



AM-37 and ST-36 Are Small Molecule Bombesin Receptor Antagonists

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While peptide antagonists for the gastrin-releasing peptide receptor (BB₂R), neuromedin B receptor (BB₁R), and bombesin (BB) receptor subtype-3 (BRS-3) exist, there is a need to develop non-peptide small molecule inhibitors for all three BBR. The BB agonist (BA)1 binds with high affinity to the BB₁R, BB₂R, and BRS-3. In this communication, small molecule BBR antagonists were evaluated using human lung cancer cells. AM-37 and ST-36 inhibited binding to human BB₁R, BB₂R, and BRS-3 with similar affinity ($K_i = 1.4\text{--}10.8 \mu\text{M}$). AM-13 and AM-14 were approximately an order of magnitude less potent than AM-37 and ST-36. The ability of BA1 to elevate cytosolic Ca²⁺ in human lung cancer cells transfected with BB₁R, BB₂R, and BRS-3 was antagonized by AM-37 and ST-36. BA1 increased tyrosine phosphorylation of the EGFR and ERK in lung cancer cells, which was blocked by AM-37 and ST-36. AM-37 and ST-36 reduced the growth of lung cancer cells that have BBR. The results indicate that AM-37 and ST-36 function as small molecule BB receptor antagonists.

Keywords: small molecule antagonists, GRPR, NMBR, bombesin receptor subtype-3, lung cancer

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INTRODUCTION

The bombesin (BB) family of peptides is biologically active in the central nervous system (CNS) and periphery. BB, a 14 amino acid peptide isolated from frog skin, has 9 of the 10 same C-terminal amino acids as does human gastrin-releasing peptide (GRP), a 27 amino acid peptide (1). GRP binds with high affinity to the BB₂R, which regulates pruritus, lung development, and gastrin secretion. Neuromedin B (NMB) is a 10 amino acid peptide with 70% sequence homology to the C-terminal of BB. NMB binds with high affinity to the BB₁R and causes satiety, hypothermia, and thyrotropin (TSH) secretion from the pituitary (2). BB receptor subtype-3 (BRS-3) is an orphan receptor with homology to the BB₁R and BB₂R, and binds the universal agonist, BB agonist (BA)1, with high affinity as does the BB₁R and BB₂R (3). Because BRS-3 knockout mice have impaired energy balance, glucose homeostasis, and increased body weight, BRS-3 agonists may function as satiety agents (4). In the CNS, GRP and NMB may act in a paracrine manner being released from brain neurons in the hypothalamus and dentate gyrus, respectively, activating BB₂R and BB₁R in adjacent cells (5).

In numerous cancers, including lung cancer, GRP and NMB function in an autocrine manner to stimulate cellular proliferation. Small cell lung cancer (SCLC), a neuroendocrine tumor, has high levels of GRP (6, 7). GRP is secreted from SCLC and binds to cell surface BB₂R resulting in increased cellular proliferation (8). NMB is present in both SCLC and non-small cell lung cancer (NSCLC) cells, and after secretion it binds to cell surface BB₁R stimulating proliferation (9). Because

many lung cancer cells have BB₁R, BB₂R, and/or BRS-3 there is a need to develop antagonists that block all three receptors of the BB family.

The human BB₁R, BB₂R, and BRS-3 contain 390, 384, and 399 amino acids and have approximately 50% sequence homology. The BB₁R, BB₂R, and BRS-3 are members of the rhodopsin β group G protein-coupled receptors (GPCR) family, and they interact with G_q causing phosphatidylinositol (PI) turnover (10). PI-4,5-bisphosphate (PIP₂) is metabolized to diacylglycerol, which activates protein kinase C and inositol-trisphosphate (IP₃) which causes elevated cytosolic Ca²⁺. Neuropeptide receptors regulate the transactivation of the epidermal growth factor (EGF) receptor leading to NSCLC proliferation (11). The proliferation of NSCLC cells caused by BA1 can be inhibited by the tyrosine kinase inhibitor (TKI) gefitinib or BBR antagonists. The actions of BA1 on BB₁R, BB₂R, and BRS-3 are antagonized selectively by PD168368, PD176252, and Bantag-1, respectively (12).

In the present study, small molecules were synthesized and their ability to antagonize BB₁R, BB₂R, and BRS-3 in lung cancer cells evaluated. The results indicate that AM-37 and ST-36 are useful agents to inhibit the growth of NSCLC cells which have BB₁R, BB₂R, or BRS-3.

MATERIALS AND METHODS

Cell Culture

Non-small cell lung cancer cell line NCI-H1299 (ATCC, Manassas, VA, USA) was stably transfected with BB₁R, BB₂R, and BRS-3. The transfected cells were grown in RPMI-1640 containing 10% fetal bovine serum (FBS) with 0.3 mg/ml geneticin (Invitrogen, Grand Island, NY, USA). The transfected cells, which contained approximately 100,000 receptors/cell, were weekly split using trypsin/EDTA (13). In addition, lung cancer cell lines NCI-H727, H1299, and H1975 were purchased from ATCC and cultured in RPMI-1640, which contained 10% FBS. The cell types were derived from different human biopsy specimens. These studies were approved by the NIDDK biospecimens and biosafety committees.

Ligand Synthesis

The small molecules were synthesized as described previously (14). **Figure 1D** shows the structural formula of AM-37, (R)-3-(1H-indol-3-yl)-2-[3-(4-methoxyphenyl)ureido]-N-[[1-(3-pyridinyl)cyclohexyl]methyl]propanamide, and of its S-enantiomer ST-36. **Figure 1E** shows the structural formula of AM-13, (R)-N-[[1-(4-fluorophenyl)cyclohexyl]methyl]-3-(1H-indol-3-yl)-2-[3-(4-methoxyphenyl)ureido]propanamide, and its S-enantiomer AM-14. The molecular weight of AM-37 and ST-36 is 525.6 Da, whereas the molecular weight of AM-13 and AM-14 is 542.2 Da.

Receptor Binding

The ability of AM-37, ST-36, AM-13, and AM-14 to inhibit specific ¹²⁵I-BA1 binding to NSCLC cells transfected stably with BB₁R, BB₂R, and BRS-3 was investigated. NSCLC cells were placed in 24 well plates. When confluent, the cells were washed three times with PBS. The cells were incubated with

binding buffer (PBS containing 0.25% bovine serum albumin and 0.025% bacitracin, Sigma-Aldrich, St. Louis, MO, USA). Various concentrations of AM-37, ST-36, AM-13, or AM-14 were added to the cells for 10 min, followed by 100,000 cpm of ¹²⁵I-BA1 (0.16 nM) and incubated at 37°C for 30 min when equilibrium of binding was reached. The cells were rinsed three times with binding buffer for 2 min at 4°C. The cells that contained bound peptide dissolved in 0.2 N NaOH and counted in a Wallac 1470 γ -counter. The K_i was calculated as described (15).

Cytosolic Ca²⁺

The ability of AM-37, ST-36, AM-13, and AM-14 to function as BBR antagonists was investigated. NSCLC cells transfected with BB₁R, BB₂R, and BRS-3 were harvested and loaded with Fura-2AM (Calbiochem, La Jolla, CA, USA) as described previously (16). The excitation ratio was determined at 340 and 380 nm with an emission wavelength of 510 nm. The lung cancer cellular calcium response was determined after the addition of AM-37, ST-36, AM-13, or AM-14 followed by 10 nM BA1.

Tyrosine Phosphorylation

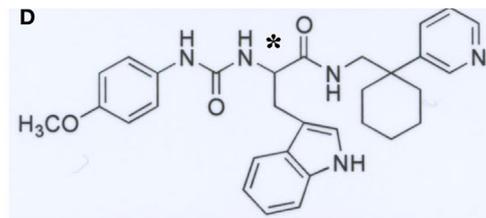
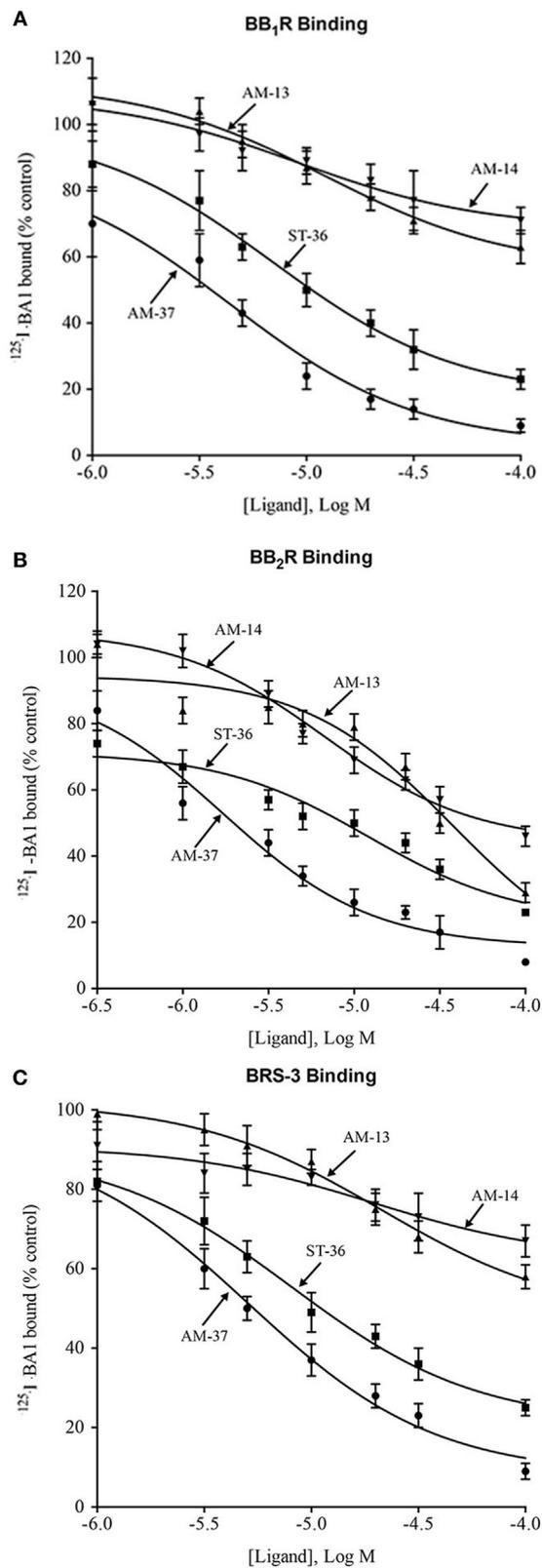
The tyrosine phosphorylation of the EGFR and ERK was investigated by western blot. NSCLC cells transfected with BB₁R, BB₂R, and BRS-3 were placed in 10 cm dishes. When the cells were confluent, they were placed in SIT medium (RPMI-1640 containing 3 \times 10⁻⁸ M sodium selenite, 5 μ g/ml bovine insulin, and 10 μ g/ml apo-transferrin; Sigma-Aldrich, St. Louis, MO, USA) for 3 h. AM-37, ST-36, AM-13, or AM-14 were added for 30 min followed by 100 nM BA1 for 2 min. Cell extracts were made as described previously (16), and 600 μ g of protein extract was immunoprecipitated with 4 μ g anti-phosphotyrosine antibody (Becton Dickinson, USA). The immunoprecipitates were fractionated using a 4–20% polyacrylamide gel (Novex, San Diego, CA, USA). Proteins were transferred to a nitrocellulose membrane and incubated with 2 μ g anti-EGFR or anti-ERK antibody (Cell Signaling Technologies, Danvers, MA, USA). After washing the blot, it was incubated with enhanced chemiluminescence detection reagent (Thermo Scientific) for 5 min and exposed to Biomax XAR film (Carestream, Rochester, NY, USA). The band intensity was determined using a Kodak image station 440 densitometer. Alternatively, 20 μ g of protein extract was loaded onto polyacrylamide gels and after transfer to nitrocellulose, the blot was probed with anti-PY^{1,068}-EGFR, anti-EGFR, anti-PY²⁰⁴ERK, or anti-ERK (Cell Signaling Technologies, Danvers, MA, USA).

Proliferation

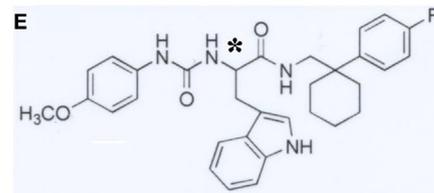
The proliferation of NSCLC cells was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl-2H-tetrazolium bromide (MTT) assay as described previously (16). NCI-H727, H1299, and H1975 cells were placed in SIT medium and varying concentration of AM-37, ST-36, AM-13, or AM-14 added. After 2 days, 0.1% MTT solution (15 μ l) was added. After 4 h, DMSO (150 μ l) was added and the absorbance at 570 nm was determined.

Statistical Analysis

The results are expressed as the mean \pm SD. Statistical significance of differences was performed by a one-way or two-way



AM-37 = (R) enantiomer
ST-36 = (S) enantiomer



AM-13 = (R) enantiomer
AM-14 = (S) enantiomer

FIGURE 1 | Binding. The ability of varying concentrations of AM-37 (●), ST-36 (■), AM-13 (▲), and AM-14 (▼) to inhibit specific ¹²⁵I-BA1 binding was investigated using (A) BB₁R-, (B) BB₂R-, and (C) BRS-3-transfected NCI-H1299 cells. The mean value ± SD of three determinations each repeated in duplicate is shown. (D) The structure of AM-37 and ST-36 is shown. (E) The structure of AM-13 and AM-14 is shown; *indicates the optically active site.

repeated measures of variance. The binding curves were drawn using PRISM.

RESULTS

Receptor Binding

The ability of the small molecules to bind to BB₁R, BB₂R, and BRS-3 was investigated. AM-37 (R-enantiomer) inhibited specific ¹²⁵I-BA1 binding to BB₁R, BB₂R, and BRS-3 in a dose-dependent manner with *K_i* values 3.6, 1.4, and 5.5 μM, respectively (Figure 1). ST-36 (S-enantiomer) inhibited specific ¹²⁵I-BA1 binding to BB₁R, BB₂R, and BRS-3 with *K_i* values of 7.9, 6.9, and 10.8 μM, respectively (Figure 1). In contrast, AM-13 (R-enantiomer) and AM-14 (S-enantiomer) inhibited specific ¹²⁵I-BA1 binding to BB₁R, BB₂R, and BRS-3 with *K_i* > 20 μM. The results indicate that AM-37 and ST-36 bind to BB₁R, BB₂R, and BRS-3 with greater affinity than does AM-13 and AM-14.

The specificity of binding was investigated. Table 1 shows that BA1 bound with high affinity (*K_i* = 0.002, 0.0005, and 0.004 μM) to BB₁R, BB₂R, and BRS-3. AM-37, ST-36, AM-13, and AM-14 inhibited specific ¹²⁵I-BA1 binding (*K_i* = 1.4, 6.9, 27, and 45 μM) to BB₂R. ST-36 inhibited specific ¹²⁵I-BA1 binding (*K_i* = 7.9 and 10.8 μM) to BB₁R and BRS-3, respectively. AM-13 and AM-14 bind with low affinity to BB₁R and BRS-3 (*K_i* > 100 μM and > 100 μM, respectively).

Cytosolic Ca²⁺

The ability of the small molecules to function as BB₁R, BB₂R, and BRS-3 antagonists was investigated. Addition of 10 nM BA1 to NCI-H1299 cells transfected with BB₁R increased the cytosolic Ca²⁺ from 160 to 178 nM within seconds (Figure 2A). The response was transient and returned to baseline after 1 min. Addition of 30 μM AM-37 to NCI-H1299 cells transfected with BB₁R had no effect on the basal cytosolic Ca²⁺ but blocked the increase in cytosolic Ca²⁺ caused by BA1 (Figure 2B). Addition of 30 μM AM-14 had no effect of basal cytosolic Ca²⁺ but partially blocked the increase caused by 10 nM BA1 (Figure 2C). Table 2 shows that AM-37 and AM-14 significantly inhibited the ability of BA1 to increase cytosolic Ca²⁺ after addition to NCI-H1299 cells transfected with BB₁R. Addition of 10 nM BA1 to NCI-H1299 cells transfected with BB₂R increased the cytosolic Ca²⁺ from 160 to 186 nM (Figure 2D). Addition of 30 μM ST-36

to NCI-H1299 cells transfected with BB₂R had no effect on the basal cytosolic Ca²⁺ but blocked the increase in cytosolic Ca²⁺ caused by BA1 (Figure 2E). Addition of 30 μM AM-14 had no effect of basal cytosolic Ca²⁺ but partially blocked the increase caused by 10 nM BA1 (Figure 2F). Table 2 shows that ST-36 and AM-14 significantly decreased the ability of 10 nM BA1 to elevate cytosolic Ca²⁺ in NCI-H1299 cells transfected with BB₂R. Addition of 10 nM BA1 to NCI-H1299 cells transfected with BRS-3 increased the cytosolic Ca²⁺ from 170 to 194 nM (Figure 2G). Addition of 30 μM ST-36 to NCI-H1299 cells transfected with BRS-3 had no effect on the basal cytosolic Ca²⁺ but blocked the increase in cytosolic Ca²⁺ caused by BA1 (Figure 2H). Addition of 30 μM AM-13 had no effect of basal cytosolic Ca²⁺ but partially blocked the increase caused by 10 nM BA1 (Figure 2I). Table 2 shows that ST-36 and AM-13 significantly decreased the ability of 10 nM BA1 to elevate cytosolic Ca²⁺ in NCI-H1299 cells transfected with BRS-3. The results indicate that AM-37 and ST-36 are antagonists for BB₁R, BB₂R, and BRS-3. In contrast, AM-13 and AM-14 are weak antagonists for the BBR family.

The specificity of AM-37, ST-36, AM-13, and AM-14 was investigated. 10 nM neurotensin (NT) or 5 μg/ml ionomycin (ION) strongly increased the cytosolic Ca²⁺ in NSCLC cells. AM-37 or ST-36 had no effect on the ability of NT to increase cytosolic Ca²⁺ in NSCLC cells. AM-13 or AM-14 had no effect on the ability of ION to increase Ca²⁺ in NSCLC cells. Therefore, AM-36 and ST-37 are antagonists for the BBR but not the NTR.

Tyrosine Phosphorylation

The ability of the small molecules to impair EGFR transactivation was investigated. Previously, we found that the BB₁R and BRS-3 regulate EGFR tyrosine phosphorylation (13, 16). Figure 3 shows that addition of 100 nM BA1 to NCI-H1299 cells transfected with BB₂R increased significantly the EGFR tyrosine phosphorylation to 326%. If the cells were pretreated with 10 μM AM-37 or ST-36, addition of BA1 had little effect. In contrast, if the cells were treated with 10 μM AM-13, BA1 increased strongly EGFR tyrosine phosphorylation. Similarly, BA1 addition to NCI-H1299 cells transfected with BB₂R increased ERK tyrosine phosphorylation to 277%. This increase in ERK tyrosine phosphorylation was decreased significantly in the cells pretreated with AM-37 or ST-36 but not AM-13. Similarly, AM-14 had little effect on EGFR or ERK tyrosine phosphorylation (data not shown). The results indicate that AM-37 and ST-36 antagonize the ability of the BB₂R to regulate tyrosine phosphorylation of the EGFR and ERK. Similar transactivation results were obtained for NSCLC cells transfected with BB₁R or BRS-3 (data not shown).

Proliferation

The ability of the small molecules to inhibit lung cancer proliferation was investigated. AM-37 inhibited NCI-H1299 proliferation in a dose-dependent manner. Figure 4 shows that AM-37 had little effect at 3 μM but strongly inhibited proliferation at 30 μM. The IC₅₀ for AM-37 was 16 μM. Similarly, ST-36 had an IC₅₀ of 22 μM, whereas AM-14 was less potent (IC₅₀ > 50 μM).

TABLE 1 | Binding to lung cancer cells transfected with human bombesin receptors.

Ligand	<i>K_i</i> , μM		
	BB ₁ R	BB ₂ R	BRS-3
BA1	0.002 ± 0.0002	0.0005 ± 0.0001	0.004 ± 0.0003
AM-37	3.6 ± 0.5	1.4 ± 0.2	5.5 ± 0.6
ST-36	7.9 ± 0.9	6.9 ± 0.3	10.8 ± 0.9
AM-13	>100	27 ± 4	>100
AM-14	>100	45 ± 8	>100

The mean value ± SD of four determinations is indicated. The structure of bombesin agonist 1 is (D-Tyr⁶, β-Ala¹¹, Phe¹³, Nle¹⁴)BB⁶⁻¹⁴. BRS-3, bombesin receptor subtype-3.

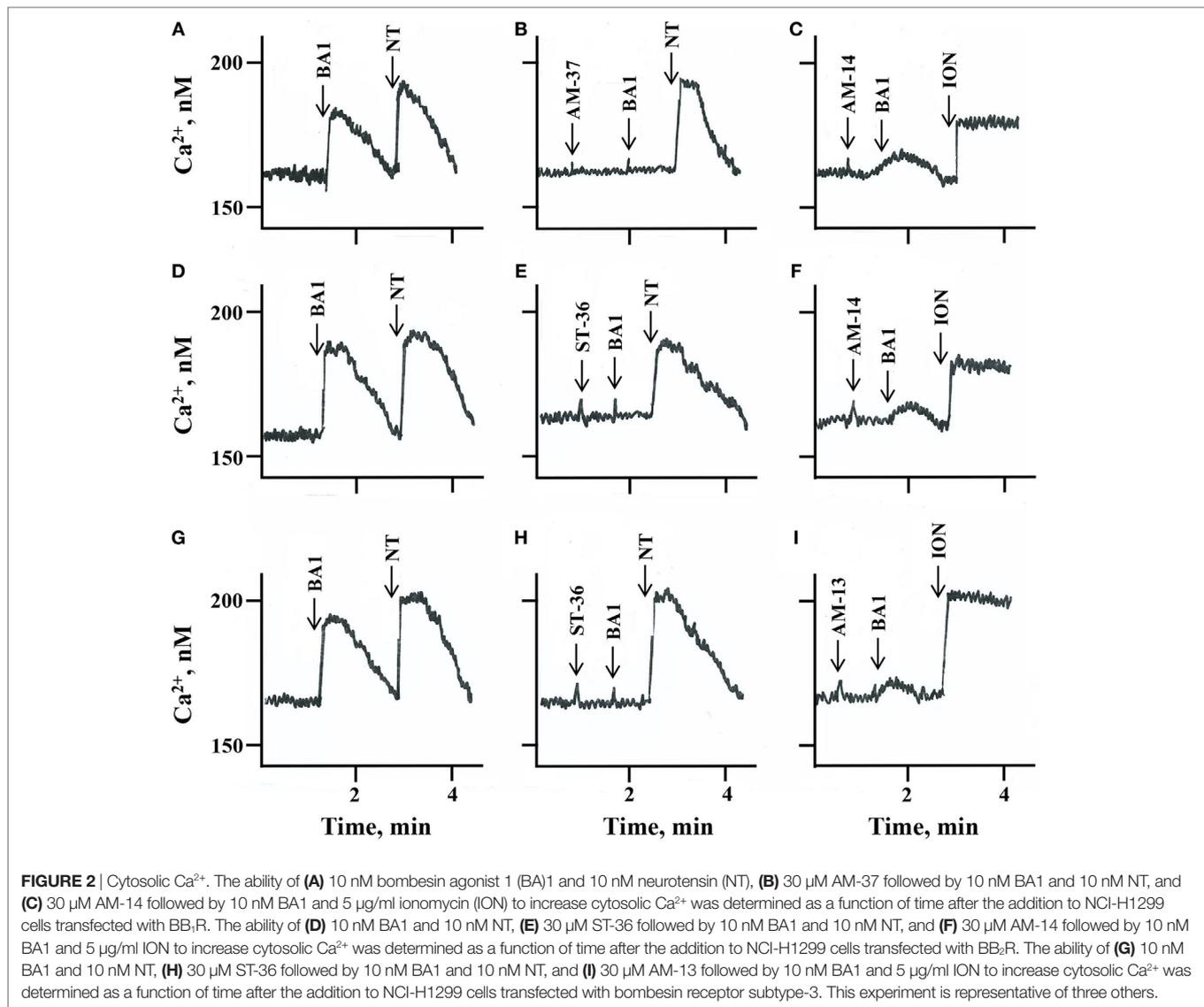


TABLE 2 | Increases in cytosolic Ca^{2+} using human lung cancer cells transfected with bombesin receptors.

Addition	Increase in cytosolic Ca^{2+} , nM		
	BB_1R	BB_2R	BRS-3
BA1, 10 nM	18.5 ± 1.1	26.3 ± 1.7	24.4 ± 2.3
BA1 + AM-37, 30 μM	1 ± 0.6 ^a	0 ^a	0 ^a
BA1 + ST-36, 30 μM	0 ^a	0 ^a	0 ^a
BA1 + AM-13, 30 μM	7 ± 0.8 ^a	5 ± 0.6 ^a	2 ± 0.3 ^a
BA1 + AM-14, 30 μM	6 ± 0.6 ^a	6 ± 0.5 ^a	3 ± 0.4 ^a

The initial increase in the cytosolic Ca^{2+} after addition of BA1 to lung cancer cells containing BB_1R , BB_2R , or BRS-3 is indicated. Addition of small molecules significantly inhibited ($p < 0.01$; ^a by ANOVA) the ability of BA1 to increase cytosolic Ca^{2+} . This experiment is representative of three others.

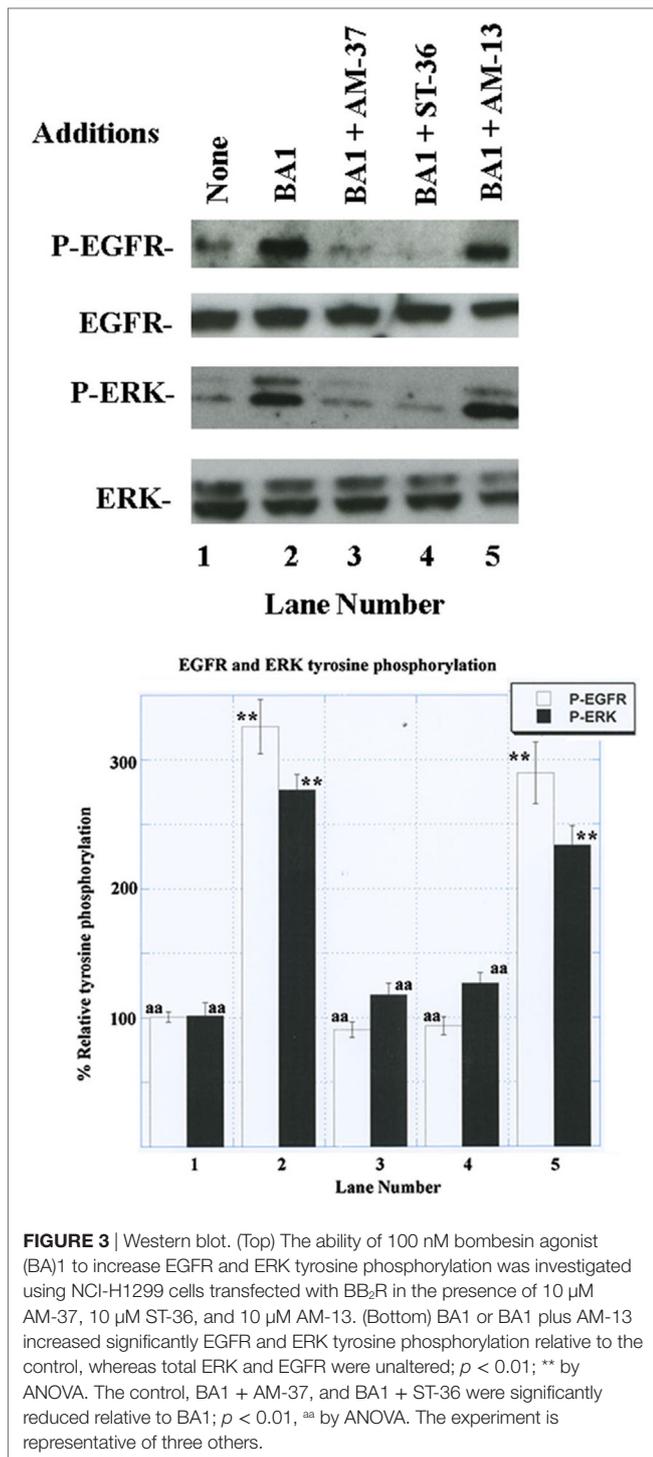
BRS-3, bombesin receptor subtype-3, BA1, bombesin agonist 1.

The specificity of the small molecules was investigated. Table 3 shows that AM-37, ST-36, AM-13, and AM-14 (50 μM) inhibited significantly the proliferation of NCI-H727 cells, which have

mRNA for BB_1R , BB_2R , and BRS-3 . In contrast, AM-37, ST-36, AM-13, and AM-14 had little effect on NCI-H1975 cells, which lack BB_1R , BB_2R , and BRS-3 . These results indicate that the BBR is essential for AM-37, ST-36, AM-13, or AM-14 to inhibit cancer cellular proliferation.

DISCUSSION

While NSCLC patients are traditionally treated with combination chemotherapy, the 5-year survival rate is only 16% (17). Some NSCLC patients (13%) have L858R EGFR mutations, and these patients respond to TKI such as gefitinib or erlotinib; however, secondary EGFR mutations can occur such as T790M resulting in TKI resistance (18). Numerous GPCR are expressed in lung cancer cell lines and biopsy specimens. BB_2R mRNA is expressed in 46–67% of the lung cancer cell lines examined (19). BB_1R mRNA is present in 81% of the NSCLC cell lines examined (20). Using autoradiographic techniques,



BRS-3 binding sites were detected in 40% of the lung cancer biopsy specimens examined (21). The EGFR is abundant on NSCLC (approximately 100,000 EGFR/cell), whereas BBR are present on most native NSCLC cells (approximately 2,000 BBR/cell) (22).

Addition of GRP to NSCLC cells causes transactivation of the EGFR (23). The effects of GRP on NSCLC tyrosine

phosphorylation of the EGFR are impaired by gefitinib, a TKI, and PD176252, a peptoid BB₂R antagonist. Because the ERK and EGFR tyrosine phosphorylation caused by GRP was impaired by marimastat, GM6001 and antibodies to TGF α , matrix metalloproteases may regulate the cellular shedding of TGF α from NSCLC cells. The TGF α may then bind to the EGFR causing its tyrosine phosphorylation. The results indicate that the BB₂R regulates EGFR transactivation in NSCLC cells.

The BB₁R regulates EGFR transactivation (16). The increase in EGFR and ERK tyrosine phosphorylation caused by NMB addition to NSCLC cells was impaired by PD168368, a BB₁R peptoid antagonist, as well as gefitinib. The increase in EGFR tyrosine phosphorylation caused by NMB was impaired by *N*-acetyl cysteine (NAC), an antioxidant, or tiron, a superoxide scavenger. NMB increased reactive oxygen species (ROS) in NSCLC cells, and the increase was inhibited by Tiron. It remains to be determined if the ROS impair protein tyrosine phosphatases in NSCLC cells, which remove phosphate from the P-EGFR. Activation of BRS-3 with BA1 increased EGFR and ERK tyrosine phosphorylation (13). The increase in EGFR tyrosine phosphorylation caused by BA1 is impaired by NAC, tiron, and diphenyleneiodonium, an inhibitor of NADPH oxidase enzymes.

ML-18 is a small molecule that prefers BRS-3 relative to BB₁R or BB₂R (24). ML-18, an *S*-enantiomer, inhibits ¹²⁵I-BA1 binding to BRS-3, BB₂R, and BB₁R with IC₅₀ values of 4.8, 16, and >100 μM, respectively, whereas the *R*-enantiomer EMY-98 is inactive. ML-18 is a BRS-3 antagonist, which inhibits the ability of BA1 to increase cytosolic Ca²⁺, increase ERK and EGFR tyrosine phosphorylation (24). Also, ML-18 inhibited NSCLC growth and increased the cytotoxicity of gefitinib. His¹⁰⁷ is important for BRS-3 to bind antagonists with high affinity (25). Tyr¹⁰¹ of the BB₂R is important for binding of non-peptide antagonists (26). Similarly, this Tyr is conserved in the BB₁R and BRS-3. It remains to be determined if this Tyr is essential for binding of AM-37 to the BB₁R, BB₂R, or BRS-3. ST-36, which is an *S*-enantiomer, inhibited specific ¹²⁵I-BA1 binding to BB₁R, BB₂R, and BRS-3 with IC₅₀ values of 7.9, 6.9, and 10.8 μM, respectively. It is surprising that AM-37, which is the *R*-enantiomer, binds with slightly higher affinity to BBR than does ST-36. Previously, the BB₁R was found to prefer PD168,368, which is an *S*-isomer, relative to the *R*-isomer (27).

(D-Arg¹, D-Trp^{5,7,9}, Leu¹¹)substance P (SP) is an inhibitor of signal transduction and growth of SCLC cells (28). (D-Arg¹, D-Trp^{5,7,9}, Leu¹¹)SP impaired the ability of BB, vasopressin, or bradykinin to increase cytosolic Ca²⁺ and ERK activity. (D-Arg¹, D-Trp^{5,7,9}, Leu¹¹)SP decreased SCLC growth *in vitro*, and (D-Arg¹, D-Trp^{5,7,9}, Leu¹¹)SP has a unique tertiary structure in with two type IV non-standard turns, which juxtapose the N- and C-terminal adjacent to one another (29). Due to this unique structure (D-Arg¹, D-Trp^{5,7,9}, Leu¹¹)SP may be able to interact with multiple GPCR. In contrast, AM-37 and ST-36 are small molecules that have a different structure from that of (D-Arg¹, D-Trp^{5,7,9}, Leu¹¹)SP.

AM-37 and ST-36 inhibited the proliferation of NSCLC cells such as NCI-H1299 and H727, which have BB₁R, BB₂R, or BRS-3.

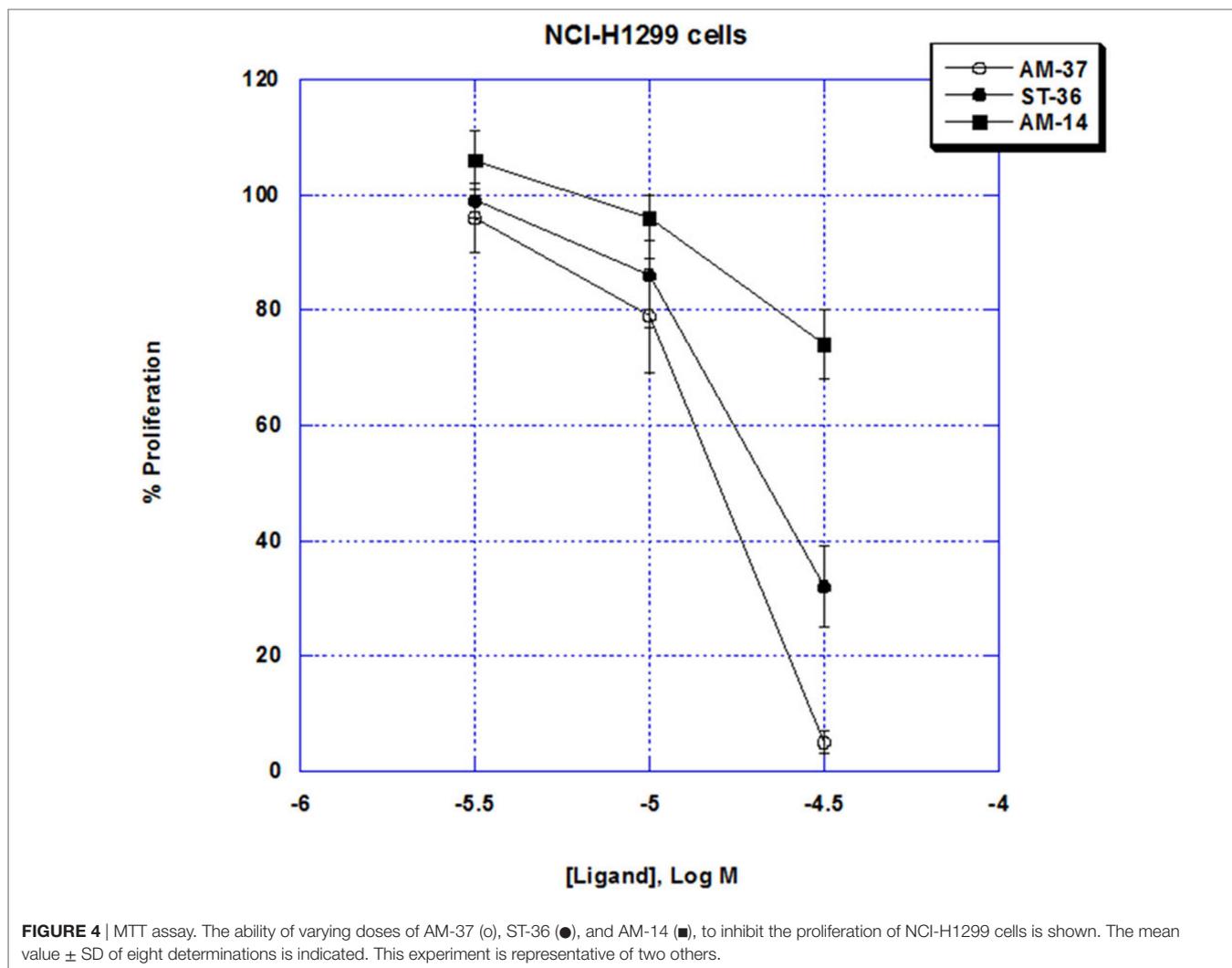


TABLE 3 | MTT proliferation assay using human lung cancer cell lines.

Addition	% proliferation	
	NCI-H727	NCI-H1975
None	100 \pm 5	100 \pm 6
AM-37, 50 μ M	13 \pm 1 ^{aa}	103 \pm 5
ST-36, 50 μ M	30 \pm 3 ^{aa}	95 \pm 5
AM-13, 50 μ M	67 \pm 4 ^a	89 \pm 5
AM-14, 50 μ M	64 \pm 3 ^a	88 \pm 6

The ability of AM-37, ST-36, AM-13, and AM-14 to inhibit non-small cell lung cancer growth was determined using NCI-H727, which express BB₁R, BB₂R, and bombesin receptor subtype-3 and H1975 cells, which do not express BBR. The mean value \pm SD of eight determinations is indicated. This experiment is representative of two others; ^a*p* < 0.05, ^{aa}*p* < 0.01, relative to no additions by ANOVA. Using a test for normality, the data points had a Gaussian distribution.

In contrast, AM-37 and ST-36 have little effect on NSCLC cell line NCI-H1975, which lacks BB₁R, BB₂R, and BRS-3. It remains to be determined if AM-37 or ST-36 are synergistic with gefitinib in inhibiting the growth of NSCLC. A goal is to identify

GPCR antagonists, which potentiate the action of TKI in NSCLC patients.

CONCLUSION

AM-37 and ST-36 are small molecules, which bind to the BB₁R, BB₂R, and BRS-3. Because AM-37 and ST-36 inhibit the ability of BA1 to increase cytosolic Ca²⁺ as well as increase EGFR and ERK tyrosine phosphorylation, they function as BB₁R, BB₂R, and BRS-3 antagonists. A particular advantage of AM-37 and ST-36 is that they will inhibit the growth of NSCLC cells if they have BB₁R, BB₂R, or BRS-3.

AUTHOR CONTRIBUTIONS

TM and SM were responsible for the receptor binding studies. TM and NT were responsible for the cell culture and calcium experiments. TM, PM, and IR-A were responsible for the trans-activation and growth experiments. ML was responsible for the synthesis of the small molecules. TM and RJ were responsible for the writing of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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