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β_2-Adrenergic receptor agonists inhibit the proliferation of 1321N1 astrocytoma cells

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Abstract

Astrocytomas and glioblastomas have been particularly difficult to treat and refractory to chemotherapy. However, significant evidence has been presented that demonstrates a decrease in astrocytoma cell proliferation subsequent to an increase in cAMP levels. The 1321N1 astrocytoma cell line, as well as other astrocytomas and glioblastomas, express β_2-adrenergic receptors (β_2-AR) that are coupled to Gs activation and consequent cAMP production. Experiments were conducted to determine whether the β_2-AR agonist (R,R')-fenoterol and other β_2-AR agonists could attenuate mitogenesis and, if so, by what mechanism. Receptor binding studies were conducted to characterize β_2-AR found in 1321N1 and U118 cell membranes. In addition, cells were incubated with (R,R')-fenoterol and analogs, to determine ability to stimulate intracellular cAMP accumulation and ability to inhibit [^{3}H]-thymidine incorporation into the cells. 1321N1 cells contain significant levels of β_2-AR as determined by receptor binding. (R,R')-fenoterol and other β_2-AR agonists, as well as forskolin, stimulated cAMP accumulation in a dose-dependent manner. Accumulation of cAMP induced a decrease in [^{3}H]-thymidine incorporation. There was a correlation between concentration required to stimulate cAMP accumulation and inhibit [^{3}H]-thymidine incorporation. U118 cells have a reduced number of β_2-AR and a concomitant reduction in the ability of β_2-AR agonists to inhibit cell proliferation. These studies demonstrate the efficacy of β_2-AR agonists for inhibition of growth of the astrocytoma cell lines. Since a significant portion of brain tumors contain β_2-AR to a greater extent than whole brain, (R,R')-fenoterol, or some analog, may be useful in the treatment of brain tumors after biopsy to determine β_2-AR expression.

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Introduction

In humans, the vast majority of malignant brain tumors are gliomas and astrocytomas, which are extremely lethal, as the median survival from diagnoses is 12-15 months (Wrensch et al., 2006). The current clinical approaches to the treatment of gliomas and astrocytomas include a combination of surgery, radiation and chemotherapy, but these approaches have not significantly improved patient survival (Stupp et al., 2006). Thus, the development of new therapies is an important area for drug development.

One such approach has been suggested by data that demonstrated that higher grades of human brain tumors are associated with lower adenylyl cyclase activity and/or cellular cAMP concentrations (Furman and Shulman, 1977; Racagni et al., 1983). In addition, data from a study in human-derived A172 glioma cells showed a decrease in proliferation, an increase in differentiation and an induction of apoptosis after treatment with a cAMP analog (dibutyryl-cAMP, 8-bromo-cAMP), an adenylate cyclase activator (forskolin), or a phosphodiesterase inhibitor (3-isobutyl-1-methyl-xanthine) (Chen et al., 1998). Similar results were obtained in studies examining the role of the chemokine CXCL12 and its cognate receptor CXCR4 in the growth of human-derived U87MG glioblastoma multiforme cells (Yang et al., 2007). In this study, the results indicated that there was an association between increased tumor grade and ligand activation of CXCR4 and that this could be linked through the inhibition of adenylyl cyclase activity and the reduction of cellular cAMP concentrations. The data also demonstrated that the treatment of U87MG cells with AMD 3465, a CXCR4 antagonist, blocked the growth of these cells *in vitro* and in a cranial xenograft model. Thus, the data suggest that the

stimulation of cAMP production might prove to be effective in the treatment of gliomas and astrocytomas.

Based upon these observations, we have investigated the possibility that selective β_2-adrenergic receptor (β_2-AR) agonists may affect the growth of gliomas and astrocytomas through the direct stimulation of cAMP and/or associated pathways. Previous studies have demonstrated that β_2-AR are expressed in glioblastomas, either maintained as established cell lines or primary cultures derived from human biopsies (Prenner et al., 2007; Annabi et al., 2009) as well in the human-derived 1321N1 astrocytoma cell line (Toews et al., 1983; Wakshull et al., 1985). We have demonstrated that U118 cells, another human astrocytoma cell line also contains β_2-AR, but to a lesser extent than 1321N1 cells. Furthermore, in the current study, the U87MG cell line was used as a negative control as our data indicated that there was no detectable expression of the β_2-AR in these cells and the sensitivity of the U87MG cell line to increased cAMP levels has been previously established (Yang et al., 2007). The agonists used in the current study were isoproterenol and a series of fenoterol analogs, Fig. 1. We have previously reported and characterized the fenoterol analogs and extensive structure activity relationship studies have demonstrated that these compounds have a broad range of properties including selective β_2-AR binding affinities relative to the β_1-AR, ability to stimulate cAMP accumulation in HEK cells transfected with β_2-AR, and efficacy in a mouse cardiomyocyte contractility model (Jozwiak et al., 2007; Woo et al., 2009).

The results of this study indicate that β_2-AR agonists inhibited cellular replication in the 1321N1 and U118 cell lines, that this effect was blocked by the β_2-AR antagonist

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propranolol and that the β_2-AR agonists had no effect on the growth of U87MG cells. Since the data in the Oncomine cancer profiling database (http://www.oncomine.org) suggests that a significant portion of gliomas and astrocytomas express β_2-ARs to a greater extent than in brain, this receptor represents a potential therapeutic target in the treatment of these tumors.

Methods

Cell culture

The 1321N1 astrocytoma cells were obtained from European Collection of Cell Cultures (Sigma-Aldrich, St. Louis, MO) and the U118 and U87MG cells from American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin in a humidified 5% CO_2 incubator. Drug treatments were carried out when cells were 70-80% confluent.

Receptor Binding

Binding to cell membranes obtained from 1321N1 cells and U118 cells was conducted in a 96-well format, as described previously (Jozwiak et al., 2007). In brief, the cells were scraped from the 150 x 25 mm plates and centrifuged at 500 x g for 5 min. The cell pellet was washed twice, homogenized in Tris-HCl [50 mM, pH 7.7] and the crude membranes were recovered by centrifugation at 27,000 x g for 10 min. The pellet was resuspended in Tris-HCl [25 mM, pH 7.4] containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 5 mM glucose. The competition binding assays contained 0.3 nM [^{3}H]CGP-12177 (30 Ci/mmol, Perkin Elmer, Waltham, MA) and 25 μg membrane protein in a volume of 1.0 mL, and binding was conducted in triplicate for 60 min at 25ºC. Nonspecific binding was determined using 1 μM propranolol. The amount

of protein in the binding assay was 270 μg per well for 1321N1 cells and U118 cells. The reaction was terminated by filtration using a Tomtec 96 harvester (Orange, CT) through glass fiber filters. Bound radioactivity was counted on a Pharmacia Biotech beta-plate liquid scintillation counter (Piscataway, NJ) and expressed in counts per minute. IC_{50} values were determined using at least six concentrations of each fenoterol analog, and calculated using Graphpad/Prism (ISI, San Diego, CA). The K_i values were determined by the method of Cheng and Prusoff ((Cheng and Prusoff, 1973).

cAMP Accumulation

β_2-AR mediated cAMP accumulation was determined as described previously (Toll et al., 1998; Jozwiak et al., 2007). 1321N1 or U118 cells were plated in 96-well plates. When the cells reached confluence, the medium was removed and each well rinsed with 0.1 mL of Krebs-HEPES buffer (130 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, and 10 mM glucose, pH 7.3). The plates were preincubated for 10 min at room temperature with buffer alone; then, test compound diluted in buffer was added to the wells for quadruplicate determinations. The plates were incubated for an additional 10 min with the test compound. After incubation, the medium was removed and 0.1 mL of 0.5 M formic acid was added. After a minimum of 1 h, the supernatant was removed and lyophilized. cAMP was quantified using the protein kinase binding assay of Gilman (Gilman, 1970). The amount of protein per well was determined using the BCA protein determination kit (Thermo Scientific Pierce, Rockford, IL) and was used to calculate the amount of cAMP/mg/well.

Mitogenesis

To measure β_2-AR mediated inhibition of mitogenesis, 1321N1, U118, or U87MG cells were seeded in a 96-well plate at approximately 5,000 cells/well. After 48 h, the wells were rinsed twice and the medium was replaced with fresh medium containing 10 µL of drug in sterile water. After another 22 h of incubation at 37˚C in the presence of the appropriate concentration of fenoterol analog or forskolin, 0.25 µCi of [^{3}H]-thymidine 12177 (10 Ci/mmol, Perkin Elmer, Waltham, MA) was added to each well. The cells were incubated for an additional 2 h at 37˚C, at which point 10 µL of 10X trypsin was added, and the resuspended cells were harvested using a Tomtec 96 harvester through glass fiber filters. DNA-associated radioactivity was counted as described above. To determine the effect of β2-AR antagonists or PKA inhibitors on (R,R')-fenoterol inhibition of [^{3}H]-thymidine incorporation, compounds were incubated with 1321N1 cells for 22 h with the (R,R')-fenoterol. At this point, [^{3}H]-thymidine was added and samples processed as described above.

Cell Cycle Analysis

Cell cycle distribution was analyzed by flow cytometry. Briefly, cells were trypsinized, washed with phosphate-buffered saline (PBS) and fixed with 95% ethanol at -20° C for 24 h. Fixed cells were washed with PBS, treated with 0.05% RNase for 30 min at 37° C and stained with propidium iodide. The stained cells were analyzed using a FACScan laser flow cytometer (FACSCaliber, BD Biosciences).

Western Blotting

Cells were lysed with a solution of 1% Triton-X100 prepared in PBS. The lysis buffer contained a cocktail of phosphatase inhibitors (BioVision, Inc., San Francisco, CA) to prevent dephosphorylation of the phosphorylated proteins. Proteins (40 ug/well) were

separated by 4-12% pre-cast gels (Invitrogen, Carlsbad, CA) using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then electrophoretically transferred onto Hybond^{TM}-P membrane (Amersham Biosciences, Piscataway, NJ). Blots were probed with the following antibodies: Cyclin D1 (sc-246, mouse polyclonal IgG), cyclin A (sc-596, rabbit polyclonal IgG), p27^{kip1} (sc-528, Rabbit polyclonal IgG) and actin (sc-1616, goat polyclonal IgG) all purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and p-Akt (Ser473, rabbit polyclonal IgG) purchased from Cell Signaling Technology (Beverly, MA). The ECL Plus Western Blotting Detection System of Amersham Biosciences (Piscataway, NJ) and the procedure recommended by the manufacturer was used for the detection of antigens. Protein bands were quantified by analyzing the images obtained using an AlphaimagerTM S-3400 (Alpha Innotech Corp., San Leandro, CA).

Statistical Analyses.

Analysis of variance (ANOVA) was used to assess the effect of each treatment as compared to control. Fisher's PLSD test was used for post-hoc analysis.

Materials

(R,R')-Fenoterol and the fenoterol analogues (Table 1) were synthesized as previously described (Jozwiak *et al*., 2007; Jozwiak *et al*., 2010a). [^{3}H]-CGP-12177 was purchased from Perkin-Elmer (Shelton, CT), DMEM was purchased from Lonza Walkersville, Inc. (Walkersville, MD), FBS was purchased from Atlas Biologicals (Fort Collins, CO), penicillin, streptomycin and geneticin (G418) were purchased from Invitrogen, NaCl and $CaCl_2$ were purchased from Mallinckrodt (Phillipsburg NJ) and (±)-

propranolol, (R)-isoproterenol, forskolin, Tris-HCl, Trizma Base, PBS, KCl, $MgSO_4$, $MgCl_2$, D-(+)-glucose, KH_2PO_4 and HEPES were purchased from Sigma-Aldrich.

Results

Receptor binding studies in 1321N1 and U118 cells

Initial RT-PCR studies indicated that the β_2-AR was expressed in 1321N1 cells while the β_1-AR was not (unpublished observation). The expression of β_2-AR in 1321N1 cells was confirmed using receptor binding studies with [^{3}H]-CGP-12177 as the marker ligand. Saturation experiments determined that the binding affinity (K_d value) of CGP-12177 was 0.23 nM, which was consistent with previously reported values, e.g. 0.3 nM obtained using C6 glioma cells (Staehelin et al., 1983) and 0.17 nM obtained using CHO-K1 cells expressing human β_2-AR (Baker et al., 2005) (Figure 1A). The expression level of the β_2-AR (B_{max}) in the 1321N1 cellular membranes was 32 fmol/mg of protein. The β_2-AR selective antagonist ICI 118-551 inhibited [^{3}H]-CGP-12177 binding with high affinity (K_i = 0.58 nM) and a Hill coefficient of ~1.0, indicating a single binding site. The results reflect the presence of functional β_2-AR in the 1321N1 cells, and are consistent with the results of Toews *et al*. (Toews et al., 1983), which had previously established the presence of β_2-AR in this cell line. [^{3}H]-CGP-12177 binding was also observed in U118 cells. However, in this cell line, there were fewer receptors. Saturation analysis indicated a Kd of 0.14 nM for [^{3}H]-CGP-12177 binding, and a Bmax of 9.6 fmol/mg protein (Figure 1B). There was no observable binding of [^{3}H]-CGP-12177 to the membranes obtained from U87MG cells indicating that the β_2-AR is not expressed, or very poorly expressed, in this cell line.

Agonist-induced cAMP accumulation in 1321N1 and U118 cells

The agonist-induced cAMP accumulation in 1321N1 cells was studied using (R)-isoproterenol and selected fenoterol derivatives. Each of the agonists with an R-configuration at the β–hydroxy carbon atom produced a significant increase in cAMP production, Fig. 2. The calculated EC_{50cAMP} value for (R)-isoproterenol was 16.5 nM, which was consistent with the previously reported EC_{50cAMP} value of 11.2 nM determined in C6-2B rat astrocytoma cells (Barovsky et al., 1984). The EC_{50cAMP} values for the fenoterol analogs ranged from 15.9 nM for (R,R')-fenoterol to greater than 1.8 μM for (S,S')-fenoterol, Table 1. However, only (R,R')-fenoterol and (R,R')-methoxyfenoterol were full agonists producing maximal cAMP accumulations of >100% relative to (R)-isoproterenol, while the cAMP accumulations produced by (R,R')-4-methoxy-1-naphthylfenoterol, (R,S')-4-methoxy-1-naphthylfenoterol and (S,S')-fenoterol were 35%, 53%, and 31%, respectively. The increase in cAMP accumulation produced by these compounds was blocked by the addition of 1 μM propranolol and ICI 118-551. The fact that (R,R')-fenoterol is a "superagonist" stimulating greater than the prototypical agonist isoproterenol (162% stimulation relative to isoproterenol, being 100%) is unusual. However, we have demonstrated this previously in HEK cells transfected with β2-AR (Jozwiak et al., 2010b). This is probably due to the fact that unlike isoproterenol, (R,R')-fenoterol activates only Gs and not both Gs and Gi, leading to cAMP stimulation greater than that induced by isoproterenol (Woo et al., 2009; Jozwiak et al., 2010b).

Forskolin is an adenylate cyclase activator that has been shown to increase intracellular cAMP concentrations in glioma cells via a β_2-AR-independent mechanism

(Chen et al., 1998; Yang et al., 2007). In these studies, the treatment of 1231N1 cells with forskolin induced the accumulation of cAMP in a dose-dependent manner, Fig. 2.

The fenoterol analogs and forskolin also induced a stimulation of cAMP accumulation in U118 cells. However, consistent with the reduced number of β_2-AR in this cell line, both the potency for stimulation of cAMP accumulation and the percent stimulation was less. In other words, the fenoterol analogs were all partial agonists in U118 cells, with (R,R')-fenoterol and (R,R')-methoxy fenoterol stimulating cAMP accumulation approximately 60% of the standard isoproterenol. Consistent with inability to detect β_2-AR in U87MG cells, neither isoproterenol nor (R,R')-fenoterol stimulated cAMP accumulation in these cells (Table 1).

β2-AR agonists inhibit proliferation of 1321N1 and U118 cells in vitro.

Previous studies have demonstrated that in A172 and U87MG cells, a forskolin-induced increase in intracellular cAMP led to a decrease in proliferation, increased differentiation and induction of apoptosis (Chen et al., 1998; Yang et al., 2007). The same effect was observed with the 1321N1 cell line, Fig. 3A, with a calculated IC_{50} value of 170.3 ± 37.2 nM for forskokin-induced inhibiton of [^{3}H]-thymidine incorporation, similar to published values obtained in A172 cells (Chen et al., 1998).

Since the results of the studies with forskolin had demonstrated that 1321N1 cells are sensitive to increases in intracellular cAMP concentrations, the effect of β_2-AR agonists on cellular proliferation in 1321N1, U118, and U87MG cells was investigated. In this study, cells were incubated with β_2-AR agonists for 22 h, at which time [^{3}H]-thymidine was added for an additional 2h, the cells were then harvested and [^{3}H]-

thymidine incorporation was determined. Significant reductions in [^{3}H]-thymidine incorporation were observed in a concentration-dependent manner for all of the compounds used in the study in 1321N1 and U118 cells. The data was used to determine IC_{50} values associated with the inhibition of [^{3}H]-thymidine incorporation and in 1321N1 cells, the values ranged from 0.05 nM observed with (R)-isoproterenol to 337 nM observed with (S,S')-4-methoxyfenoterol, Table 2. The inhibitory effect of (R,R')-fenoterol was blocked by the addition of the β_2-AR antagonist propranolol (1 μM) and by the selective β_2-AR antagonist ICI 118-551. Schild analysis of ICI-118-551 inhibition demonstrated competitive inhibition with a pA2 of 8.9 and slope of -1.24 ± 0.3. When the calculated EC_{50cAMP} and IC_{50} values for inhibition of mitogenesis were compared for a subset of compounds, a log-log correlation of the data revealed an excellent correlation between the two values with an $r^2 = 0.93652$ (Table 2).

β_2-AR agonists also reduced [^{3}H]-thymidine incorporation in U118 cell, but again, consistent with the receptor number, they were less efficient in inhibiting [^{3}H]-thymidine incorporation. IC_{50} values ranged from 2.0 nM for isoproterenol to 815 nM for (S,S')-fenoterol, and generally maximal inhibition was only approximately 50%, compared to 80-90% inhibition of [^{3}H]-thymidine incorporation found in the 1321N1 cells. The incubation of U87MG cells with β_2-AR agonists had no effect on [^{3}H]-thymidine incorporation, which is consistent with the lack of β_2-ARs in this cell line. It is interesting to note that β_2-AR agonists also had no effect on the proliferation of HEK cells transfected with β_2-AR (data not shown), even though the compounds used in this study are highly active in the stimulation of cAMP accumulation in these cells (Jozwiak et al., 2007). In both cell lines, fenoterol analogs were considerably more potent for inhibition

of [^{3}H]thymidine incorporation than for stimulation of cAMP accumulation. This suggests a small increase in cAMP can induce a large decrease in cell proliferation, or conversely, perhaps inhibition of mitogenesis is not completely due to cAMP accumulation. To test the importance of cAMP accumulation, we incubated cells with the protein kinase A (PKA) inhibitor H-89 prior to addition of the fenoterol analog. H-89 was able to dose dependently reverse the (R,R')-fenoterol-induced decrease in [^{3}H]thymidine incorporation (Figure 4), with 3 and 10 μM inhibiting by 6.5 and 83 fold respectively, indicating the importance of cAMP for the inhibition of mitogenesis.

If fenoterol analogs are to be effective as chemotherapeutic agents for brain tumors, they must cross the blood brain barrier. Preliminary studies indicate that 4-methoxyfenoterol crosses the blood brain barrier after i.v. administration to rats. A comparison of brain to plasma levels showed that after 15 minutes the ratio appeared to stabilize around 0.5 (supplementary Figure S1), indicating reasonable penetration.

(R,R')-Fenotrol induces cell cycle arrest at G_1 in 1321N1 cell line.

The effect of (R,R')-fenoterol on cell cycling was determined by treating the 1321N1 cells with various concentrations of (R,R')-fenoterol for 20 h followed by flow cytometric analysis. Untreated cells were used as controls. (R,R')-Fenoterol induced G_1 arrest with an associated decrease in the proportion of cells in G_2 and S phase, as the proportion of cells in G_1 phase increased from 49.8% (controls) to 60.6-76% in treated cells, Table 3. The results also demonstrated that (R,R')-fenoterol arrested the cell cycle at doses as low as 0.1 nM, which is consistent with the compound's ability to inhibit [^{3}H]-thymidine incorporation, Table 1. The results are also consistent with the data obtained from the treatment of A172 cells in which activation of PKA by cAMP

analogs induced cell growth arrest by blocking the cell cycle during the G_1 or G_2 phase (Chen et al., 1998).

(R,R')-Fenoterol modulates levels of proteins involved in cell division

The effect of (R,R')-fenoterol on selected molecular events associated with G_1 arrest in 1321N1 cells was examined using Western blot analysis. The data indicate that (R,R')-fenoterol significantly increased protein levels of the cyclin-dependent kinase inhibitor $p27^{kip1}$ and inhibited phosphorylation of Akt, at Ser-473, in a dose-dependent manner at nanomolar concentrations (Figure 5). At the same range of concentrations, (R,R')-fenoterol down-regulated the protein expression of cyclin D1 and cyclin A, but had only a modest effect on phosphorylation of mitogen-activated kinases ERK1/2, reaching significance at only a single concentration (Figure 5). (R,R')-fenoterol induced very similar changes in cell cycles protein expression in U118 cells (data not shown). Interestingly, in this cell line, a similar protein level produced a greatly reduced Western blot expression for each of the cell cycle proteins tested. In addition, consistent with a previous report (Cobbs et al., 2008), only ERK1 is phosphorylated by β_2-AR stimulation in this cell line.

Discussion

Previous *in vitro* and *in vivo* studies utilizing the A172 and U87MG cell lines have demonstrated that increased intracellular cAMP levels decreased proliferation, increased differentiation and induced apoptosis (Chen et al., 1998; Yang et al., 2007). Forskolin, an adenylate cyclase activator, was a common agent in these studies. The treatment of 1321N1 cells with this compound increased the basal intracellular

concentration of cAMP from below 0.5 nmol/mg protein to ~4 nmol/mg protein and induced cell cycle arrest in G_1 phase, indicating that the proliferation of 1321N1 cells is also sensitive to changes in intracellular cAMP levels. The results of this study also demonstrate that treatment of 1321N1 cells with β_2-AR agonists similarly increased intracellular cAMP levels and inhibited mitogenesis.

The connection between β_2-AR stimulation and the inhibition of mitogenesis was supported by data from studies utilizing U118 and U87MG cells. The treatment of U87MG cells, which do not express functional β_2-AR, with the same series of β_2-AR agonists did not increase cAMP levels and had no effect on [^{3}H]-thymidine incorporation or cell proliferation. In addition, studies with the human-derived U118 glioma cell line indicate that there is a low, but significant expression of β_2-AR in these cells. Treatment of U118 cells with (R,R')-fenoterol inhibited [^{3}H]-thymidine incorporation with an IC_{50} value that was 50-fold higher than the value calculated in the 1321N1 cells suggesting that the level of β_2-AR expression affected the quantitative inhibitory activity of (R,R')-fenoterol.

The inhibitory effect of β_2-AR agonists on the growth of 1321N1 cells is also consistent with previous reports that isoproterenol suppresses the growth of MDA-MB-231 human breast cancer cells through increased cAMP production, and that this effect was blocked by propranolol (Slotkin et al., 2000). It has also been demonstrated that pirbuterol (a β_2-AR agonist) inhibited the growth of human breast cancer cells *in vivo* by blocking the Raf-1/ERK1/2 pathway (Carie and Sebti, 2007). In fact, activation of GPCRs might be a reasonable strategy as chemotherapy for a variety of tumors.

Recently histamine was approved in Europe for treatment of acute myeloid leukemia to be used in combination with IL-2.

In contrast, β_2-AR agonists have also been shown to have the opposite effect on cellular proliferation in certain cell types, as β_2-AR activation increased proliferation, migration and invasiveness in several cancer cell models (Thaker and Sood, 2008). For example, activation of β_2-AR increased tumor angiogenesis and enhanced the expression of vascular endothelial growth factor and metalloproteinases in a mouse model of ovarian carcinoma through activation of the cAMP/PKA signaling pathway (Thaker et al., 2006). An association between β_2-AR agonism and the promotion of tumor growth has also been demonstrated in human hepatocellular carcinoma cells (Yuan et al., 2010), pancreatic cancer cells (Weddle et al., 2001; Hu et al., 2010) and gastric cancer cells (Shin et al., 2007).

A number of cellular mechanisms have been proposed for the cAMP-associated inhibition or promotion of cell growth. In astrocytomas, data indicate that cAMP can reduce cell growth by inhibiting the growth factor-mediated cell proliferation signaling pathways such as ERK and phosphoinositide 3-kinase (PI3K) (Cook and McCormick, 1993; Sevetson et al., 1993; Kim et al., 2001; Stork and Schmitt, 2002), by elevating the levels of cell cycle inhibitor proteins $p21^{cip1}$ (Lee et al., 2000) and $p27^{kip1}$ (van Oirschot et al., 2001) and/or by decreasing the level of cyclin D1 protein (L'Allemain et al., 1997). In contrast, isoproterenol promoted tumor growth in human hepatocellular carcinoma cells by both ERK1/2 dependent and independent mechanisms (Yuan et al., 2010). In pancreatic cancer cells, agonist binding to β_2-AR transactivated the epidermal growth factor receptor activating the Akt and ERK1/2 cascade in a PKA-dependent manner (Hu

et al., 2010) and β_2-AR-dependent growth was promoted by the conversion of arachidonic acid to prostaglandins and other metabolites (Weddle et al., 2001).

In this study, the data from experiments utilizing (R,R')-fenoterol indicated that ERK1/2 activity, reported to be crucial for cyclin D1 induction (Mebratu and Tesfaigzi, 2009), was only slightly increased by (R,R')-fenoterol, although cyclin D1 was nevertheless down regulated, as was Akt phosphorylation. Conversely, the cell cycle inhibitor $p27^{kip1}$ was up-regulated. It is quite likely that in the 1321N1 cells, the inhibition of cyclin D1 production by cAMP is at least in part due to the inhibition of PI3K/Akt pathway. $P21^{cip1}$ and $p27^{kip1}$ are known to be inhibited by activated Akt (Toyoshima and Hunter, 1994; Fujita et al., 2002), therefore, since (R,R')-fenoterol inactivated Akt , it is possible that the increase in $p27^{kip1}$ and decrease in cyclin D1 is a reflection of both direct action of cAMP on these proteins as well as an indirect action through inactivation of Akt. In addition, the finding that (R,R')-fenoterol decreased the level of cyclin A suggests that the fenoterol compounds cause growth inhibition through modulation of multiple phases of cell cycle. This is based upon the data that cyclin A is required at two points in the human cell cycle (Pagano et al., 1992) and that by binding to Cdk2 and Cdk1, cyclin A gives rise to two distinct kinase activities, one required in S phase and the other in G_2, respectively (Pagano et al., 1992).

Previously we had found all of the fenoterol analogs to be full β_2-AR agonists in HEK-β_2-AR cells, with EC_{50cAMP} values ranging from 0.2 nM to 580 nM, (Jozwiak et al., 2007; Jozwiak et al., 2010b). In this study, the EC_{50cAMP} values were determined in the 1321N1 and U118 cells. The compounds were weaker agonists in the 1321N1 cell line as compared to the HEK-β_2-AR cells, and weaker still in the U118 cells (Table 1), which

is consistent with there being fewer β_2-AR in these cell lines, and, therefore, a far greater receptor reserve in the transfected HEK-β_2-AR cells. However, although there were quantitative differences in the agonist activities of the tested compounds, the EC_{50cAMP} values from each of the cell lines were correlated with the observed inhibition of [^{3}H]-thymidine incorporation. One potential problem with chronic agonist treatment could be receptor desensitization. This does not appear to attenuate the activity of fenoterols in vivo, as preliminary studies with 4-methoxyfenoterol, administered daily over a period of 42 days, reduced tumor growth of 1321N1 cells implanted in the back of male SCID mice (data not shown).

The quantitative difference between the results obtained with the same functional test in three cell line is reflected in the observed activity of (S,S')-fenoterol, which is a weak partial β_2-AR agonist in the 1321N1 and U118 cells (Fig. 2, Table 1), a full agonist (>100% accumulation) in HEK-β_2-AR cells (Jozwiak et al., 2010b) and an effective inhibitor of mitogenesis (Table 2). The results obtained with (S,S')-fenoterol suggest that a very small increase in cAMP accumulation is sufficient to block cell division in 1321N1 cells, but do not rule out the potential involvement of additional mechanisms that are not related to the stimulation of cAMP accumulation. One such mechanism may be the ability of the various fenoterol analogs to stabilize or induce different conformations of the β_2-AR, which in turn, affect different intracellular cascades. This potential "ligand-directed signaling" is suggested by the data obtained from previous functional studies involving the fenoterol stereoisomer analogs used in this study. In an earlier study utilizing a rat cardiomyocyte contractility model (R,R')-ethylfenoterol and (S,S')-fenoterol were essentially inactive with $EC_{50cadio}$ values of 8,551 nM and 55,000

nM, respectively (Jozwiak et al., 2010b) while in this study, both compounds were active inhibitors of mitogenesis in 1321N1 cells with IC_{50} values of 1.44 nM and 184.2 nM, respectively. It has also been demonstrated that (R,R')-fenoterol and (R,R')-4-methoxyfenoterol preferentially activate G_s signaling while the corresponding (S,R')-isomers activated both G_s and G_i proteins (Woo et al., 2009) and that (R,R')- and (R,S')-fenoterol bind to the β_2-AR in an entropy-driven process while (S,R')- and (S,S')-fenoterol bind in an enthalpy-driven process (Jozwiak et al., 2010a). Thus, the observed functional consequences of the interaction of a fenoterol analog with the β_2-AR appear to reflect the contributions of the molecular structures of the agonist and the receptor as well as the cellular environment in which that interaction occurs. These factors are currently being investigated using comparative molecular field analysis (CoMFA).

Conclusions

The data from this study indicate that an increase in intracellular cAMP in astrocytoma cells has negative effects on growth. This inhibitory effect can be mediated by β_2-AR agonists, and the magnitude of this effect is dependent upon receptor levels in these cells. The results suggest that the use of a β_2-AR agonist in treatment of patients with gliomas and astrocytomas that express the β_2-AR may represent a new clinical approach. The potential clinical utility of β_2-AR agonism is suggested by the analysis of the expression profile of *ADRB2* in six data sets contained in the Oncomine cancer profiling database (http://www.oncomine.org). These sets have profiled astrocytoma, glioblastoma or meningioma tumor samples and normal brain tissue of the same type (see Table 1, Supplemental data). The results indicate that there is a heterogeneous expression of *ADRB2* in human brain tumors, leading to differences in β_2-AR levels

among pathological human brain cancer subtypes. Thus, the use of β_2-AR agonists in the treatment of glioblastomas and astrocytomas appears to be a reasonable clinical approach once the *ADRB2* profile is established for a particular tumor.

In addition, the results indicate that fenoterol analogs are a potential source of effective compounds for the treatment of astrocytomas and glioblastomas. The compounds represent a broad range of chemical and stereochemical structures with an array of pharmacological properties. This is exemplified by (R,R')-fenoterol and (R,R')-ethylfenoterol, where the former compound is currently undergoing clinical trials for the treatment of congestive heart failure, while the relative lack of cardiovascular effects of (R,R')-ethylfenoterol make it a reasonable lead drug candidate for the treatment of gliomas and astrocytomas.

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We would like to thank Mr. Juan Orduna for the preliminary xenograft studies with (R,R')-fenoterol on SCID mice.

Authorship Contributions

Participated in research design: Toll, Waleh, Wainer, Bernier, Jozwiak, Xiao, Woo

Conducted Experiments: Jimenez, Waleh,

Performed data analysis: Jimenez, Waleh, Toll

Wrote or contributed to the writing of the manuscript: Toll, Waleh, Wainer, Bernier

Other: Wainer acquired funding for the research

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Footnotes

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Figure Legends

Figure 1. Saturation binding of [^{3}H]CGP 12177 to (A) 1321N1 and (B) U118 cells. Binding was conducted to 1321N1 and U118 cell membranes as described in Materials and Methods. Data for each cell line shown are mean and SD from two experiments of triplicate determinations. Data in the text show mean and SD for the Kd and Bmax values from four consistent experiments.

Figure 2. Stimulation of cAMP accumulation by fenoterol analogs. (R,R')-fenoterol (■) is a full agonist for stimulation of cAMP accumulation in 1321N1 cells, exhibiting greater stimulation of cAMP than the standard isoproterenol (▲) or forskolin (●). (S,S')-Fenoterol (▼) is a partial agonist in these cells. Data shown are from a single experiment conducted in quadruplicate that was repeated two additional times for n = 3. Error bars indicate mean ± SD from a single experiment.

Figure 3. Inhibition of [^{3}H]-thymidine incorporation by fenoterol isomers and forskolin. A. (R,R')-fenoterol (■) is 1000 times more potent than both (S,S')-fenoterol (●) and forskolin (▲). B. The selective β_2-AR antagonist ICI 118-551 at 1 nM (●) and 3 nM (▲) induces a parallel rightward shift in the (R,R')-fenoterol (■) dose response curve. Data shown are from a single experiment conducted in quadruplicate that was repeated two additional times for n = 3. Error bars indicate mean ± SD from a single experiment.

Figure 4. Effect of PKA inhibitors on fenoterol-induced inhibition of [^{3}H]-thymidine incorporation. H-89 (3 μM, ● and 10 μM, ▲), or vehicle (■) was incubated with 1321N1 cells for 22 h in the presence of (R,R')-fenoterol, as described in Materials and Methods.

Data shown are from one of two consistent experiments, each conducted in quadruplicate.

Figure 5. Effect of R-R'-fenoterol on cell cycle marker proteins. 1321N1 cells were incubated with various concentrations of (R,R')-fenoterol for 20h. Cells were lysed and total protein lysates were collected and analyzed by Western blot for $p27^{Kip1}$ (panel A), cyclin D1 (panel B), phosphoAKT (panel C), ERK1/2 (panel D), and cyclin A (panel E). Band intensities were measured and normalized to β-actin. In each case: 1) Control, 2) 10^{-10} M, 3) 10^{-8} M and 4) 10^{-6} M of (R,R')-fenoterol. Bar graphs show mean ± SEM from at least 3 individual experiments. (*): $p<0.05$, different treatment groups compared with control. Blots from a representative experiment are shown.

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Table 1. The activity of (R)-isoproterenol and fenoterol analogs presented as IC_{50} values and percent inhibition associated with the inhibition of [^{3}H]-thymidine incorporation, stimulation of cAMP accumulation presented as EC_{50} and percent stimulation. The values were determined in 1321N1 and U118 cells are presented as mean ± SD for n = 3 or greater.

Compound	Mitogenesis Inhibition		Stimulation of cAMP Accumulation	
	IC_{50} (nM)	Percent Inhibition	EC_{50} (nM)	Percent Stimulation
1321N1 Cells				
(R)-isoproterenol	0.05 ± 0.01	88.7±1.89	17.0 ± 5.62	100
(R,R')-fenoterol	0.15 ± 0.07	84.3±6.95	15.9 ± 2.04	162.1±16.9
(S,S')-fenoterol	184± 26.1	85.0±12.0	1856 ± 925	31.1±21.2
(R,R')-4-methoxyfenoterol	0.17 ± 0.02	85.7±4.19	20.9 ± 6.21	71.8±5.00
(R,R')-4-methoxy-1-naphthylfenoterol	3.98 ± 0.28	87.7±1.70	68.9 ± 13.1	34.6±6.48
(R,S')-4-methoxy-1-naphthylfenoterol	4.37 ± 0.70	84.0±0.82	88.2 ± 19.6	53.3±23.2
U118 Cells				
(R)-isoproterenol	1.75 ± 0.39	68.3±5.63	55.6 ± 12.0	100
(R,R')-fenoterol	5.40± 3.59	61.9±6.34	57.3 ± 14.36	67.1 ± 7.53
(S,S')-fenoterol	815± 126	52.0±2.94	4809 ± 1619	20.1 ± 8.73
(R,R')-4-methoxyfenoterol	7.05 ± 2.18	59.7±6.80	64.6± 12.9	60.7±2.28
(R,R')-4-methoxy-1-naphthylfenoterol	41.1 ± 6.01	51.0±4.32	56.70± 15.67	22.8±7.21
(R,S')-4-methoxy-1-naphthylfenoterol	25.8 ± 8.03	49.3±8.35	46.77± 9.02	16.1 ± 1.88

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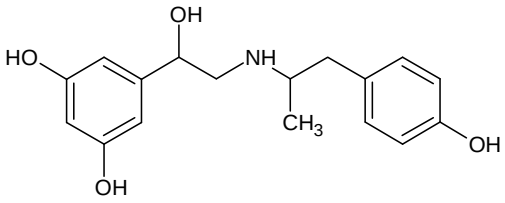
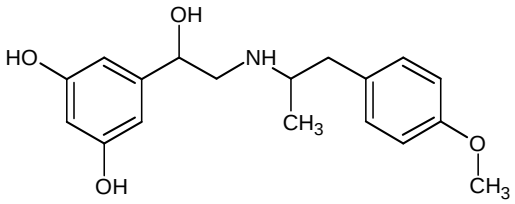
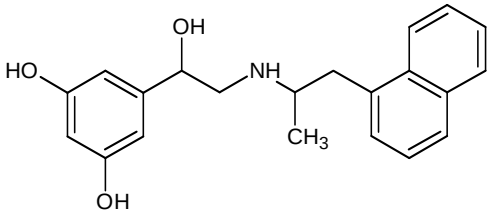
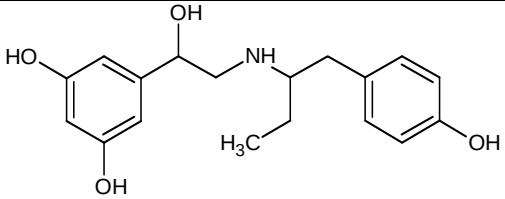
Table 2. The inhibition of mitogenesis in 1231N1 cells by the stereosiomers of fenoterol and fenoterol analogs, where R and S denote the configuration at the chiral β-hydroxy carbon and R' and S' denote the configuration at the aminoalkyl chiral center. See paper for experimental details, n = 4. Synthesis of fenoterols, 4-methoxyfenoterols and 1-naphthylfenoterols was described in Jozwiak et al., (2007). Synthesis of ethylfenoterol, 2-naphthylfenoterols, and 4-methoxy-1-naphthylfenoterols were described in Jozwiak et al., (2010b).

Compound	Structure	Stereochemistry /Substitution	Mitogenesis Inhibition IC_{50} (nM)
fenoterol		(R,R')	0.14 ± 0.07
		(R,S')	6.09 ± 1.93
		(S,R')	6.74 ± 2.18
		(S,S')	184.2 ± 26.1
4-methoxyfenoterol		(R,R')	0.17 ± 0.02
		(R,S')	2.01 ± 0.76
		(S,R')	3.16 ± 0.71
		(S,S')	337.2 ± 97.2
1-naphthylfenoterol		(R,R')	1.57 ± 0.34
		(R,S')	1.19 ± 0.38
		(S,R')	14.8 ± 5.59
		(S,S')	229.1 ± 57.4
ethylfenoterol		(R,R')	1.44 ± 0.27
		(R,S')	17.88 ± 4.56

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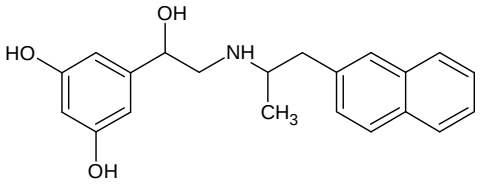
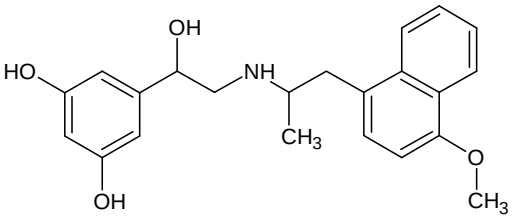
2-naphthylfenoterol		(R,R')	1.91 ± 0.57
		(R,S')	72.6 ± 29.31
4-methoxy-1-naphthylfenoterol		(R,R')	3.98 ± 0.28
		(R,S')	4.37 ± 0.70

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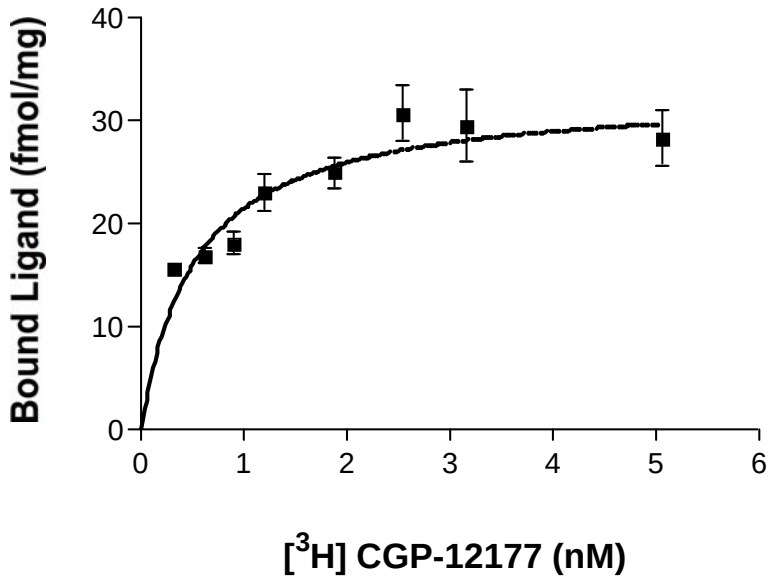
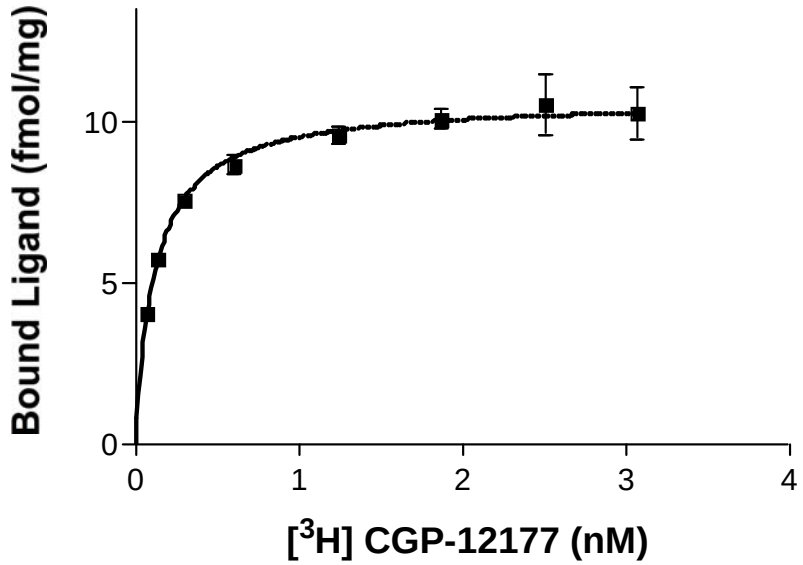
Table 3. Effect of (R,R')-Fenoterol on 1321N1 Cell Cycle Kinetics. The cells were harvested after 20 h treatment with 0, 0.1, 10.0, or 1000 nM (R,R')-fenoterol and used for FACS analysis. Cell cycle phase proportions were determined by flow cytometry. Values show mean ± SD for three independent experiments.

	% Phase Distribution		
(R,R')-fenoterol [nM]	G1	G2	S
Control	49.8 ± 1.0	6.5 ± 1.6	43.7 ± 0.2
0.1	60.6 ± 3.7	1.8 ± 0.1	37.6 ± 1.6
10.0	76.0 ± 2.0	2.1 ± 0.3	21.9 ± 0.9
1000	74.7 ± 3.2	0.7 ± 0.1	24.6 ± 1.8

Figure 1



A



B

Figure 2

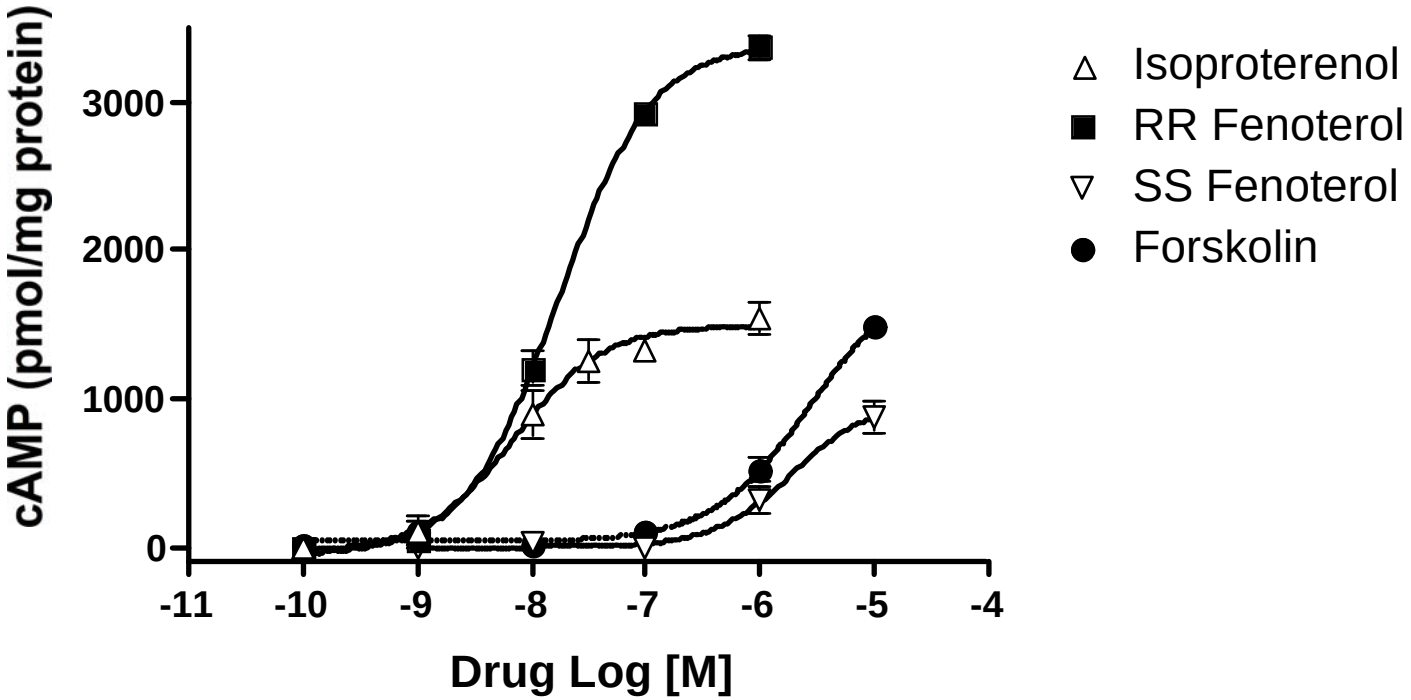


Figure 3

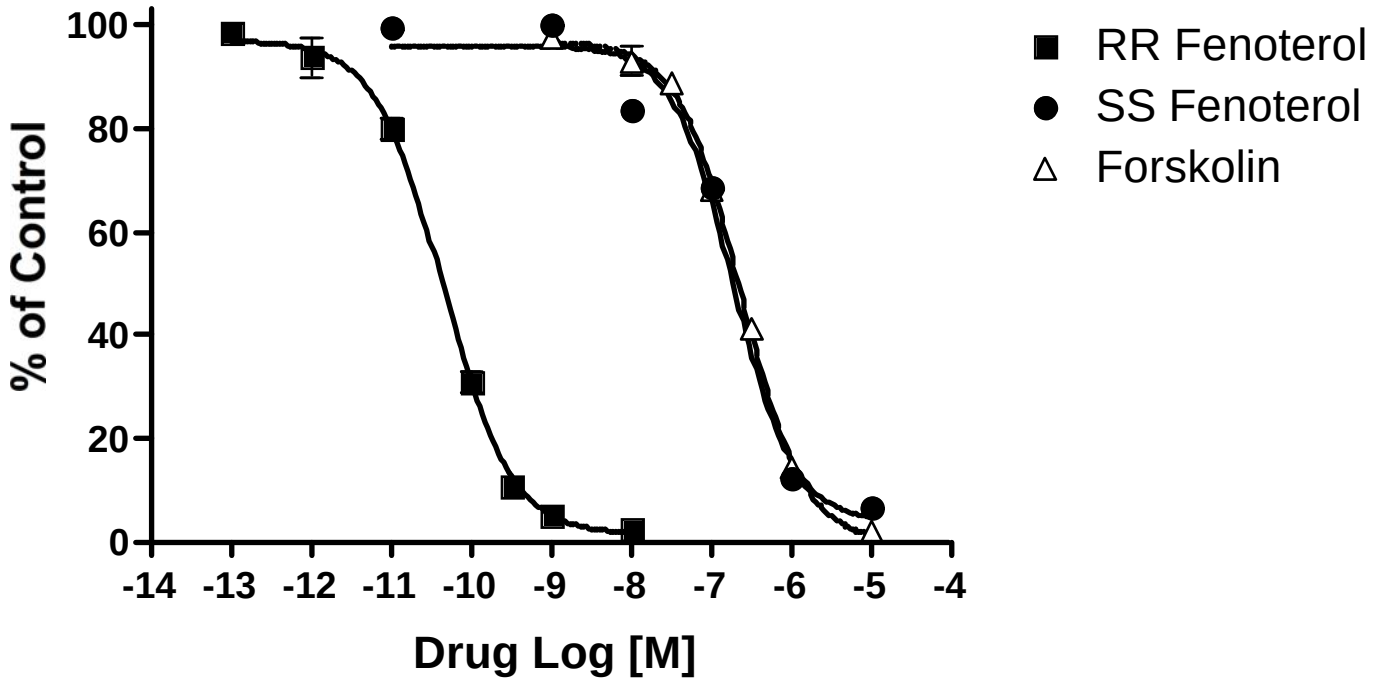
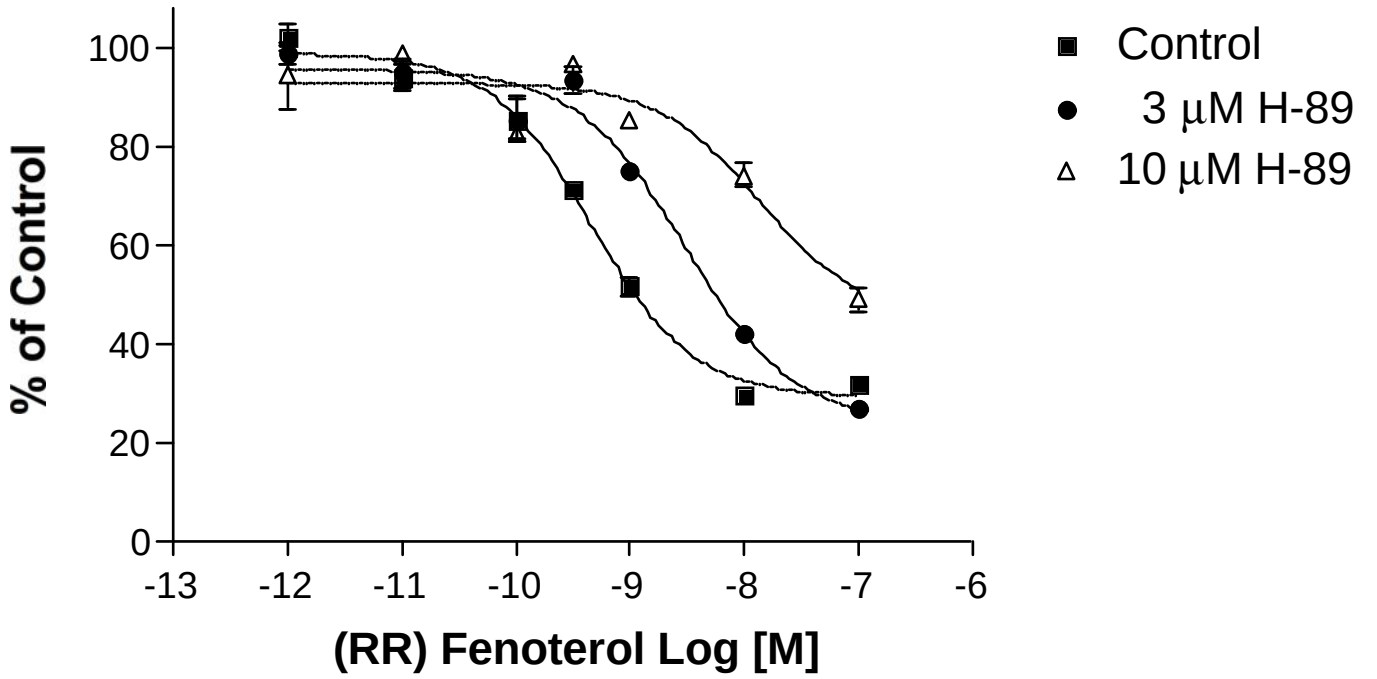


Figure 4



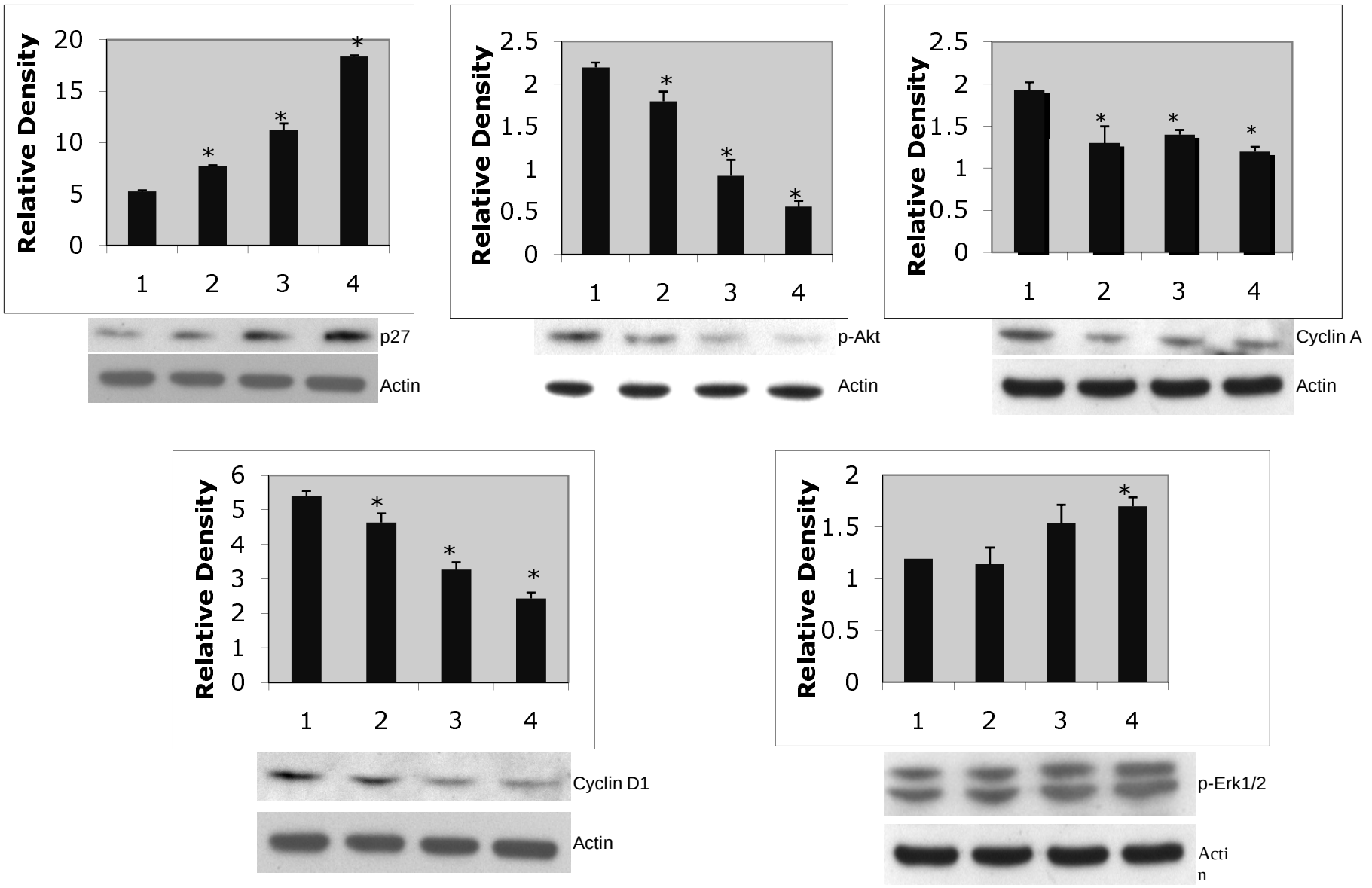


Figure 5