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Nonpeptide gastrin releasing peptide receptor antagonists inhibit the proliferation of lung cancer cells

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Abstract

The ability of nonpeptide antagonists to interact with gastrin releasing peptide receptors on lung cancer cells was investigated. PD176252 (3-(1H-Indol-3-yl)-*N*-[1-(5-methoxy-pyridin-2-yl)-cyclohexylmethyl]-2-methyl-2-[3-(4-nitro-phenyl)-ureido]-propionamide) and PD168368 (3-(1H-Indol-3-yl)-2-methyl-2-[3(4-nitro-phenyl)-ureido]-*N*-(1-pyridin-2-yl-cyclohexylmethyl)-propionamide) inhibited specific ^{125}I-gastrin releasing peptide binding to NCI-H1299 cells with IC_{50} values of 20 and 1500 nM, respectively. Similar binding results were obtained using NCI-H157, H345 and N592 human lung cancer cells. PD176252 inhibited the ability of 1 nM bombesin to cause elevation of cytosolic calcium in Fura-2 loaded NCI-H345 or H1299 cells, whereas it had no effect on basal cytosolic calcium. PD176252 antagonized the ability of 10 nM bombesin to cause elevation of c-*fos* mRNA in NCI-H1299 cells. Also, PD176252 inhibited the ability of 100 nM bombesin to cause tyrosine phosphorylation of focal adhesion kinase in NCI-H1299 cells. Using a [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay, PD176252 was more potent than PD168368 at inhibiting NCI-H1299 proliferation. Also, 1 μM PD176252 significantly inhibited lung cancer colony number in vitro. PD176252 in a dose-dependent manner inhibited NCI-H1299 xenograft growth in nude mice in vivo. These results indicate that PD176252 is a gastrin releasing peptide receptor antagonist, which inhibits the proliferation of lung cancer cells.

Keywords: PD176252; Gastrin releasing peptide receptor; Antagonist; Lung cancer; Proliferation

1. Introduction

Gastrin releasing peptide and neuromedin B are members of the bombesin family of peptides (Anastasi et al., 1973; McDonald et al., 1979; Minamino et al., 1983). Gastrin releasing peptide, a 27 amino acid peptide, and neuromedin B, a 10 amino acid peptide, have sequence homologies with the carboxyl 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 (Brown et al., 1978; Gibbs et al., 1979; Merali et al., 1983; Westendorf and Schonbrunn, 1982). Bombesin and gastrin releasing peptide bind with high affinity to the gastrin releasing peptide receptor, a 384 amino acid protein which contains 7 transmembrane domains and is coupled to guanine nucleotide binding protein (Spindel et al., 1990; Battey et al., 1991). Neuromedin B suppresses feeding behavior and gastric emptying, increases pituitary hormone secretion, causes excitation of dorsal raphe serotonin neurons and increases sensory transmission in the spinal cord (Rettori et al., 1989; Cridland and Henry, 1992; Pinnock et al., 1994; Varga et al., 1995). Neuromedin B binds with high affinity to the neuromedin B receptor, a 390 amino acid protein which has approximately 50% sequence homology with the gastrin releasing peptide receptor (Wada et al., 1991). 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). Gastrin releasing

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peptide and neuromedin B are autocrine growth factors for some small cell lung cancer cells (Cuttitta et al., 1985, Moody et al., 1992).

After bombesin binds with high affinity to gastrin releasing peptide receptors, phosphatidylinositol is metabolized (Sausville et al., 1988). The resulting products, diacylglycerol and inositol-1,4,5-trisphosphate, cause protein kinase C activation and release of calcium (Ca^{2+}) from intracellular organelles (Moody et al., 1987; Bunn et al., 1990). Protein kinase C phosphorylates serine and threonine amino acids on protein substrates leading to the phosphorylation of mitogen activated protein kinase (Koh et al., 1999). The phosphorylated mitogen activated protein kinase can enter the nucleus leading to increased nuclear oncogene expression (Draoui et al., 1995). Bombesin or gastrin releasing peptide causes c-*fos* mRNA elevation and stimulates the clonal growth of small cell lung cancer cells (Carney et al., 1985). The signal transduction mechanisms for neuromedin B appear similar to those of gastrin releasing peptide (Moody et al., 2000).

Numerous peptides have been identified which block gastrin releasing peptide receptors including bombesin analogues (Heimbrook et al., 1989; Radulovic et al., 1991; Mahmoud et al., 1991) and substance P receptor antagonists (Jensen et al., 1985). Recently, nonpeptide antagonists for the gastrin releasing peptide (PD 176252) and neuromedin B (PD168368) receptors were developed (Eden et al., 1996; Ashwood et al., 1999). Here the effects of PD168368 (3-(1H-Indol-3-yl)-2-methyl-2-[3(4-nitro-phenyl)-ureido]-*N*-(1-pyridin-2-yl-cyclohexylmethyl)-propionamide) and PD176252 (3-(1H-Indol-3-yl)-*N*-[1-(5-methoxy-pyridin-2-yl)-cyclohexylmethyl]-2-methyl-2-[3-(4-nitro-phenyl)-ureido]-propionamide) were investigated on human lung cancer cells. Our results indicate that PD176252 inhibits specific ^{125}I-gastrin releasing peptide binding to NCI-H345 and H1299 cells with approximately two-orders of magnitude greater affinity than does PD168368. PD176252 antagonized the ability of BB to cause elevated cytosolic calcium, increased focal adhesion kinase tyrosine phosphorylation and increased c-*fos* mRNA. Also, PD176252 inhibited the growth of NCI-H1299 cells in vitro and in vivo. These results indicate that PD176252 is a nonpeptide lung cancer gastrin releasing peptide receptor antagonist, which inhibits proliferation of lung cancer cells.

2. Materials and methods

2.1. Cell culture

NCI-H157, H345, N592 and H1299 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% heat-inactivated fetal bovine serum (Life Technologies, Rockville, MD). The NCI-H345 and N592 cells grew as floating aggregates, whereas the NCI-H157 and H1299 cells were adherent (Carney et al., 1985). NCI-H157 and H1299 cells were split weekly 1/20 with trypsin-ethylenediaminotetraaceticacid, whereas NCI-H345 and N592 cells were diluted 1/1 into new media. 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 PD168368 and PD176252 to inhibit specific ^{125}I-gastrin releasing peptide binding to NCI-H345, N592, H157 and H1299 cells was investigated (Mahmoud et al., 1991). PD176252 and PD168368 were dissolved in dimethylsulfoxide (Sigma, St. Louis, MO) at a concentration of 10 mM. Gastrin releasing peptide was purchased (Phoenix Pharmaceuticals, Belmont, CA) and iodinated to a specific activity of 2200 Ci/mmol (Amersham, Arlington Heights, IL). Neuromedin B and bombesin were purchased from (Phoenix Pharmaceuticals), whereas 3-Phenylpropanoyl-His-Trp-Ala-Val-Dala-His-Dpro-psi-Phe-NH_2 (BW2258U89) was a gift from Dr. J. McDermed (Univ. of North Carolina). NCI-H157 or H1299 cells, which are adherent, were placed in 24-well plates and washed three times in SIT medium (RPMI-1640 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 (Sigma) and ^{125}I-gastrin releasing peptide (100,000 cpm) added as well as various concentrations of PD168368 or PD176252. After incubation at 37 °C for 30 min, free ^{125}I-gastrin releasing peptide was removed by washing three times in buffer and the cells which contained bound ^{125}I-gastrin releasing peptide dissolved in 0.2 N NaOH and counted in a gamma counter. NCI-H345 or N592 cells, which are non-adherent, were washed three times in SIT medium and placed in SIT buffer containing 0.25% bovine serum albumin and 250 μg/ml bacitracin (Sigma) and ^{125}I-gastrin releasing peptide (100,000 cpm) added as well as various concentrations of unlabeled competitor. After incubation at 25 °C for 60 min, free ^{125}I-gastrin releasing peptide was removed after centrifugation of the cells and the pellet rinsed three times with buffer. The pellet, which contained bound ^{125}I-gastrin releasing peptide, was counted in a gamma counter.

2.3. Cytosolic calcium

The ability of PD176252 to inhibit the increase in cytosolic calcium caused by gastrin releasing peptide or bombesin was investigated. NCI-H1299 cells were harvested (2.5×10^6/ml) and incubated with 5 μM Fura 2 (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 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 gastrin releasing peptide, bombesin or PD176252.

2.4. C-Fos mRNA

The ability of PD176252 to alter c-*fos* gene expression induced by bombesin was investigated (Draoui et al., 1995). For the c-*fos* experiments, NCI-H1299 cells were cultured with SIT medium containing 0.5% fetal bovine serum. After 4 h, the cells were treated with 10 nM bombesin in the presence or absence of PD176252 for 60 min. Total RNA was isolated using guanidinium isothiocyanate (Fluka Biochemicals). Ten micrograms 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.

2.5. Focal adhesion kinase

The ability of PD176252 to inhibit tyrosine phosphorylation of focal adhesion kinase induced by bombesin was determined (Leyton et al., 2001). NCI-H1299 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 SIT media. Cells were treated with 100 nM bombesin for 2.5 min, washed twice with 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 s at 4 °C and centrifuged at 10,000 × *g* for 15 min. Protein concentration was determined using Bio-Rad protein assay reagent, and 150 μg/ml of protein was incubated with 4 μg of anti-focal adhesion kinase monoclonal antibody, 4 μg of goat anti-mouse immunoglobulin and 30 μl of protein A-agarose overnight at 4 °C. The immunoprecipitates were washed three times with phosphate buffered saline and analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and Western blotting. Immunoprecipitates were fractionated using 10% polyacrylamide gels (Novex, San Diego, CA). Proteins were transferred to nitrocellulose membranes and the 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 1 μg/ml anti-focal adhesion kinase monoclonal antibody or anti-phospho-tyrosine antibody followed by anti-mouse immunoglobulinG-horseradish peroxidase conjugate (Upstate Biotechnologies, Lake Placid, NY). 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.

2.6. Proliferation

Growth studies in vitro were conducted using the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide colorimetic assays. NCI-H1299 cells (10^4/well) were placed in SIT medium and various concentrations of PD176252 or PD168368 added. After 4 days, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma) was added. After 4 h, 150 μl of dimethylsulfoxide was added. After 16 h, the optical density at 570 nm was determined.

The effects of PD176252 on the growth of NCI-H1299 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 lung cancer 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 PD176252 to inhibit NCI-H1299 tumor proliferation was 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. NCI-H1299 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. Polyethylene glycol (PEG, 100 μl) or PD176252 (10 or 1 μg in 100 μl of PEG 400) were injected daily by gavage. The tumor volume (height × width × depth) was determined weekly by calipers and recorded. 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 gastrin releasing peptide receptor antagonists inhibited specific ^{125}I-gastrin releasing peptide

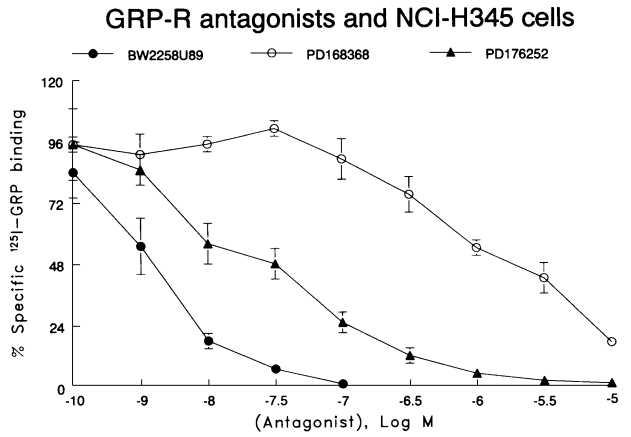

Fig. 1. Gastrin releasing peptide (GRP) receptor binding. Specific binding of ^{125}I-gastrin releasing peptide to NCI-H345 cells was determined as a function of BW2258U89 (●), PD168368 (○) and PD176252 (▲) concentration. The mean value ± S.D. of four determinations is indicated and this experiment was repeated three times.

binding to NCI-H345 cells in a concentration-dependent manner. Little specific binding was inhibited by 0.1 nM PD176252, whereas almost all specific bindings were inhibited by 1000 nM PD176252. Specific ^{125}I-gastrin releasing peptide binding was half maximally inhibited (IC_{50}) by 30 nM PD176252. In contrast, BW2258U89 and PD168368 had IC_{50} values of 2 and 2000 nM, respectively. Table 1 shows that bombesin, gastrin releasing peptide and neuromedin B inhibited specific ^{125}I-gastrin releasing peptide binding to NCI-H345 cells with IC_{50} values of 2, 3 and 100 nM, respectively. The order of potency was bombesin = BW2258U89 = gastrin releasing peptide>PD176252>neuromedin B>PD168368. Similar results were obtained using NCI-H1299, NCI-H157 and N592 cells (Table 1).

Table 1
Binding to lung cancer cells

Ligand	IC_{50}, nM			
	NCI-H345 cells	NCI-H1299 cells	NCI-H157 cells	NCI-N592 cells
Bombesin	2 ± 1	3 ± 1	2 ± 1	4 ± 1
BW2258U89	2 ± 0	2 ± 1	1 ± 0	3 ± 1
Gastrin releasing peptide	3 ± 1	4 ± 1	2 ± 0	3 ± 1
Neuromedin B	100 ± 10	200 ± 20	150 ± 10	250 ± 30
PD168368	2000 ± 160	1500 ± 120	1200 ± 90	2500 ± 200
PD176252	30 ± 3	20 ± 2	20 ± 3	30 ± 4

The mean IC_{50} (nM) to inhibit specific ^{125}I-gastrin releasing peptide binding is shown. The mean value ± S.E. of three determinations each repeated in quadruplicate is indicated. The structures of bombesin-like peptides are shown below where — represents the peptide bond and = the psi reduced peptide bond. All amino acids are of the L-isomer unless indicated.

Bombesin: Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2.

Gastrin releasing peptide: Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2.

Neuromedin B: Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2.

BW2258U89: 3-Phenylpropanoyl-His-Trp-Ala-Val-Dala-His-Dpro=Phe-NH_2.

3.2. Cytosolic calcium

Fig. 2 (top, left) shows that 1 nM bombesin caused the cytosolic calcium to increase from 150 to 170 nM, within 15 s after addition to Fura-2 loaded NCI-H1299 cells. The increase in cytosolic calcium caused by bombesin was transient, being maximal after 0.5 min and slowly returning to basal levels. Fig. 2 (top, middle) shows that 1 μM PD176252 strongly inhibited the increase in cytosolic calcium caused by 1 nM bombesin and 10 μM PD176252 totally inhibited the increase in cytosolic calcium caused by 1 nM bombesin (Fig. 2, top, right). Fig. 2 (bottom) shows that 10 μM PD176252 had little effect on basal cytosolic calcium; but totally inhibited the increase in cytosolic calcium caused by 1 nM bombesin. The effects of PD176252 were reversible, however, in that 100 nM bombesin increased the cytosolic calcium and overcame the gastrin releasing peptide receptor blockade caused by PD176252 (Fig. 2, bottom). In contrast to PD176252, 10 μM PD168368 had little effect on antagonizing the cytosolic

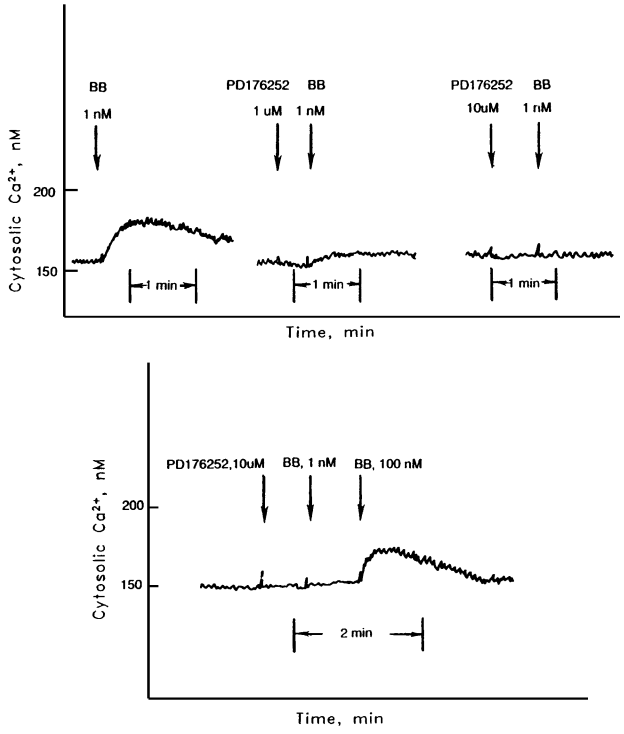

Fig. 2. Cytosolic calcium (Ca^{2+}). (Top) The ability of 1 nM bombesin (BB) to elevate cytosolic calcium in the absence (left) or presence of 1 μM PD176252 (middle) and 10 μM PD176252 (right) was determined using Fura-2 loaded NCI-H1299 cells. These experiments are representative of four others. (Bottom) PD176252 (10 μM) antagonized the ability of 1 but not 100 nM bombesin to elevate the cytosolic calcium using NCI-H1299 cells. These experiments are representative of three others.

calcium increase caused by addition of 1 nM bombesin to NCI-H1299 cells; similar cytosolic calcium results were obtained using Fura-2 loaded NCI 345 cells (data not shown).

3.3. C-Fos mRNA

The effects of PD176252 on c-*fos* mRNA were investigated. Fig. 3 (top) shows addition of 10 nM bombesin to NCI-H1299 cells caused the c-*fos* mRNA to increase after 1 h. PD176252 had little effect on basal c-*fos* expression but 1 μM PD176252 weakly and 10 μM PD176252 strongly inhibited the increase in c-*fos* mRNA caused by 10 nM bombesin. In contrast, 10 μM PD168368 had little effect on the basal c-*fos* mRNA (data not shown). Equal amounts of RNA were loaded onto the gel based on ethidium bromide staining of the 18S and 28S rRNA bands (Fig. 3, bottom).

3.4. Focal adhesion kinase

Fig. 4 (top) shows that the 125 kDa focal adhesion kinase was tyrosine phosphorylated in NCI-H1299 cells. The amount of phosphorylated focal adhesion kinase increased twofold 2.5 min after addition of 100 nM bombesin to NCI-H1299 cells. PD176252 at a 10 μM concentration strongly inhibited the increase in focal adhesion kinase tyrosine phosphorylation caused by 100 nM bombesin, whereas 10 μM PD176252 had little effect on basal focal adhesion kinase tyrosine phosphorylation. 10 μM of PD168368 had little effect on the ability of bombesin to cause tyrosine phosphorylation of focal adhesion kinase (data not shown). As a control, equal amounts of focal adhesion kinase were loaded onto the gel (Fig. 4, bottom).

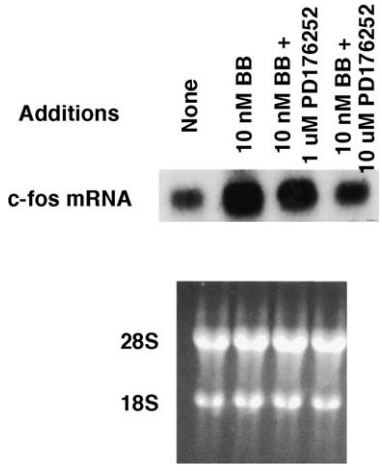

Fig. 3. C-*fos* mRNA. (Top) PD176252 (10 μM) but not 1 μM strongly inhibited the increase in c-*fos* mRNA caused by 10 nM bombesin using NCI-H1299 cells. (Bottom) Equal amounts of RNA were loaded onto the gel based on ethidium bromide staining of the 18S and 28S rRNA bands. This experiment is representative of two others.

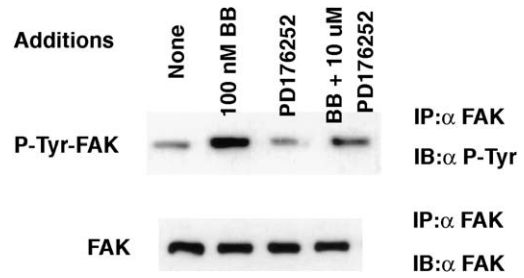

Fig. 4. Focal adhesion kinase (FAK) phosphorylation. (Top) Focal adhesion kinase tyrosine phosphorylation was increased by 100 nM bombesin and was decreased by 10 μM PD176252, whereas PD176252 had little effect on basal focal adhesion kinase phospho-tyrosine (P-Tyr); the immunoprecipitating (IP) and immunoblotting (IB) antibodies are indicated. (Bottom) Equal amounts of focal adhesion kinase protein were loaded onto the gel. This experiment is representative of three others.

3.5. Proliferation

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

The effects of PD176252 and PD168368 on NCI-H1299 proliferation were investigated. Fig. 5 shows that using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay, 1 μM PD176252 had little

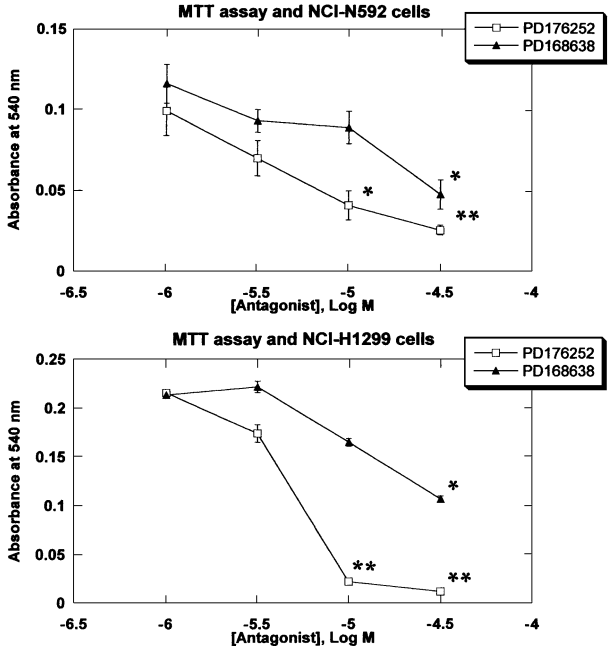

Fig. 5. [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT) assay. The proliferation of NCI-N592 (top) and NCI-H1299 (bottom) cells was determined as a function of PD176252 (□) and PD168638 (▲) concentration. The mean value ± S.D. of eight determinations is indicated. This experiment is representative of two others; $p < 0.01$, **; $p < 0.05$, * using Student's *t*-test relative to control.

effect on NCI-H345 or H1299 growth, whereas 10 or 30 μM PD 176252 significantly inhibited the growth. The IC_{50} for PD176252 was 5 and 7 μM using NCI-H1299 and H345 cells, respectively. In contrast, PD 168368 was less potent with IC_{50} values of 20 and 30 μM using NCI-H345 and H1299 cells, respectively. Similar results were obtained for NCI-H157 and N592 cells (data not shown).

3.5.2. Clonogenic assay

NCI-H1299 cells formed colonies using a soft agar clonogenic assay. Fig. 6 shows that PD176252 (0.001 μM) had little effect on colony formation, whereas 1 μM PD176252 significantly inhibited almost all colony proliferation. The IC_{50} for PD176252 was 0.2 and 0.3 μM using NCI-N592 and H345 cells, respectively. Similar results were obtained for NCI-H157 and H1299 cells (data not shown).

3.5.3. Nude mice xenografts

The growth effects of PD176252 were investigated in vivo. Fig. 7 (top) shows that after 1 week, small palpable NCI-H1299 xenografts formed in nude mice. PD176252 (10 μg), which was injected by gavage daily, significantly slowed xenograft proliferation at weeks 2 and 3. At week 3, the tumor volumes for the control were 1645 mm^3, whereas animals receiving 1 μg and 10 μg/day of PD176252 had tumor volumes of 1210 and 847 mm^3, respectively. Large NCI-H1299 tumors at week 2 were treated with PD176252 daily by gavage (Fig. 7, bottom). PD176252 significantly inhibited xenograft proliferation at weeks 3 and 4. At week 4, the tumor volumes for the control were 6500 mm^3, whereas animals receiving 1 μg and 10 μg/day of PD176252 had tumor volumes of 5183 and 3214 mm^3, respectively. These results indicate that PD176252 inhibits the growth of large and small NCI-H1299 xenografts in nude mice.

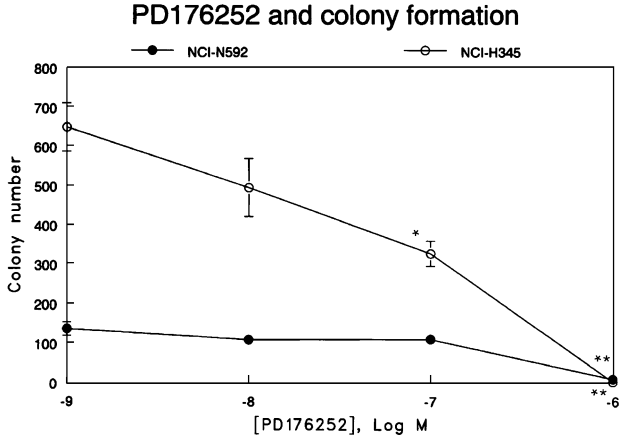

Fig. 6. Clongenic assay. The ability of PD176252 to inhibit NCI-H345 (○) and N592 (●) colony formation was determined as a function of PD176252 concentration. The mean value ± S.D. of three determinations was calculated; $p < 0.05$, *; $p < 0.01$, ** using Student's *t*-test. This experiment is representative of two others.

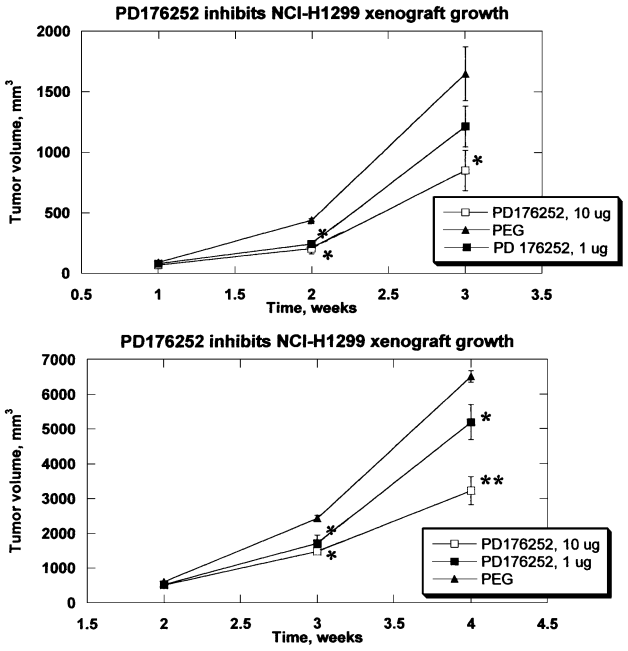

Fig. 7. NCI-H1299 xenografts. (Top) Small NCI-H1299 tumors formed after 1 week and nude mice were then injected with 100 μl of polyethylene glycol (PEG)-400 (▲) containing 1 μg (■) or 10 μg (□) of PD176252 daily by gavage. (Bottom) Large NCI-H1299 tumors formed after 2 weeks and nude mice were injected with polyethyleneglycol-400 (▲), 1 μg (■) or 10 μg (□) of PD176252 daily by gavage. These experiments are representative of two others; $p < 0.05$, *; $p < 0.01$, ** using Newman–Keul's multiple comparison test. The mean value ± S.D. of six determinations is indicated.

4. Discussion

Previously, BW2258U89 was identified as a gastrin releasing peptide receptor antagonist for lung cancer cells. BW2258U89, which is a peptide, bound with high affinity to NCI-H1299 cells (Moody et al., 1995). BW2258U89 inhibited the increase in cytosolic calcium and c-*fos* mRNA caused by 10 nM bombesin using NCI-H1299 cells. Also, BW2258U89 inhibited the growth of NCI-H1299 cells in vitro and in vivo (Marquez et al., 2000). BW2258U89 was metabolized by mouse blood enzymes, however, resulting in deamidated BW2258U89. When BW2258U89 was deamidated, its biological activity was reduced by approximately two-orders of magnitude (Marquez et al., 2000). Here the effects of the nonpeptide gastrin releasing peptide receptor antagonist, PD176252, were investigated on lung cancer cells.

PD176252, similar to PD168368, had limited solubility in water and was dissolved in dimethylsulfoxide for in vitro experiments at a final concentration of 10 mM (Ryan et al., 1999). PD176252 was then diluted for various experiments and inhibited specific ^{125}I-gastrin releasing peptide binding

to NCI-H345 cells with high affinity (IC_{50} = 30 nM). In contrast, BW2258U89 and PD168638 inhibited ^{125}I-gastrin releasing peptide binding with IC_{50} values of 2 and 2000 nM, respectively. These results indicate the PD176252 binds with approximately two-orders of magnitude greater affinity to NCI-H1299 cells than does PD168368. Similarly, using xenopus oocytes transfected with human gastrin releasing peptide receptors, Ashwood et al. (1999) showed that PD168368 bound with low affinity (IC_{50} value of 273 nM), whereas PD176252 bound with high affinity (IC_{50} value of 1 nM). In contrast, PD168368 and PD176252 inhibited specific [^{125}I-Tyr^{0}]neuromedin B binding to C6 rat glioma cells, which have neuromedin B receptors, with IC_{50} values of 20 and 30 nM (Moody et al., 2000). Also, PD168368 and PD176252 bound to oocytes transfected with human neuromedin B receptors with IC_{50} values of 0.15 and 0.17 nM (Ashwood et al., 1999). These results indicate that both PD176252 and PD168368 bind with high affinity to neuromedin B receptors, whereas PD176252 but not PD168368 binds with high affinity to gastrin releasing peptide receptors.

PD176252 is the first nonpeptide antagonist described for gastrin releasing peptide receptors (Ashwood et al., 1999). Numerous peptide antagonists for the gastrin releasing peptide receptor have been described including [D-Phe^{12}]bombesin (Heinz-Erian et al., 1987) and substance P receptor antagonists such as [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]substance P (Jensen et al., 1985). Subsequently, high affinity gastrin releasing peptide receptor antagonists were identified such as [D-Phe^{6}]bombesin$^{6-13}$ methylester, [Psi^{13,14},Leu^{14}]bombesin and BW2258U89 which have IC_{50} values of 5, 30 and 2 nM, respectively (Mahmoud et al., 1991; Moody et al., 1995). Numerous amino acids such as Gln^{121}, Pro^{199}, Arg^{288} and Ala^{308} are important for high affinity agonist binding to the gastrin releasing peptide receptor, whereas Thr^{297}, Phe^{302} and Ser^{305} are important for high affinity binding of gastrin releasing peptide receptor antagonists (Moody and Jensen, 1998). It remains to be determined which amino acids of the gastrin releasing peptide receptor are important for high affinity binding of PD176252. Previously, Tyr^{220} of the neuromedin B receptor was identified as essential for high affinity binding of PD168368 (Tokita et al., 2001).

PD176252 inhibited second messenger productions regulated by the gastrin releasing peptide receptor. PD176252 had no effect on basal cytosolic calcium but antagonized the increase in cytosolic calcium caused by 1 nM bombesin. In contrast, the cytosolic calcium increased after the addition of 100 nM bombesin to NCI-H1299 cells that had been blocked by 10 μM PD176252. These results indicate that PD176252 is a reversible gastrin releasing peptide receptor antagonist.

When phosphatidylinositol is metabolized by phospholipase C, diacylglycerol is released, which activates protein kinase C leading to phosphorylation of mitogen activated protein kinase (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). Ten but not 1 μM PD176252 strongly inhibited the increase in c-*fos* mRNA caused by addition of 10 nM bombesin to NCI-H1299 cells. In contrast, PD176252 had little effect on basal c-*fos* mRNA. These results indicate that PD176252 antagonizes gastrin releasing peptide receptors in a concentration-dependent manner.

In a different signal transduction mechanism, bombesin stimulated focal adhesion kinase tyrosine phosphorylation (Leyton et al., 2001). Bombesin (100 nM) increased focal adhesion kinase phosphorylation by approximately 50% and the increase caused by bombesin was inhibited by 10 μM PD176252. In contrast, PD176252 had little effect on basal focal adhesion kinase tyrosine phosphorylation. 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).

Gastrin releasing peptide receptor antagonists inhibited the proliferation of lung cancer cells. In the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay, PD176252 was approximately an order of magnitude more potent than PD168638 at inhibiting lung cancer growth in a concentration-dependent manner. In the clonogenic assay, 1 but not 0.01 μM PD176252 inhibited the colony forming efficiency of NCI-H1299 cells. Because the NCI-H1299 cells are exposed to PD176252 for 14 days in the clonogenic assay as apposed to 4 days for the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay, lower concentrations of PD176252 are needed to inhibit NCI-H1299 proliferation in the clonogenic as apposed to the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. Because NCI-H1299 cells make endogenous bombesin-like peptides, PD176252 may antagonize the growth effects on endogenous autocrine growth factors.

It is possible that PD176252 causes a G1 to S cell cycle phase block of lung cancer cells. Previously we found that chemotherapeutic drugs such as taxol caused a G2 to M phase block of lung cancer cells (Moody et al., 2001b). Nonpeptide receptor antagonists such as SR48692 were synergistic with taxol at inhibiting lung cancer cellular growth (Moody et al., 2001a). Currently we are investigating if PD176252 is synergistic with taxol at inhibiting lung cancer growth.

Daily injection of 10 μg of BW2258U89 subcutaneously strongly inhibited lung cancer xenograft proliferation in nude mice (Moody et al., 1995). Unfortunately, clinical trials with BW2258U89 are not anticipated because the peptide is deamidated by blood proteases (Marquez et al., 2000). An advantage of nonpeptide receptor antagonists is their resistance to degradation by proteases (Betancur et al., 1998). For the nude mouse studies, PD176252 was dissolved in PEG and administered daily (1 or 10 μg) by

gavage. Because PD176252 is nonpeptide, it is resistant to degradation by stomach proteases and readily distributes throughout various organs of the body (Ashwood et al., 1999). PD176252 at a 10-μg dose significantly inhibited NCI-H1299 xenograft proliferation in nude mice. Preliminary data (T. Moody, unpublished) indicates that PD176252 significantly decreased tumor but not body weight of the nude mice, suggesting that PD176252 has little toxicity.

Gastrin releasing peptide receptor antagonists may be of utility in cancers other than lung. Using in vitro autoradiographic techniques, gastrin releasing peptide receptors were detected in biopsy specimens from breast, prostate, renal and colon cancer patients (Guger and Reubi, 1999; Markwalder and Reubi, 1999; Reubi et al., 2002) Also, (Psi^{13,14})-bombesin analogs have been found to inhibit the growth of human gastric, colon and pancreatic cancers (Pinski et al., 1994; Radulovic et al., 1991). It remains to be determined if PD176252 will be useful at inhibiting the growth of human cancer cells other than lung.

In summary, lung cancer gastrin releasing peptide receptors bind PD176252 with high affinity. PD176252 is a potent gastrin releasing peptide receptor antagonist, which inhibits the proliferation of NCI-H1299 and H345 cells. It remains to be determined if nonpeptide gastrin releasing peptide receptor antagonists will function as therapeutic agents in the treatment of lung cancer.

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