the labeled compound was found to be 2000 μCi/μg.

Binding studies. Binding of [^{125}I-Tyr^4]BN and displacement by BN analogs was conducted using confluent Swiss 3T3 cells in 24-well plates (Costar) by a modification of the method of Kris *et al.* (16). Three to 5 days after seeding, the confluent cells were washed twice with Hanks' balanced salt solution (HBSS) and incubated for 30 min at 37°C with 50 pM [^{125}I-Tyr^4]BN in the absence or presence of several concentrations of unlabeled competitors in a total volume of 0.5 ml of binding buffer [DMEM containing 25 mM Hepes (GIBCO), 0.1% bovine serum albumin, 5 mM $MgCl_2$, and bacitracin (100 μg/ml) at pH 7.4]. Nonspecific binding was determined in the presence of 1 μM unlabeled ligand. After three washings with ice-cold HBSS containing 0.1% bovine serum albumin (pH 7.4), the cells were detached with a solution of 0.05% trypsin plus 0.53 mM EDTA (GIBCO) and transferred to tubes. Radioactivity was measured with a γ counter (Micromedic Systems, Huntsville, AL). Binding data were evaluated using radioligand binding analysis programs by McPherson (17). K_i values were calculated according to the formula of Cheng and Prusoff (18).

RESULTS

Ten reduced bond nonapeptides were synthesized by allowing [$^{13}\psi^{14}$,CH_2-NH,Cys^{14}]BN-(6–14) or [$^{13}\psi^{14}$,CH_2-NH,Pen^{14}]BN-(6–14) to react with formaldehyde or acetaldehyde as described in *Materials and Methods*. D-Phe, D-Cpa (4-chlorophenylalanine), and D- or L-Tpi were chosen as the N-terminal residues of these modified compounds. The structures of the resulting peptides and their retention times on analytical HPLC are presented in Table 1. The compounds were also analyzed by fast atom bombardment MS in a Finnigan MAT-95 mass spectrometer using a cesium ion gun; the matrix used was nitrobenzyl alcohol. The molecular mass data listed in Table 2 correspond well to the character of the different N- and C-terminal residues of the compounds.

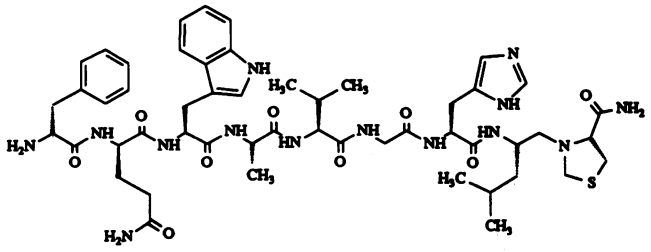
RC-3950-II was examined by NMR for assignment of the ^{1}H and ^{13}C spectra recorded on a Bruker AMX500 spectrometer (one-dimensional spectra and two-dimensional H,H and H,C correlated spectroscopy; data not included). The NMR data (in DMSO-d_6, chemical shifts given in ppm relative to tetramethylsilane as internal reference) of the C-terminal pseudodipeptide residue Leu-ψ(CH_2-N)-Tac were Leu-ψ-CH_2-^{1}H NMR δ = 2.27/2.40 (2H, 2dd); ^{13}C δ = 60.2, t and Tac-^{1}H NMR δ = 4.04 (1H, d, Tac-α); 2.83/3.37 (2H, 2dd, Tac-β); 3.83/3.88(2H, 2d, Tac-δ); ^{13}C NMR δ = 71.8(d,

Table 1. Peptide structures and retention times on analytical HPLC

Code no.	Structure*	Retention time†, min
RC-3950-I	[D-Phe^6,$^{13}\psi^{14}$,CH$_2$-NH,Cys^{14}]BN-(6–14)	7.36
RC-3950-II	[D-Phe^6,$^{13}\psi^{14}$,CH$_2$-N,Tac^{14}]BN-(6–14)	14.63
RC-3960	[D-Phe^6,$^{13}\psi^{14}$,CH$_2$-N,MeTac^{14}]BN-(6–14)	15.81
RC-3985-I	[D-Phe^6,$^{13}\psi^{14}$,CH$_2$-NH,Pen^{14}]BN-(6–14)	7.30
RC-3985-II	[D-Phe^6,$^{13}\psi^{14}$,CH$_2$-N,Me$_2$Tac^{14}]BN-(6–14)	13.83
RC-3925-II	[D-Cpa^6,$^{13}\psi^{14}$,CH$_2$-N,Tac^{14}]BN-(6–14)	16.54
RC-3930	[D-Cpa^6,$^{13}\psi^{14}$,CH$_2$-N,MeTac^{14}]BN-(6–14)	17.94
RC-3990	[D-Cpa^6,$^{13}\psi^{14}$,CH$_2$-N,Me$_2$Tac^{14}]BN-(6–14)	16.03
RC-3905-II	[Tpi^6,$^{13}\psi^{14}$,CH$_2$-N,Tac^{14}]BN-(6–14)	19.95
RC-3995	[Tpi^6,$^{13}\psi^{14}$,CH$_2$-N,Me$_2$Tac^{14}]BN-(6–14)	19.17
RC-3910-II	[D-Tpi^6,$^{13}\psi^{14}$,CH$_2$-N,Tac^{14}]BN-(6–14)	17.16
RC-4000	[D-Tpi^6,$^{13}\psi^{14}$,CH$_2$-N,Me$_2$Tac^{14}]BN-(6–14)	16.81

*The positions of the amino acid residues were numbered relative to BN. The amino acid sequence of BN-(6–14) is Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$.
†The purified peptides were analyzed on analytical HPLC (Hewlett-Packard model HP-1090 with a Phenomenex 4.6 × 250 mm W-Porex 5-μm C$_{18}$ column) using a gradient of 20–60% B in 40 min.

Tac-α); 29.6 (t, Tac-β); 60.0 (t, Tac-δ). The structure of RC-3950-II characterized by ^{1}H and ^{13}C NMR spectra is shown below.



The compounds evaluated *in vitro* for receptor binding affinity (K_i) and antagonistic activity (IC_{50}) are listed in Table 3. Peptides were tested for their ability to displace specific binding of [^{125}I-Tyr^4]BN to Swiss 3T3 cells as compared to BN. Displacement curves of these compounds are shown in Fig. 1.

Variations in C-terminal residues led to great changes in binding affinity. Four [$^{13}\psi^{14}$,CH$_2$-N,Tac^{14}]BN-(6–14) compounds, RC-3950-II, RC-3925-II, RC-3910-II, and RC-3905-II, had binding affinities with K_i values in the range 0.001–0.078 nM. Four [$^{13}\psi^{14}$,CH$_2$-N,Me$_2$Tac^{14}]BN-(6–14) compounds, RC-3985-II, RC-3990, RC-3995, and RC-4000, exhibited binding affinities with K_i values of 0.07–13 nM; and two [$^{13}\psi^{14}$,CH$_2$-N,MeTac^{14}]BN-(6–14) compounds, RC-3930 and RC-3960, showed K_i values of 4.3 and 13 nM.

In all cases, displacement of the [^{125}I-Tyr^4]BN by the analogs with C-terminal Leu-ψ(CH$_2$-N)-Tac-NH$_2$ was better than that of the analogs with C-terminal Leu-ψ(CH$_2$-N)-Me$_2$Tac-NH$_2$, Leu-ψ(CH$_2$-N)-MeTac-NH$_2$, Leu-ψ(CH$_2$-NH)-Cys-NH$_2$, or Leu-ψ(CH$_2$-NH)-Pen-NH$_2$. In most cases, the binding affinities of the compounds with C-terminal Leu-ψ(CH$_2$-N)-Me$_2$Tac-NH$_2$ were higher than those of the compounds with C-terminal Leu-ψ(CH$_2$-N)-MeTac-NH$_2$. RC-3950-II had a K_i that was 450 times lower than that of RC-3950-I, indicating a much greater potency. Similarly, RC-3985-II showed an affinity 17 times higher than that of RC-3985-I, based on K_i values (Table 3).

Antagonistic activity of these compounds was investigated by a method based on inhibition of stimulation of DNA

Table 2. The elemental formulas, molecular masses, and mass spectra of BN antagonists

Code no.	Formula*	Molecular mass*	FAB MS†
RC-3950-I	$C_{50}H_{72}N_{14}O_9S_1$	1044.5	1045.0‡
RC-3950-II	$C_{51}H_{72}N_{14}O_9S_1$	1056.5	1057.9
RC-3960	$C_{52}H_{74}N_{14}O_9S_1$	1070.5	1072.1
RC-3985-I	$C_{52}H_{76}N_{14}O_9S_1$	1072.6	ND
RC-3985-II	$C_{53}H_{76}N_{14}O_9S_1$	1084.6	1086.0
RC-3925-II	$C_{51}H_{71}N_{14}O_9S_1Cl_1$	1090.5	1091.8
RC-3930	$C_{52}H_{73}N_{14}O_9S_1Cl_1$	1104.5	1105.9
RC-3990	$C_{53}H_{75}N_{14}O_9S_1Cl_1$	1118.5	1119.9
RC-3905-II	$C_{54}H_{73}N_{15}O_9S_1$	1107.5	1109.0
RC-3995	$C_{56}H_{77}N_{15}O_9S_1$	1135.6	ND
RC-3910-II	$C_{54}H_{73}N_{15}O_9S_1$	1107.5	1109.0
RC-4000	$C_{56}H_{77}N_{15}O_9S_1$	1135.6	1137.0

ND, not determined.
*The elemental formulas and molecular masses are based on the composition of the designed compounds.
†[M + H]$^+$; there is an uncertainty in the mass determination of approximately 0.5 Da. Fast atom bombardment (FAB) MS was performed by E. Busker (Degussa AG, Hanau-Wolfgang, Germany) with a Finnigan MAT-95 mass spectrometer.
‡Analyzed by Finnigan-MAT 4600.

Table 3. Inhibition of [^{125}I-Tyr^4]BN binding and GRP-(14–27)-induced stimulation of [^{3}H]thymidine uptake into Swiss 3T3 cells by BN analogs

Code no.	Receptor binding (K_i),* nM	Antagonistic activity (IC_{50}),† nM
RC-3950-I	35	ND
RC-3950-II	0.078	1.0
RC-3960	13	120
RC-3985-I	16	ND
RC-3985-II	0.93	57
RC-3925-II	0.007	6.1
RC-3930	4.3	100
RC-3990	13	62
RC-3905-II	<0.001	60
RC-3995	0.07	41
RC-3910-II	<0.001	37
RC-4000	0.074	73
RC-3095	0.20‡	63
BN	0.28§	

ND, not determined.
*Varying doses of unlabeled peptide were used to determine the ability to displace [^{125}I-Tyr^4]BN binding; mean values of two or three independent tests (each performed in three replicates) are indicated.
†IC_{50} is defined as the concentration that inhibited by 50% the stimulation of [^{3}H]thymidine uptake caused by 1 nM GRP-(14–27); mean values of two to four independent tests (each performed in six replicates) are indicated.
‡Mean value of six independent tests.
§Mean value of 11 independent tests.

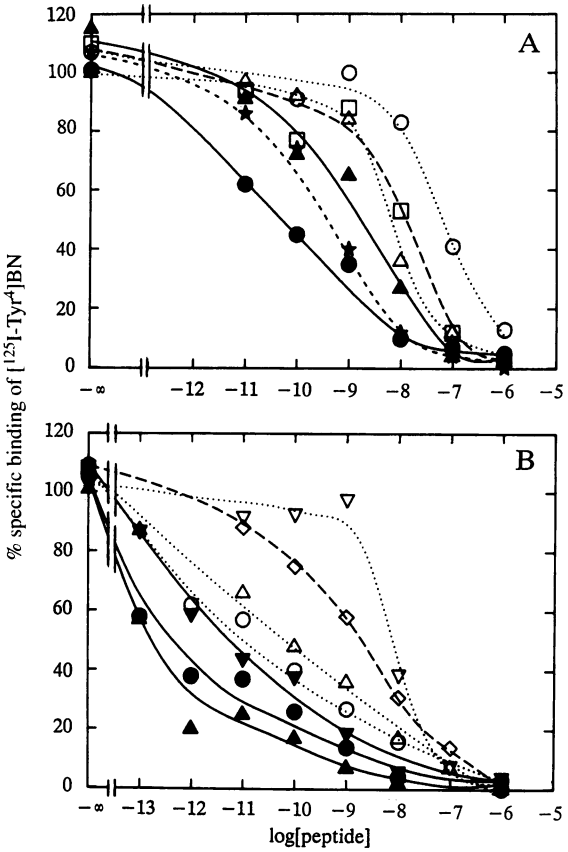


FIG. 1. Displacement of [^{125}I-Tyr^4]BN binding in Swiss 3T3 cells by BN and BN antagonists. (*A*) ○, RC-3950-I; ●, RC-3950-II; □, RC-3960; △, RC-3985-I; ▲, RC-3985-II; ⋆, BN. (*B*) ▼, RC-3925-II; ◇, RC-3930; ▽, RC-3990; ●, RC-3905-II; ○, RC-3995; ▲, RC-3910-II; △, RC-4000. The points indicate the mean value of specific binding from triplicate measurements of a representative experiment.

synthesis—i.e., the incorporation of [^{3}H]thymidine in quiescent Swiss 3T3 fibroblasts produced by GRP-(14–27). This system would also detect any agonistic activity of various analogs. Swiss 3T3 cells were incubated first with the analogs alone at concentrations from 1 nM to 1 μM to detect any agonistic activity and then with the analogs in combination with 1 nM GRP-(14–27) for determining antagonistic activity. None of the compounds tested alone showed any stimulation of the [^{3}H]thymidine uptake. The stimulatory effect of GRP-(14–27) on 3T3 cells varied depending on the number of cell passages. In our tests, 1 nM GRP-(14–27) produced 100–350% stimulation of [^{3}H]thymidine incorporation above the controls (data not shown), and antagonists inhibited this effect. Fig. 2 shows the inhibition curves for selected compounds. The best BN antagonist in the group was RC-3950-II with an IC_{50} value of 1 nM (Table 3). RC-3925-II was second best with an IC_{50} value of 6.1 nM.

In general, the [$^{13}\psi^{14}$,CH_2-N,Tac^{14}]BN-(6–14) compounds with N-terminal D-Phe, D-Cpa, or D-Tpi were more potent antagonists than the [$^{13}\psi^{14}$,CH_2-N,Me_2Tac^{14}]BN-(6–14) analogs with the corresponding N-terminal groups (Table 3 and Fig. 2). However, the RC-3905-II, which contains an N-terminal L-Tpi, was 30% less potent as an antagonist than RC-3995. Two [$^{13}\psi^{14}$,CH_2-N,MeTac^{14}]BN-(6–14) compounds with N-terminal D-Phe or D-Cpa (RC-3960 and RC-3930) were slightly less potent antagonists than [$^{13}\psi^{14}$,CH_2-N,Me_2Tac^{14}]BN-(6–14) compounds.

Antagonistic activity (IC_{50} values) and receptor binding (K_i values) were not always correlated. For example, RC-3905-II and RC-3910-II showed higher binding affinities than RC-3950-II, but they were less potent antagonists than RC-3950-II.

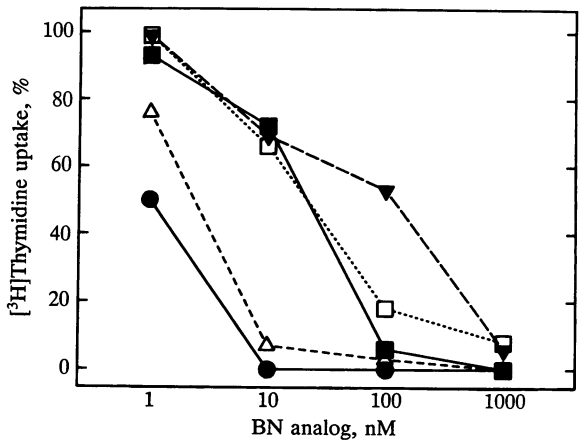


FIG. 2. Inhibition of 1 nM GRP-(14–27)-stimulated [^{3}H]thymidine incorporation into Swiss 3T3 cells by BN analogs. ●, RC-3950-II; □, RC-3910-II; △, RC-3925; ■, RC-3985-II; ▼, RC-3960. The points indicate the mean value of six replicates of a representative experiment.

DISCUSSION

One of the approaches to the synthesis of BN antagonists is the modification of the peptide bond in positions 13–14 to a reduced form, which affects its backbone conformation and produces enhanced antagonistic properties (8). It has also been found that in short-chain [$^{13}\psi^{14}$,CH_2-NH,Leu^{14}]BN-(6–14) or [$^{13}\psi^{14}$,CH_2-NH,Phe^{14}]BN-(6–14) antagonists, the incorporation of either D-Phe or D-Cpa as an N-terminal residue resulted in an increase of the antagonistic potency (9). On the basis of these reduced bond [$^{13}\psi^{14}$,CH_2-NH]BN-(6–14) antagonists, we synthesized a series of compounds with D- or L-Tpi as the N-terminal residue and Tpi as the C-terminal residue (12, 14).

Tpi is a constrained Trp analog in which the nitrogen of the α-amino group of Trp becomes part of a six-membered ring. The secondary amine of Tpi cannot form the reduced bond by the alkylation reaction. Leu-ψ(CH_2-N)-Tpi is formed by the reaction of formaldehyde with Leu-ψ(CH_2-NH)-Trp on benzhydrylamine resin. This paper reports synthesis of antagonists with Tac, MeTac, and Me_2Tac, which are constrained analogs of cystine containing a secondary amine. Again, the alkylation reaction could not be used to form the reduced peptide bonds of these cystine analogs. [$^{13}\psi^{14}$,CH_2-N,Tac^{14}]BN-(6–14), [$^{13}\psi^{14}$,CH_2-N,MeTac^{14}]BN-(6–14), and [$^{13}\psi^{14}$,CH_2-N,Me_2Tac^{14}]BN-(6–14) were synthesized by incubating [$^{13}\psi^{14}$,CH_2-NH,Cys^{14}]BN-(6–14) or [$^{13}\psi^{14}$,CH_2-NH,Pen^{14}]BN-(6–14) with formaldehyde or acetaldehyde. In the resulting compounds, the nitrogen of the α-amino group of Cys or Pen is a part of a five-membered thiazolidine ring.

We found that the replacement of Cys or Pen by the constrained Cys analogs produced a substantially increased binding affinity in radioreceptor assays. The order of relative binding affinities was [$^{13}\psi^{14}$,CH_2-N,Tac^{14}]BN-(6–14) > [$^{13}\psi^{14}$,CH_2-N,Me_2Tac^{14}]BN-(6–14) > [$^{13}\psi^{14}$,CH_2-N,MeTac^{14}]BN-(6–14) > [$^{13}\psi^{14}$,CH_2-NH,Cys^{14}]BN-(6–14) and [$^{13}\psi^{14}$,CH_2-NH,Pen^{14}]BN-(6–14). Antagonistic activities of [$^{13}\psi^{14}$,CH_2-NH,Cys^{14}]BN-(6–14) and [$^{13}\psi^{14}$,CH_2-NH,Pen^{14}]BN-(6–14) in growth inhibition tests with 3T3 cells were not determined. In most cases, the order of relative antagonistic activities was also [$^{13}\psi^{14}$,CH_2-N,Tac^{14}]BN-(6–14) > [$^{13}\psi^{14}$,CH_2-N,Me_2Tac^{14}]BN-(6–14) > [$^{13}\psi^{14}$,CH_2-N,MeTac^{14}]BN-(6–14). It appears that an increase in both receptor binding and antagonistic activity occurs with com-

pounds containing Tac as a C-terminal peptide residue. However, extra carbons in the thiazolidine ring as in MeTac and Me_2Tac do not enhance either binding affinity or antagonistic activity.

In our present test system, the IC_{50} values (nanomolar) were considerably higher than those obtained previously in the growth inhibition studies (12, 14). Part of this difference could be due to changes in the methodology for IC_{50} determination. Present tests were based on shorter term cell incubation with the compounds (24 hr vs. 48 hr) (12, 14), and also a short [^{3}H]thymidine labeling time (4 hr) was used as compared to previous continuous exposure to [^{3}H]thymidine during the 48-hr incubation period with the test compound (12, 14).

The results show that the compounds RC-3925-II, RC-3905-II, and RC-3910-II exhibit higher binding affinities than RC-3950-II; however, RC-3950-II is a better antagonist than RC-3925-II, RC-3905-II, and RC-3910-II in growth inhibition assays. It is possible that these three compounds with very nonpolar N-terminal residues might adhere more powerfully than RC-3950-II to the surface of test wells during the 24-hr incubation for the determination of antagonistic activity. This would reduce the concentration of these peptides in solution and their corresponding inhibitory activities. On the other hand, binding assays require only a 30-min incubation period, and the adsorption of peptide may not be significant.

The BN antagonist RC-3095 was synthesized and characterized earlier in our laboratory (14). In the present study, the K_i of RC-3095 was 0.20 nM, which is nearly the same as that of BN. The binding affinity of RC-3905-II was 200 times higher than that of RC-3095, but the antagonistic activities of both analogs were nearly equal (IC_{50} about 60 nM). RC-3095 has been shown to be a potent inhibitor of tumor growth in various experimental cancer models including MXT breast cancers in mice (19), nitrosamine-induced pancreatic cancers in hamsters (20), and human tumor lines such as HT-29 colon cancer (21), PC-82 prostate cancers (22), MKN45 gastric cancers (23), and CFPAC-1 ductal pancreatic cancer (24) xenografted into nude mice.

The modified BN antagonist RC-3950-II has a higher binding affinity to Swiss 3T3 cells, PC-3 and DU-145 human prostate cancer cell lines (25), and Hs746T human gastric cancer cells (26) than RC-3095. RC-3950-II also has a greater antagonistic activity in Swiss 3T3 cells and appears to be more efficacious than RC-3095 in other oncological tests such as growth inhibition of MCF-7 MIII human breast cancers (27) and H69 small cell lung carcinoma tumors in nude mice (28).

RC-3950-II was also compared to [D-Phe^6,$^{13}\psi^{14}$,CH_2-NH,Phe^{14}]BN-(6–14), which was originally reported by Coy *et al.* (9) and later also synthesized in our laboratory and designated with code no. RC-3110. It was found that the binding affinity of RC-3950-II was higher than that of RC-3110 in Swiss 3T3 cells, Hs746 human gastric cancer cells, and human prostate cancer cell lines PC-3 and DU-145 (25, 26).

The difference between RC-3950-II and RC-3110 resides in the C-terminal residue, which in RC-3950-II is Tac-NH_2 and in RC-3110 is Phe-NH_2. The C-terminal residue in BN is Met-NH_2, which is important for good binding but not essential for antagonistic activity. Replacement of Met by Leu or Phe in a BN antagonist with reduced peptide bonds resulted in a higher binding affinity. In addition, the displacement of Met eliminates the oxidation during the synthesis and storage (9). Tac is a closed ring analog of Met and is more hydrophobic than Met. The replacement of Phe with Tac in reduced bond BN antagonists produced improved K_i and IC_{50} values. BN antagonists such as RC-3950-II may have important therapeutic applications for the treatment of various malignancies such as prostatic, gastric, and pancreatic cancer, small cell lung carcinoma, and other tumors.

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