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Nonpeptide neuromedin B receptor antagonists inhibit the proliferation of C6 cells

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Abstract

The ability of nonpeptide antagonists to interact with neuromedin B receptors on C6 cells was investigated. 2-[3-(2,6-Diisopropyl-phenyl)-ureido]3-(1*H*-indol-3-yl)-2-methyl-*N*-(1-pyridin-2-yl-cyclohexylmethyl)-proprionate (PD165929), 3-(1*H*-indol-3-yl)-2-methyl-2-[3(4-nitro-phenyl)-ureido]-*N*-(1-pyridin-2-yl-cyclohexylmethyl)-propionamide (PD168368) and 3-(1*H*-indol-3-yl)-*N*-[1-(5-methoxy-pyridin-2-yl)-cyclohexylmethyl]-2-methyl-2-[3-(4-mitro-phenyl)-ureido]-propionamide (PD176252) inhibited (^{125}I-Tyr^{0})neuromedin B binding with IC_{50} values of 2000, 40 and 50 nM, respectively. Because neuromedin B is a G-protein coupled serpentine receptor, the effects of neuromedin B antagonists on second messenger production and proliferation were investigated. PD168368 inhibited the ability of 10 nM neuromedin B to cause elevation of cytosolic Ca^{2+}, whereas it had no effect on basal cytosolic Ca^{2+}. PD168368 inhibited the ability of 100 nM neuromedin B to cause elevation of c-*fos* mRNA. Also, PD168368 in a dose-dependent manner inhibited the ability of 100 nM neuromedin B to cause phosphorylation of focal adhesion kinase. Using a [3-(4,5 dimethylthiazol-2-yl)-2.5-diphenyl-2*H*-tetrazolium bromide] assay, the order of antagonist potency to inhibit C6 proliferation was PD168368 = PD176252 > PD165929. Also, 1 μM PD168368 and PD176252 significantly inhibited colony number using a proliferation assay in vitro. PD168368 significantly inhibited C6 xenograft growth in nude mice in vivo. These results indicate that PD168368 is a C6 cell neuromedin B receptor antagonist, which inhibits proliferation.

Keywords: Neuromedin B receptor antagonist, nonpeptide; Ca^{2+}, cytosolic; c-*fos* mRNA; Focal adhesion kinase; Proliferation

1. Introduction

Neuromedin B and gastrin releasing peptide are members of the bombesin family of peptides (McDonald et al., 1979; Minamino et al., 1983; Anastasi et al., 1973). Gastrin releasing peptide, a 27-amino acid peptide, and neuromedin B, a 10 amino acid peptide, have sequence homologies with the C-terminal of bombesin. Bombesin, which contains 14 amino acids, is biologically active in the central nervous system and periphery where it functions as a neuromodulator, decreasing food intake, inducing grooming behavior, increasing growth hormone secretion and elevating blood glucose levels (Gibbs et al., 1979; Merali et al., 1983; Westendorf and Schonbrunn, 1982, 1983; Brown et al., 1978). Neuromedin B suppresses feeding behavior and gastric emptying, increases pituitary hormone secretion, causes excitation of dorsal raphe serotonin neurons, controls potassium secretion by the blood–brain barrier and increases sensory transmission in the spinal cord (Rettori et al., 1989; Cridland and Henry, 1992; Pinnock et al., 1994; Vigne et al., 1997; Varga et al., 1995). In addition to being active in the central nervous system, bombesin increases the proliferation of normal epithelial and lung cells (Rozengurt and Sinett-Smith, 1983; Willey et al., 1984). Neuromedin B and gastrin releasing peptide are autocrine growth factors for some small cell lung cancer (SCLC) cells (Cuttitta et al., 1985; Moody et al., 1992).

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Four types of bombesin receptors have been cloned. The gastrin releasing peptide receptor is a 384-amino acid protein that contains seven transmembrane domains and is coupled to guanine nucleotide binding protein (Battey et al., 1991; Spindel et al., 1990). The gastrin releasing peptide receptor and the neuromedin B receptor, which is a 390-amino acid protein, stimulate phosphatidylinositol turnover (Sausville et al., 1988; Wada et al., 1991). The bombesin receptor subtype-3 is a 399-amino acid protein which has approximately 50% amino acid sequence homology to the gastrin releasing peptide and neuromedin B receptors, however, the endogenous ligand for the bombesin receptor subtype 3 is unknown (Fathi et al., 1993b). The gastrin releasing peptide receptor prefers gastrin releasing peptide relative to neuromedin B, whereas the neuromedin B receptor prefers neuromedin B relative to gastrin releasing peptide; bombesin receptor subtype-3 binds (D-Phe^{6}, β-Ala^{11}, Phe^{13}, Nle^{14})bombesin$^{6-14}$ but not gastrin releasing peptide or neuromedin B with high affinity (Moody et al., 1989; Moody and Jensen, 1996; Mantey et al., 1997). The bombesin$_4$ receptor was cloned and it prefers bombesin relative to neuromedin B or gastrin releasing peptide (Nagalla et al., 1995).

Gastrin-releasing peptide receptors have been identified in many cells whereas neuromedin B receptors are present on C6 glioma and SCLC cells (Wang et al., 1992; Moody et al., 1992). In addition, neuromedin B receptors have been transfected into BALB-3T3 and NCI-H1299 cells (Ryan et al., 1999). After neuromedin B binds with high affinity to its receptor, phosphatidylinositol is metabolized. The resulting products, diacylglycerol and inositol-1,4,5-trisphosphate, cause protein kinase C activation and release of Ca^{2+} from intracellular organelles. The latter leads to a transient increase in the cytosolic Ca^{2+}. The protein kinase C phosphorylates serine and threonine amino acids on protein substrates leading to the phosphorylation of mitogen activated protein kinase. The phosphorylated mitogen activated protein kinase can enter the nucleus leading to increased nuclear oncogene expression. Neuromedin B causes c-*fos* mRNA elevation and stimulates the clonal growth of SCLC cells. The signal transduction mechanisms for gastrin releasing peptide receptors and bombesin receptor subtype-3 appear similar to those of the neuromedin B receptor.

While numerous peptide receptor antagonists have been identified which block gastrin releasing peptide receptors (Heimbrook et al., 1989; Radulovic et al., 1991), neuromedin B receptors are blocked with low affinity by substance P antagonists (Zachary and Rozengurt, 1986; Woll and Rozengurt, 1988) and somatostatin agonists (Orbuch et al., 1993). Recently, neuromedin B receptor nonpeptide antagonists such as 2-[3-(2,6-diisopropyl-phenyl)-ureido]3-(1*H*-indol-3-yl)-2-methyl-*N*-(1-pyridin-2-yl-cyclohexylmethyl)-proprionate (PD165929) were developed (Eden et al., 1996). Here, the effect the effects of nonpeptide neuromedin B receptor antagonists were investigated on rat C6 glioma cells. Our results indicate that 3-(1*H*-indol-3-yl)-2-methyl-2-[3(4-nitro-phenyl)-ureido]-*N*-(1-pyridin-2-yl-cyclohexylmethyl)-propionamide (PD168368) inhibits the proliferation of C6 cells in vitro and in vivo.

2. Materials and methods

2.1. Cell culture

C6 cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (Life Technologies, Rockville, MD). The cells were split weekly with trypsin-ethylene diamino tetraacetic acid. The cells were mycoplasma-free and were used when they were in exponential growth phase after incubation at 37°C in 5% CO_2/95% air.

2.2. Receptor binding

The ability of PD165929, PD168368 and 3-(1*H*-indol-3-yl)-*N*-[1-(5-methoxy-pyridin-2-yl)-cyclohexylmethyl]-2-methyl-2-[3-(4-nitro-phenyl)-ureido]-propionamide (PD176252) to inhibit specific (^{125}I-Tyr^{0})neuromedin B binding to C6 cells was investigated. For the in vitro studies, PD165929, PD168368 and PD176252 were dissolved in dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO) at a concentration of 10 mM. (Tyr^{0})neuromedin B was purchased (Peninsula Lab., San Carlos, CA) and iodinated to a specific activity of 2200 Ci/mmol (Amersham, Arlington Heights, IL). C6 cells in 24-well plates were washed three times in SIT medium (DMEM containing 3×10^{-8} M sodium selenite, 5 μg/ml bovine insulin and 10 μg/ml transferrin (Sigma)). The cells were incubated in SIT buffer containing 0.25% bovine serum albumin and 250 μg/ml bacitracin. After incubation at 37°C for 30 min, free (^{125}I-Tyr^{0})neuromedin B was removed and the cells which contained bound (^{125}I-Tyr^{0})neuromedin B were counted in a gamma counter.

2.3. Cytosolic Ca^{2+}

The ability of PD168368 to inhibit the increase in cytosolic Ca^{2+} caused by neuromedin B was investigated. C6 cells were harvested (2.5×10^{6}/ml) and incubated with 5 μM Fura 2 AM (Calbiochem, LaJolla, CA) at 37°C for 30 min (Moody et al., 1987). The cells, which contained loaded Fura 2, were centrifuged at $1500 \times g$ for 10 min and resuspended at the same concentration in new SIT medium, the SIT medium had Ca^{2+} but similar results were obtained if 1 mM ethyleneglycoltetraaceticacid was added. The fluorescence intensity was continuously monitored using a Perkin-Elmer LS2 spectrofluorometer equipped with a magnetic stirring mechanism and temperature (37°C) regulated cuvette holder prior to and after the addition of peptide.

2.4. Focal adhesion kinase

The ability of PD168368 to inhibit tyrosine phosphorylation of focal adhesion kinase induced by neuromedin B was determined (Garcia et al., 1997). C6 cells were cultured in 15-cm dishes. When a monolayer of cells formed they were placed in SIT media containing 0.5% fetal bovine serum overnight. Three hours before treatment, cells were placed in fresh ST media. Cells were treated with neuromedin B for 2.5 min, washed twice with phosphate-buffered saline (PBS) and lysed in buffer containing 50 mM Tris.HCl (pH 7.5), 150 mM sodium chloride, 1% Triton X-100, 1% deoxycholate, 1% sodium azide, 1 mM ethyleneglycoltetraacetic acid, 0.4 M ethylenediaminotetraacetic acid, 1.5 μg/ml aprotinin, 1.5 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride and 0.2 mM sodium vanadate (Sigma). The lysate was sonicated for 5 at 4°C and centrifuged at 10,000 × *g* for 15 min. Protein concentration was measured by Bio-Rad protein assay reagent, and 150 μg/ml of protein was incubated with 4 μg of anti-phosphotyrosine monoclonal antibody (Upstate Biotechnologies, Lake Placid, NY), 4 μg of goat anti-mouse immunoglobulin IgG and 30 μl of protein A-agarose overnight at 4°C. The immunoprecipitates were washed 3 times with PBS and analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and Western blotting. Immunoprecipitates were fractionated using 10% polyacrylamide gels (Novex, San Diego, CA). Membranes were blocked overnight at 4°C using blotto (5% non-fat dried milk in solution containing 50 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM sodium chloride, 0.05% Tween 20 and 0.02% sodium azide) and incubated for 2 h at 25°C with anti-mouse immunoglobulinG-horseradish peroxidase conjugate. The membrane was washed for 10 min with blotto and twice for 10 min with washing solution (50 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM sodium chloride, 0.05% Tween 20 and 0.02% sodium azide). The blot was incubated with enhanced chemiluminescence detection reagent for 5 min and exposed to Hyperfilm ECL. The density of bands was determined using a densitometer.

2.5. C-Fos mRNA

The ability of PD168368 to alter c-*fos* gene expression induced by NMB was investigated (Draoui et al., 1995). For the c-*fos* experiments, C6 cells were cultured with SIT medium containing 0.5% fetal bovine serum. After 4 h, the cells were treated with 100 nM neuromedin B in the presence or absence of competitor for 60 min. Total RNA was isolated using guanidinium isothiocyanate (Fluka Biochemicals). Ten μg of denatured RNA was separated in a 0.66 M formaldehyde 1% agarose gel. The gel was treated with ethidium bromide to assess RNA integrity. The RNA was blotted onto a nytran membrane overnight and the membrane hybridized with DNA probes labeled with ^{32}P-dCTP using a Bethesda Research Laboratories random priming kit. The membrane was apposed to Kodak XAR-2 film at −80°C for 1 day and the autoradiogram developed. The autoradiograms were analyzed using a Molecular Dynamics densitometer.

2.6. Proliferation

Growth studies in vitro were conducted using the a [3-(4,5 dimethylthiazol-2-yl)-2.5-diphenyl-2 *H*-tetrazolium bromide] colorimetic assays. C6 cells (10^4/well) were placed in SIT medium and varying concentrations of PD165929, PD168368 and PD176252 added. After 4 days, a [3-(4,5 dimethylthiazol-2-yl)-2.5-diphenyl-2 *H*-tetrazolium bromide] (Sigma) was added. After 4 h, 150 μl of DMSO was added. After 16 h, the optical density at 570 nm was determined using an ELISA reader.

The effects of PD168368 on the growth of C6 cells were investigated using a clonogenic assay (Mahmoud et al., 1991). The base layer consisted of 3 ml of 0.5% agarose in SIT medium containing 5% fetal bovine serum in 6-well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose (FMC, Rockford, ME), PD168368 and 5×10^4 C6 cells. Triplicate wells were plated and after 2 weeks, 1 ml of 0.1% *p*-iodonitrotetrazolium violet was added and after 16 h at 37°C, the plates were screened for colony formation; the number of colonies larger than 50 μm in diameter were counted using an Omnicon image analysis system.

The ability of the neuromedin B antagonists to inhibit C6 growth was also investigated in vivo. Female athymic Balb/c nude mice (Taconic Farms), 4–5 weeks old, were housed in a pathogen-free temperature controlled isolation room, with a diet consisting of autoclaved rodent chow and autoclaved water given ad libitum. C6 cells (1×10^7) were injected into the right flank of each mouse by subcutaneous injection. Palpable tumors were observed in approximately 90% of the mice after 1 week. PBS (100 μl) or PD168368 (0.12 or 1.2 mg/kg in polyethylene glycol 400) were injected daily during weeks 1.5 through 2.5. The tumor volume (height × width × depth) was determined weekly by calipers and recorded. When the tumor became necrotic (> 2000 mm^3 in volume), the growth studies were terminated. The animal studies were approved by the NCI animal care and use committee and are in accordance with the Declaration of Helsinki accepted principles in the care and use of experimental animals.

3. Results

3.1. Receptor binding

Fig. 1 shows that neuromedin B receptor antagonists inhibited specific (^{125}I-Tyr^0)neuromedin B binding to C6 cells in a dose-dependent manner. Little specific binding

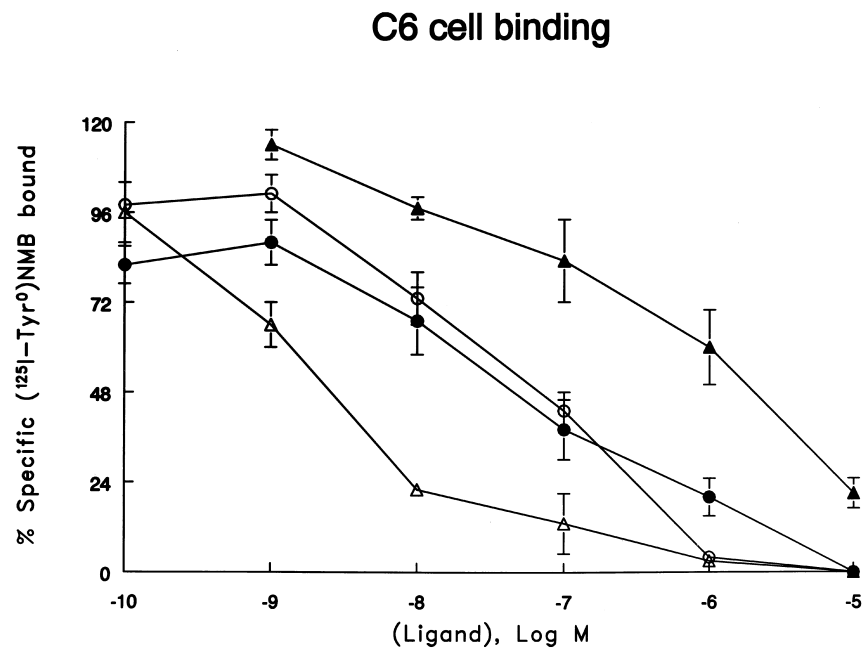

Fig. 1. Neuromedin B receptor binding. Specific binding of (^{125}I-Tyr^{0})neuromedin B to C6 cells was determined as a function of neuromedin B (△), PD165929 (▲), PD168368 (●) and PD176252 (○) concentration. The mean value ± S.D. of four determinations is indicated and this experiment was repeated four times.

was inhibited by 1 nM PD168368 whereas almost all specific binding was inhibited by 10,000 nM PD168368. Specific (^{125}I-Tyr^{0})neuromedin B binding was half maximally inhibited (IC_{50}) by 40 nM PD168368, 50 nM PD176252 and 2000 nM PD165929. In contrast, neuromedin B bound with high affinity (IC_{50} value of 2 nM) to C6 cells. These results indicate that neuromedin B, PD168368 and PD176252 bind with high affinity to C6 neuromedin B receptors.

3.2. Cytosolic Ca^{2+}

Fig. 2 (left) shows that administration of 10 nM neuromedin B caused the cytosolic Ca^{2+} to increase from 150 to 180 nM 10 s after addition to Fura-2AM loaded C6

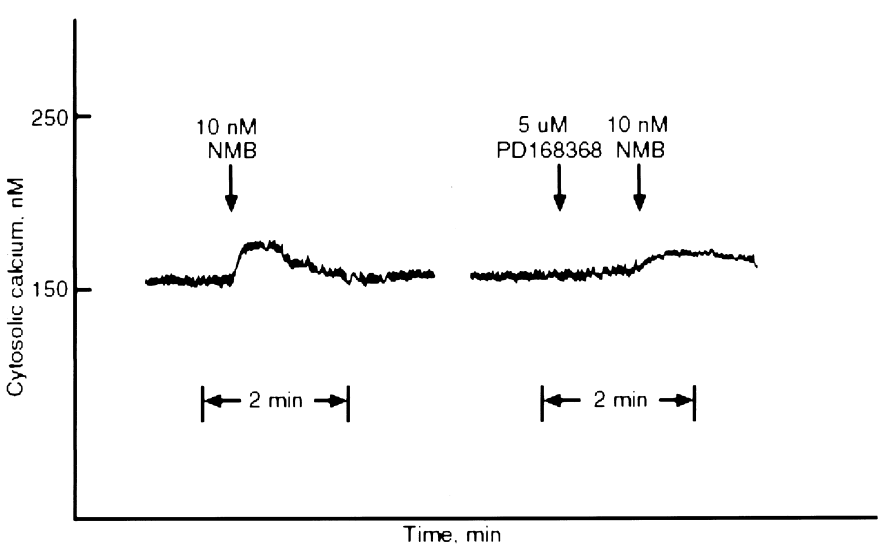

Fig. 2. Cytosolic Ca^{2+}. The ability of 10 nM neuromedin B to elevate cytosolic Ca^{2+} in the absence (left) or presence (right) of 5 μM PD168368. This experiment is representative of two others.

cells. The increase in cytosolic Ca^{2+} caused by neuromedin B was transient, being maximal after 0.5 min and slowly returning to basal levels after 2 min. Fig. 2 (right) shows that 5 μM PD168368 decreased the elevation in cytosolic Ca^{2+} caused by 10 nM neuromedin B; 0.5 μM PD168368 did not alter the elevation in cytosolic Ca^{2+} caused by 10 nM neuromedin B (data not shown). Also, 20 μM PD176252 and PD165929 blocked the increase in cytosolic Ca^{2+} caused by 10 nM neuromedin B (data not shown). These results indicate that PD168368 antagonizes the ability of neuromedin B to elevate cytosolic Ca^{2+} in C6 cells.

3.3. C-fos mRna

The effects of PD168368 on c-*fos* mRNA were investigated. Fig. 3 (top) shows the addition of 100 nM neuromedin B to C6 cells caused the c-*fos* mRNA to increase after 1 h. PD168368 had little effect on basal c-*fos* expression but 1 μM PD168368 weakly and 10 μM PD168368 strongly inhibited the increase in c-*fos* mRNA caused by 100 nM neuromedin B. Equal amounts of RNA were loaded onto the gel based on ethidium bromide staining of the 18S and 28S rRNA bands (Fig. 3, middle). Neuromedin B increased the c-*fos* mRNA 2.8-fold (Fig. 3, bottom). 10 μM PD168368 + 100 nM neuromedin B had little effect on the c-*fos* mRNA relative to the control.

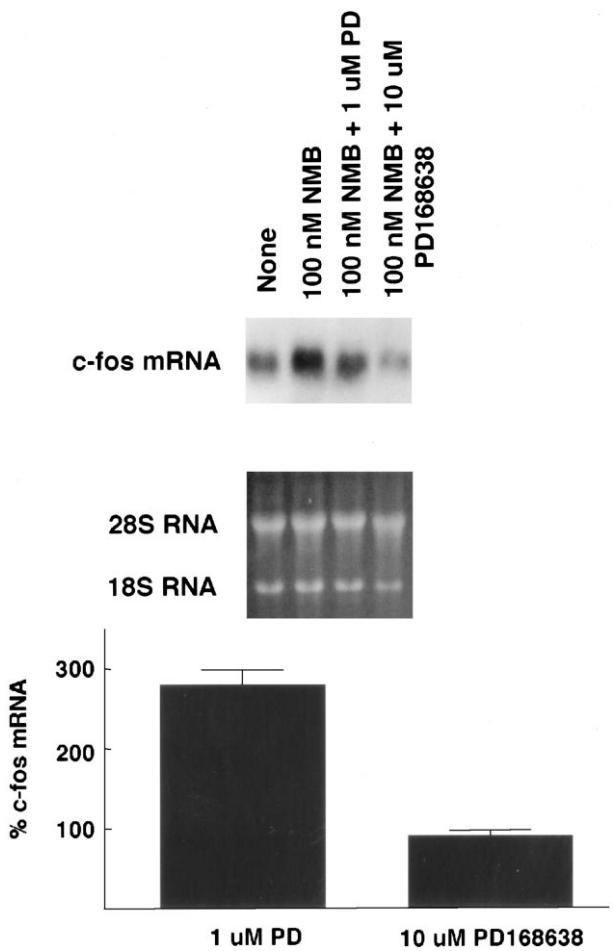

Fig. 3. C-*fos* mRNA. Top: PD168368 (PD) inhibited the increase in c-*fos* mRNA caused by 100 nM neuromedin B in a dose-dependent manner using C6 cells. Middle: Equal amounts of RNA were loaded onto the gel based on ethidium bromide staining of the 18S and 28S rRNA bands. Bottom: The c-*fos* mRNA was quantitated on a densitometer and the % c-*fos* mRNA calculated relative to basal values. The mean value ± S.D. of three determinations is indicated.

3.4. Focal adhesion kinase

Fig. 4 (top) shows that the 125-kDa focal adhesion kinase was tyrosine phosphorylated in C6 cells. The amount of phosphorylated focal adhesion kinase increased by 50% 1 min after addition of 100 nM neuromedin B to C6 cells. PD168368 at a 10 and 100 μM concentration moderately and strongly, respectively, inhibited the increase in focal adhesion kinase tyrosine phosphorylation caused by 100 nM neuromedin B. In contrast, lower concentrations of PD168368, e.g. 0.1 and 1 μM had little effect (Fig. 4, bottom). The results indicate that PD168368 antagonized in a concentration-dependent manner the ability of neuromedin B to tyrosine phosphorylate C6 focal adhesion kinase.

3.5. Proliferation

3.5.1. [3-(4,5 Dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide] assay

The effects of PD165929, PD168368 and PD176252 on C6 proliferation were investigated. Fig. 5 shows that using the [3-(4,5 dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide] assay, 0.1 μM PD168368 had little effect on C6 growth whereas 10 μM PD168368 maximally inhibited growth. The IC_{50} for both PD168368 and PD176252 was 2 μM whereas PD165929 was less potent with an IC_{50} value of 8 μM. These results indicate that nonpeptide neuromedin B antagonists inhibit the proliferation of C6 cells in a dose-dependent manner. As a control, 0.1% DMSO had little effect on proliferation (data not shown).

3.5.2. Clonogenic assay

C6 cells formed colonies using a soft agar clonogenic assay. Table 1 shows that in the presence of 1 μM PD168638 and PD176252 the number of colonies was reduced significantly by 51% and 34%, respectively. In contrast, 0.1 μM PD168368 or 0.1 μM PD176252 had little effect on colony proliferation. Neuromedin B (0.01 μM) significantly increased colony number and the increase caused by neuromedin B was reversed by 1 μM PD168368 or 1 μM PD176252. These results indicate that

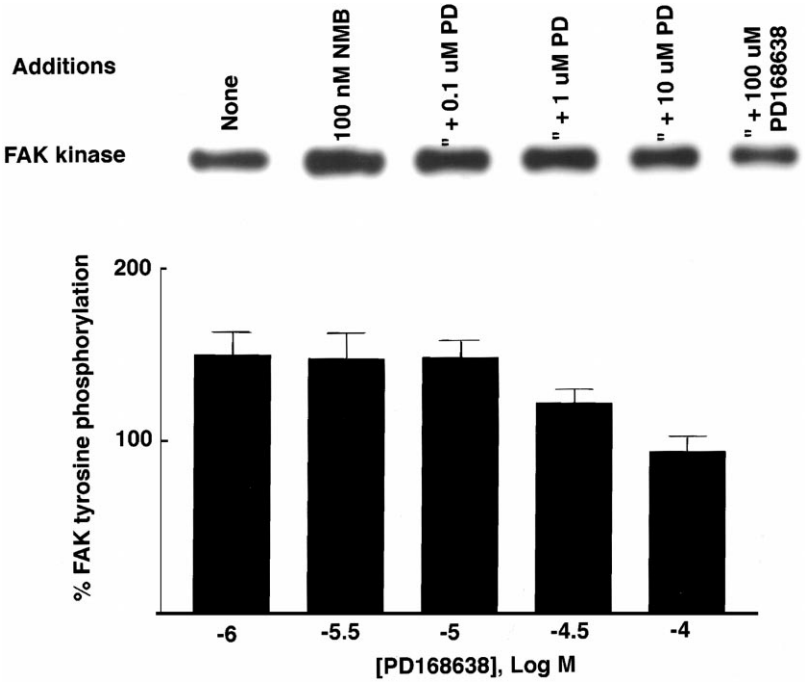

Fig. 4. Focal adhesion kinase phosphorylation. Top: Focal adhesion kinase tyrosine phosphorylation was increased by 100 nM neuromedin B and was decreased by PD168368 in a dose-dependent manner. Bottom: The % focal adhesion kinase tyrosine phosphorylation was calculated relative to basal. The mean value ± S.D. of three experiments is indicated.

nonpeptide neuromedin B receptor antagonists inhibit whereas neuromedin B increases C6 colony forming efficiency.

3.5.3. Nude mice xenografts

The growth effects of PD168368 were investigated in vivo. Fig. 6 shows that after 1.5 weeks palpable C6

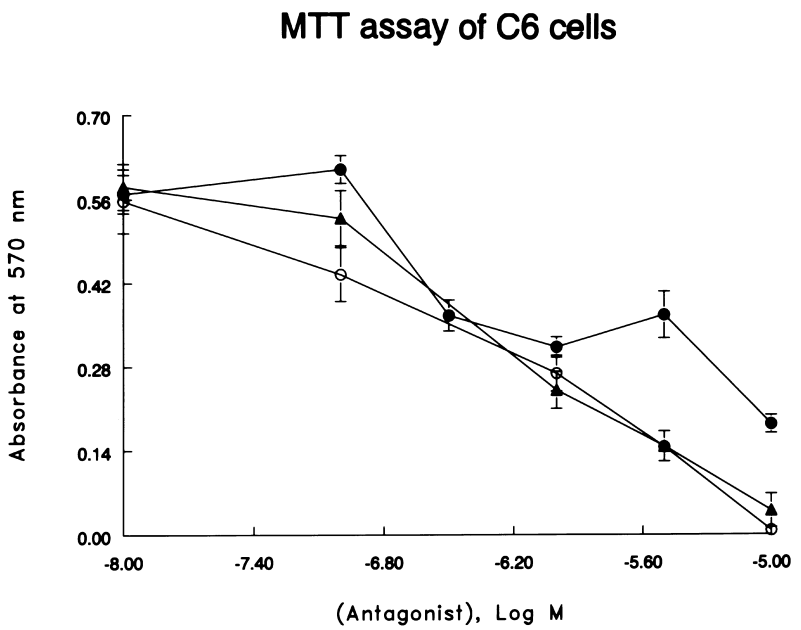

Fig. 5. [3-(4,5-Dimethylthiazol-2-yl)-2.5-diphenyl-2 *H*-tetrazolium bromide] assay. The proliferation of C6 cells was determined as a function of PD165929 (●), PD168368 (○) and PD176252 (▲) concentration. The mean value ± S.D. of eight determinations is indicated. This experiment was repeated three times.

Table 1
Clonogenic assay

Addition	Colonies
None	97 ± 16
PD168368, 0.1 μM	79 ± 12
PD168368, 1 μM	48 ± 14*
PD176252, 0.1 μM	92 ± 9
PD176252, 1 μM	67 ± 8*
Neuromedin B, 0.01 μM	135 ± 14*
Neuromedin B + PD168368, 1 μM	88 ± 9
Neuromedin B + PD176252, 1 μM	104 ± 12

The mean value ± S.D. of three determinations is indicated. This experiment is representative of 2 others. The structures of NMB, PD165929, PD168368 and PD176252 are as follows: NMB, Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2; PD165929, 2-[3-(2,6-Diisopropyl-phenyl)-ureido]3-(1*H*-indol-3-yl)-2-methyl-*N*-(1-pyridin-2-yl-cyclohexylmethyl)-proprionate; PD168368, 3-(1*H*-Indol-3-yl)-2-methyl-2-[3(4-nitro-phenyl)-ureido]-*N*-(1-pyridin-2-yl-cyclohexylmethyl)-propionamide; PD176252, 3-(1*H*-Indol-3-yl)-*N*-[1-(5-methoxy-pyridin-2-yl)-cyclohexylmethyl]-2-methyl-2-[3-(4-mitro-phenyl)-ureido]-propionamide.

* $p < 0.05$ using Student's *t*-test.

xenografts formed in nude mice. PD168368 was injected by gavage daily (0.12 mg/kg, s.c., and 1.2 mg/kg) and PD168368 significantly slowed xenograft proliferation at weeks 2 and 2.5. At week 2.5, the tumor volumes for animals receiving PBS and 0.12 mg/kg/day PD168368 were similar at 1093 and 1141 mm^3, respectively. In contrast, animals receiving 1.2 mg/kg/day PD168368 had a significantly smaller xenograft volume at 644 mm^3. These results indicate that PD168368 slowed tumor growth in a dose-dependent manner

4. Discussion

Previously, we found that C6 cells bound neuromedin B with high affinity ($IC_{50} = 2$ nM; Wang et al., 1992). Neuromedin B elevated cytosolic Ca^{2+} and increased phosphatidylinositol turnover. The effects of neuromedin B were antagonized with low affinity by the peptide antagonist (D-Arg^1, D-$Trp^{7,9}$, Leu^{11})substance P. Here, the effects of nonpeptide receptor antagonists for neuromedin B were investigated on C6 cells.

A major problem was the limited solubility of PD165929, PD168368 and PD176252 in physiological buffers. Previously it was found that PD168368 was soluble in cyclodextrin and DMSO but not physiological buffers (Ryan et al., 1999). For the in vitro studies, we dissolved PD165929, PD168368 and PD176252 in DMSO and then diluted a stock of 10 mM into physiological buffer. We found that PD165929, PD1687368 and PD176252 inhibited (^{125}I-Tyr^0)neuromedin B binding to rat glioma C6 cells with variable affinities (IC_{50} values of 2000, 40 and 50 nM), respectively. When PD168368 was dissolved in cyclodextrin it was found to bind to C6 cells, which have neuromedin B but not gastrin releasing peptide receptors, with high affinity ($IC_{50} = 39$ nM; Ryan et al., 1999). In

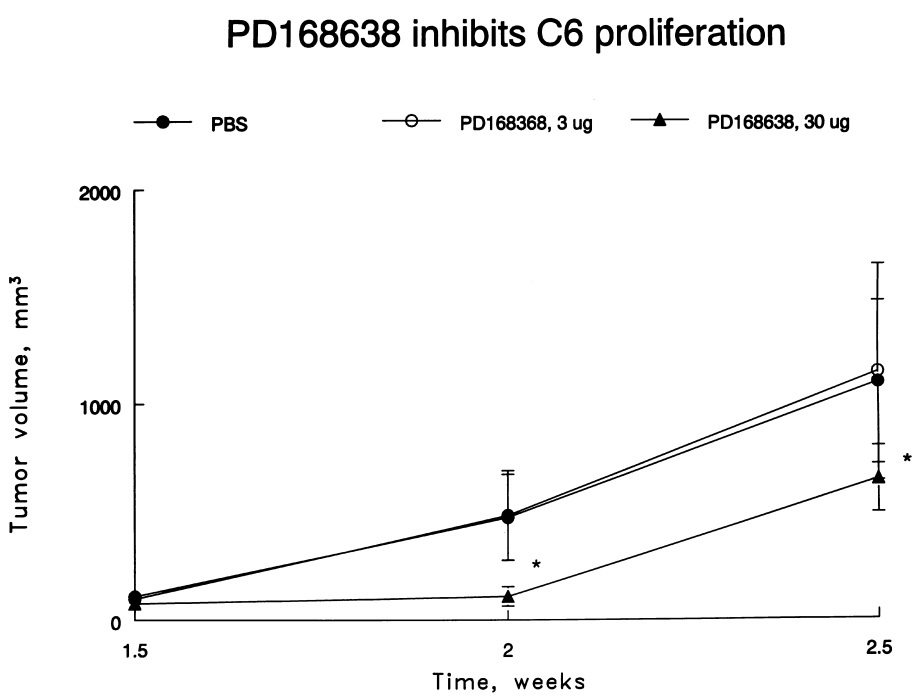

Fig. 6. C6 xenografts. C6 tumors formed after 1.5 weeks. Nude mice bearing C6 xenografts were injected s.c. with PBS (●), 0.12 mg/kg (○) or 1.2 mg/kg (▲) of PD168368. These experiments are representative of 2 others; $p < 0.05$ using Newman Keul's multiple comparison *t*-test, *. The mean value ± S.D. of four determinations is indicated. These experiments are representative of three others.

contrast human SCLC cells have both neuromedin B and gastrin releasing peptide receptors (Mahmoud et al., 1991; Moody et al., 1992). Preliminary data (T. Moody, unpublished) indicate that PD168368 inhibits (^{125}I-Tyr^{0})neuromedin B binding to NCI-H345 SCLC cells with high affinity (IC_{50} value of 20 nM). In contrast, PD165929 is more potent at inhibiting binding to Xenopus oocytes tranfected with the human neuromedin B receptor and has an IC_{50} value of 7 nM (Eden et al., 1996). These results suggest that PD165929 may bind with higher affinity to the human than rat neuromedin B receptor. Also, PD168368 and PD176252 bound to human neuromedin B receptors with IC_{50} values of 0.15 and 0.17 nM (Ashwood et al., 1999); neuromedin B may bind with higher affinity in these latter studies due to differences in the methodology used. While both PD165929 and PD168368 bind with low affinity to the gastrin releasing peptide receptor (IC_{50} values of $>$ 10,000 and 273 nM, respectively; Ashwood et al., 1999), PD176252 had an IC_{50} values of 1 nM for the human gastrin releasing peptide receptor. These results indicate that PD165929 and PD168368 are highly specific for the neuromedin B receptor, whereas PD176252 blocks both neuromedin B and gastrin releasing peptide receptors.

Somatostatin analogs such as (Nal^{6},Thr^{8})cyclo-somatostatin-octapeptide were found to antagonize neuromedin B receptors with low affinity (IC_{50} value of 1000 nM; Orbuch et al., 1993). Also, (D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11})substance P binds to neuromedin B receptors with low affinity (IC_{50} value of 2000 nM). The nonpeptide neuromedin B receptor antagonists such as PD168368 bind with over an order of magnitude greater affinity to neuromedin B receptors than do somatostatin or substance P analogs. An essential amino acid residue for high affinity binding on the neuromedin B receptor has been defined. Substitution of Ser^{216} for Ile^{216} in the neuromedin B receptor abolished high affinity neuromedin B binding and the ability of neuromedin B to cause phosphatidyl turnover (Fathi et al., 1993a). It is unknown which amino acids in the neuromedin B receptor are important for high affinity PD168368 binding.

The NMB receptor may interact with guanine nucleotide binding protein (Gq) causing phosphatidylinositol turnover. The inositol-1,4,5-trisphosphate released causes release Ca^{2+} from intracellular organelles such as the endoplasmic reticulum. PD168368 antagonized the increase in cytosolic Ca^{2+} caused by 10 nM neuromedin B. Because high concentrations of neuromedin B (10 nM) were used, high concentrations of PD168368 (10 μM) were needed to inhibit the increase in cytosolic Ca^{2+} caused by neuromedin B. PD168368 had little effect on basal cytosolic Ca^{2+}.

When phosphatidylinositol is metabolized by phospholipase C, diacylglycerol is released which activates protein kinase C. Previous studies have shown that neuromedin B causes increased phospholipase C, and phospholipase D activity (Wang et al., 1992; Hou et al., 1998). Protein kinase C may phosphorylate protein substrates which activates mitogen activated protein kinase. Gastrin releasing peptide increased phosphorylation of the mitogen activated protein kinase substrate myelin basic protein (Koh et al., 1999). Activated mitogen activated protein kinase can enter the nucleus and activate elk-1, leading to increased nuclear oncogene expression (Draoui et al., 1995). Here neuromedin B increased c-*fos* mRNA and the increase in c-*fos* expression caused by neuromedin B is inhibited by PD168368.

In a different signal transduction mechanism, neuromedin B stimulated focal adhesion kinase tyrosine phosphorylation. Neuromedin B (100 nM) increased focal adhesion kinase phosphorylation by approximately 50% and the increase caused by neuromedin B was inhibited by 10 μM PD168638. Previously, gastrin releasing peptide was found to stimulate focal adhesion kinase activity in Swiss 3T3 cells and the increase caused by gastrin releasing peptide was reversed by gastrin releasing peptide receptor antagonists such as (Psi^{13,14}, Leu^{14})bombesin (Zachary et al., 1992). In contrast (Psi^{13,14}, Leu^{14})bombesin has little effect on neuromedin B receptors. Activation of the mitogen activated protein kinase and/or focal adhesion kinase pathways may increase the proliferation of C6 cells.

Neuromedin B receptor antagonists inhibited the proliferation of C6 cells. In the [3-(4,5 dimethylthiazol-2-yl)-2.5-diphenyl-2*H*-tetrazolium bromide] assay, PD165929, 168368 and 176252 inhibited C6 growth in a concentration-dependent manner. The order of antagonist potency was PD168368 = PD176252 > PD165929. Preliminary data (T. Moody, unpublished) indicates that 10 μM PD168368 causes apoptosis of C6 cells. In the clonogenic assay, 1 but not 0.1 μM PD168368 inhibited the colony forming efficiency of C6 cells. Because the C6 cells are exposed to PD168368 for 14 days in the clonogenic assay as apposed to 4 days for the [3-(4,5 dimethylthiazol-2-yl)-2.5-diphenyl-2*H*-tetrazolium bromide] assay, lower concentrations of PD168368 are needed to inhibit C6 proliferation in the clonogenic as apposed to the [3-(4,5 dimethylthiazol-2-yl)-2.5-diphenyl-2*H*-tetrazolium bromide] assay. These results suggest that C6 cells may make an endogenous neuromedin-like peptide and use it as an autocrine growth factor. Also, neuromedin B increased C6 colony formation

For the nude mouse studies, PD168368 was dissolved in polyethylene glycol 400 and injected daily (0.1 and 1.2 mg/kg. Because PD168368 is nonpeptide, it is resistant to degradation by stomach proteases and readily distributes throughout various organs of the body including the brain. PD168368 significantly inhibited C6 xenograft proliferation in nude mice. Preliminary data (T. Moody, unpublished) indicates that PD168368 significantly decreased tumor but not body weight of the nude mice, suggesting that PD168368 has little toxicity.

In summary, C6 neuromedin B receptors interact with nonpeptide neuromedin B receptor antagonists. PD168368

is a potent neuromedin B receptor antagonist, which inhibits the proliferation of C6 cells. It remains to be determined if nonpeptide neuromedin B receptor antagonists inhibit the proliferation of cancer cells, such as SCLC, which are enriched in neuromedin B receptors.

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