Molecular Pharmacology Fast Forward. Published on August 6, 2008 as doi:10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

The β_3-adrenoceptor agonist L755507 and antagonist L748337 activate different signaling pathways in CHO-K1 cells stably expressing the human β_3-adrenoceptor

Masaaki Sato, Dana S. Hutchinson, Bronwyn A. Evans & Roger J. Summers

Monash Institute of Pharmaceutical Sciences and Department of Pharmacology

PO Box 13E

Monash University

Victoria 3800

Australia

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Agonist and antagonist signaling pathways of β_3-adrenoceptors

Corresponding author:

Prof. Roger J. Summers
Department of Pharmacology
PO Box 13E
Monash University VIC 3800
Australia
Tel: +61 3 99051440
Fax: +61 3 99058192
Email: Roger.Summers@med.monash.edu.au

Number of text pages: 29
Number of tables: 1
Number of figures: 13
Number of references: 33

Abstract word count: 249
Introduction word count: 687
Discussion word count: 1484

Abbreviations

AR	adrenoceptor
CHO	Chinese Hamster Ovary
ECAR	Extracellular acidification rate
PI3K	phosphoinositide 3-kinase
p38 MAPK	p38 mitogen-activated protein kinase
Erk1/2	extracellularly regulated protein kinase
cAMP	cyclic adenosine monophosphate
PTX	Pertussis toxin

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

This study identifies signaling pathways activated by the β_2-/β_3-AR agonist zinterol, the selective β_3-AR agonist L755507 and the selective β_3-AR antagonist L748337 in CHO-K1 cells expressing human β_3-adrenoceptors. Zinterol and L755507 caused a robust concentration-dependent increase in cAMP accumulation (pEC_{50} 8.5 and 12.3 respectively), while L748337 had low efficacy. Maximal cAMP accumulation with zinterol and L755507 was increased after pre-treatment with pertussis toxin, indicating that the human β_3-AR couples to Gi as well as Gs. In contrast to cAMP, zinterol, L755507 and L748337 increased phosphorylation of Erk1/2 with very high potency (pEC_{50} 10.9, 11.7 and 11.6). These compounds also stimulated phosphorylation of p38 MAPK, but with much lower potency than Erk1/2 (pEC_{50} 5.9, 5.5 and 5.7 respectively). Pertussis toxin completely blocked Erk1/2 and p38 MAPK phosphorylation in response to L748337, demonstrating a requirement for Gi/o coupling, whereas L755507-stimulated p38 MAPK phosphorylation was not inhibited by pertussis toxin, and Erk1/2 phosphorylation was inhibited by only 30%. We found that high levels of cAMP interfered with agonist-activated p38 MAPK phosphorylation. L748337 increased extracellular acidification rate (ECAR) in the cytosensor microphysiometer with efficacy similar to zinterol and L755507, albeit with lower potency (pEC_{50} 7.2 compared to zinterol, 8.1, and L755507, 8.6). The ECAR response to L748337 was largely via activation of p38 MAPK, demonstrated by 65% inhibition with RWJ67657. We conclude that the β_3-AR agonist L755507 couples to both Gs and Gi to activate adenylate cyclase and MAPK signaling, whereas the β_3-AR antagonist L748337 couples predominantly to Gi to activate MAPK signaling.

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

β-Adrenoceptors (ARs) mediate responses to circulating adrenaline and to noradrenaline released from sympathetic nerve terminals. The β_1-, β_2- and β_3-AR subtypes have distinct patterns of expression in heart, lung, blood vessels, the gastrointestinal tract, adipose tissue and the central nervous system. Agonists at the β_3-AR stimulate lipolysis in white adipocytes, thermogenesis in brown adipocytes, and reduce contractility in gastrointestinal tract and the uterus. Consequently the β_3-AR is of interest as a therapeutic target for the treatment of obesity, irritable bowel syndrome, and pre-term labour. Agonists selective for the β_2-AR already have widespread clinical use in the treatment of asthma, chronic obstructive pulmonary disease and pre-term labour.

In contrast, β_1-AR antagonists are used in the treatment of high blood pressure and heart failure. These act via inhibition of the renin-angiotensin system, central inhibition of sympathetic nervous system outflow, and slowing of heart rate, however the wider mechanisms of β-AR antagonist action are not understood due to complex effects in multiple cell types. There is emerging evidence that β-AR antagonists have agonist actions independent of their ability to block cAMP signaling. To date, studies have focussed on stimulation of Erk1/2 signaling via β_1- and β_2-ARs (Azzi et al., 2003; Baker et al., 2003; Galandrin & Bouvier, 2006; Wisler et al., 2007; Galandrin et al., 2008). For example, the antagonist carvedilol stimulates Erk1/2 phosphorylation in cells expressing the human β_2-AR via receptor phosphorylation and recruitment of β-arrestin (Wisler et al., 2007). In cells expressing the human β_1-AR, bucindolol is a partial agonist and propranolol is a weak inverse agonist for cyclic AMP relative to (-)-isoproterenol (ISO), but both drugs stimulate Erk1/2 phosphorylation (Galandrin et al., 2008). Only the ISO-stimulated Erk1/2 response is PTX-sensitive, and none of these responses involve β-arrestins. Bioluminescence resonance

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

energy transfer (BRET) demonstrates that ISO induces a conformational change in the β_1-AR that is spatially distinct from those induced by bucindolol and propranolol.

We recently found that the β_3-AR antagonist SR59230A can activate signaling in CHO-K1 cells expressing mouse β_3-ARs at physiological levels. SR59230A is a competitive antagonist for cAMP accumulation yet increases extracellular acidification rate (ECAR) in the cytosensor microphysiometer by activating p38 MAPK, acting as a full agonist relative to the recognized β_3-AR agonist CL316243 (Hutchinson et al., 2005). Both CL316243 and SR59230A induce p38 MAPK phosphorylation but the effect is more pronounced in cells that express physiological rather than high levels of receptors, and SR59230A has higher efficacy than CL316243. Both ligands also stimulate Erk1/2 phosphorylation, but again SR59230A has higher efficacy than CL316243 (Sato et al., 2007).

The signaling properties of the mouse β_3-AR differ from those of the human receptor. For example, agonists activate Erk1/2 signaling by the human β_3-AR partly via Gi/o, whereas mouse β_3-AR mediated Erk1/2 phosphorylation is PTX insensitive (Soeder et al., 1999; Gerhardt et al., 1999; Hutchinson et al., 2002). The potential of antagonists to stimulate MAPK signaling at the human β_3-AR has not been examined previously. Given the widespread clinical use of β-AR antagonists, it is important to understand their action at each of the human β-ARs. We have examined actions of two structurally-related drugs, L748337 and L755507 (Fig 1), compared to the β_2-AR/β_3-AR agonist zinterol (Hutchinson et al., 2006), at the human β_3-AR expressed in CHO-K1 cells (CHOhβ_3). L748337 is a selective β_3-AR antagonist that competitively blocks cAMP responses to agonists in CHOhβ_3 cells, and inhibits lipolytic responses in primate adipocytes (Candelore et al., 1999). L755507 is a

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

potent agonist at the human β_3-AR, with 440-fold selectivity for β_3-AR compared to β_1 or β_2-ARs (Parmee et al., 1998). It elevates cAMP in CHOhβ_3 cells, causes thermogenesis in transgenic mice expressing human β_3-ARs (Hu et al., 2001), and induces lipolysis and an elevation of metabolic rate in rhesus monkeys (Fisher et al., 1998).

We have examined the agonist and antagonist actions of these compounds at the human β_3-AR by measuring cAMP accumulation, ECAR, Erk1/2 phosphorylation and p38 MAPK phosphorylation. We find that L748337 has very weak partial agonist activity for cAMP accumulation but acts as an antagonist of responses to zinterol and L755507. L748337 strongly activates Erk1/2 phosphorylation by signaling predominantly through Gi.

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and methods

Generation of human β_3-adrenoceptor clones

An insert carrying the coding region of the human β_3-AR was generated by reverse transcription-polymerase chain reaction on RNA extracted from human SK-N-MC cells, using *Pfx* polymerase (Life Technologies). The primers used were, forward (5'-CGCAAGCTTCGCCATGGCTCCGTGG-3') and reverse (CTTCTAGACCTTCAGG CCTAAGAAACTCCC-3'), and included *Hind* III and *Xba* I sites for subcloning fragments into the mammalian vector pcDNA3.1(+) (Invitrogen). The complete insert and junctions with pcDNA3.1(+) were checked by DNA sequencing on both strands (Micromon, Monash University, Australia).

Cell culture and transfection of the human β_3-AR in CHO-K1 cells

CHO-K1 cells were grown as monolayers in 50:50 Dulbecco's modified Eagle Medium (DMEM): Ham's F-12 medium containing 10% (v v^{-1}) foetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). All cells were maintained under 5% CO_2 at 37ºC. For transfection, CHO-K1 cells were seeded overnight at 12 x 10^6 cells per 150 cm^2 flask. Plasmid DNA (2-5 μg) containing the coding region of the human β_3-AR and additional pcDNA3.1(+) to a DNA total of 21 μg was added to 1.75 ml of OPTIMEM^{TM} (Life technologies). This was then added to a solution containing 170 μl of lipofectamine^{TM} (Life Technologies) in 1.75 ml of OPTIMEM^{TM} and incubated for 30 min at room temperature. An additional amount of OPTIMEM^{TM} (14 ml) was then added to the lipid-DNA complexed solution to create the transfection mix. Media was removed from the flask, the cells were washed with 10 ml OPTIMEM^{TM} and the transfection mix layered onto the cells and left for 4 h. 17.5 ml of DMEM/Ham's F-12 (50:50) containing 20% (v v^{-1}) foetal

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

bovine serum was then added and incubated overnight. Media was replaced 24 h later with standard (50:50) DMEM/Ham's F-12, and another 24 h later, stable transformants were selected in medium containing 800 μg ml^{-1} G418. Clonal cell lines were obtained by limiting dilution of mixed cell populations, and were expanded and analysed by a single point [^{125}I]-(-) cyanopindolol (ICYP, 800 pM) binding screen. Suitable clones were grown further for a full saturation binding analysis.

Radioligand binding assay

Cells were grown to 90% confluence as a monolayer before membranes were harvested for binding studies. Cells were washed with PBS, and scraped from flask with lysis buffer (25 mM Tris pH 7.5 room temperature, 1 mM EDTA, 10 mg ml^{-1} bacitracin, 10 mg ml^{-1} leupeptin, 10 mg ml^{-1} pepstatin A, 0.5 mg ml^{-1} aprotinin). Cells were homogenised with a Dounce homogeniser (approximately 10 strokes per pestle), and centrifuged at low speed (800 x g, 10 min) to remove cell debris. The supernatant containing membranes were retained and the pellet re-homogenised and centrifuged again. Supernatants were pooled and centrifuged (39,000 x g, 20 min, 4ºC). The pellet was homogenised in binding buffer (50 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 10 mg ml^{-1} bacitracin, 10 mg ml^{-1} leupeptin, 10 mg ml^{-1} pepstatin A, 0.5 mg ml^{-1} aprotinin) and placed on ice for use on the same day. Experiments were performed at room temperature in a volume of 100 μl of binding buffer (pH 7.4) in 96 well microtiter plates. Homogenate (~10-20 μg protein) was incubated with ICYP (100-2000 pM) for 60 min at room temperature in the absence or presence of (-)-alprenolol (1 mM) to define non-specific binding. Reactions were terminated by rapid filtration through GF/C filters pre-soaked for 30 min in 0.5% (v v^{-1}) polyethyleneimine using a Packard Cell Harvester. Filters were washed three times with cold wash buffer (50 mM Tris, pH 7.4, 4ºC), dried and radioactivity measured using a Packard Top Count. Experiments were

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

performed in duplicate with *n* referring to the number of different membrane homogenate samples used.

Cyclic AMP accumulation studies

Cells ($1x10^4$ per well) were grown in 96-well plates in DMEM/Ham's F-12 medium containing 0.5% (v v^{-1}) FBS for 2 days. On the day of experiment, the medium was aspirated and appropriate drugs diluted in stimulation buffer (1mg ml^{-1} BSA, 0.5 mM IBMX, 0.5 M Hepes, pH 7.4 in Hank's balanced salt solution (HBSS)) added in a final volume of 100 µl. After 30 min of incubation at 37˚C, the medium was removed and 100 µl of lysis buffer (1mg ml^{-1} BSA, 0.3% (v v^{-1}) Tween-20, 0.5 M Hepes, 0.5 mM IBMX, pH 7.4) added. Samples were rapidly frozen at -70°C and then thawed before assay to lyse cells prior to measurement of cAMP.

To examine the role of Gi/o coupling, cells were pre-treated with pertussis toxin (PTX) at 100 ng/ml for 16 h. For all other inhibitor experiments, cells were treated for 30 min before stimulation with appropriate drugs. cAMP accumulation was measured utilising the cAMP Alphascreen method (Perkin-Elmer, Victoria, Australia). Samples were defrosted and cAMP standards (10 pM to 1 µM) prepared in detection buffer (0.4% (v v^{-1}) HBSS, 3mM Hepes, 0.2% (v v^{-1}) Tween-20, 0.1% (v v^{-1}) BSA, pH 7.4) and 5 µl of unknown samples or cAMP standards transferred into a white 384-well plate. 5 µl of acceptor beads (anti-cAMP acceptor beads diluted in detection buffer) were aliquoted to each well and incubated for 30 min in the dark. 15 µl of donor bead mix (streptavidin donor beads diluted in detection buffer, 133 Units/µL biotinylated cAMP) solution was added to each well, the plate sealed and incubated in the dark overnight. cAMP accumulation was detected utilising a Fusion™ α microplate reader (Perkin-Elmer, Victoria, Australia). All results are expressed as a % of the response to

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

100 μM forskolin, to correct for variability in cell number or viability between individual samples.

Cytosensor microphysiometer studies

The cytosensor microphysiometer (Molecular Devices Corp., California, U.S.A.) was used to measure β_3-AR-mediated increases in ECAR as previously described (Hutchinson et al., 2002, 2005). Briefly, CHOhβ_3 cells were seeded into 12 mm transwell inserts (Costar, NY, USA) at $5x10^5$ cells per cup and left to adhere overnight. On the day of experiment, cells were equilibrated for 2 h, and cumulative concentration-response curves to L755507, zinterol, or L748337 constructed in paired sister cells with each concentration of drug exposed to cells for 14 min. Results are expressed as a percentage of the maximal response to L755507. In experiments examining the effect of inhibitors, cells were treated for 30mins before stimulation with appropriate drugs for 30 min. All drugs were diluted in modified RPMI1640. These results are expressed as a % of the maximal response to L755507, zinterol, or L748337 over basal.

Western blotting

Cells were grown in 12-well plates at 1 x 10^5 per well in DMEM/Ham's F-12 medium containing 0.5% FBS for 2 days, and the medium replaced to non-serum medium 2h before experiment. In time course studies, cells were exposed to agonist for 0-30 min. Cell were lysed directly in each well by the addition of 40 μl of 65ºC SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue). Cells were scraped, transferred to an Eppendorf tube on ice, and sonicated for 10 s followed by heating to 95ºC for 5 min. Aliquots of the samples were separated on a 12% polyacrylamide gel and electrotransferred to a PVDF membrane (Bio-Rad) with a semidry

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

electroblotter. After transfer, the membranes were allowed to soak in Tris-buffered saline for 5 min, followed by quenching of nonspecific binding (1 h at room temperature in 5% nonfat dry milk, 0.1% Tween 20 in Tris-buffered saline). Membranes were incubated overnight at 4ºC with primary antibody phospho-p38 MAPK (Thr180/Tyr184) or phosphor-p44/42 MAPK (Thr202/Tyr204) diluted 1:1000 added. Antibody bound was detected using a secondary antibody (HRP linked anti-rabbit IgG) diluted 1:2000 and enhanced chemiluminescence (ECL). The membranes were then stripped with 10 M urea, 50 mM sodium phosphate, 100 mM β-mercaptoethanol for 30 min at 50°C and reprobed with the appropriate p38 MAPK or p44/42 MAPK antibody, detected with the same secondary antibody. Results are expressed as the ratio of phosphorylated to total p38 MAPK or Erk1/2 protein over basal. All experiments were performed in duplicate with *n* referring to the number of independent experiments performed. We checked that the observed p38 MAPK or Erk1/2 phosphorylation was mediated by the β_3-AR by showing that untransfected CHO-K1 cells did not respond to 10 μM L755507 or L748337 (Fig 2). To examine the role of Gi/o coupling, cells were pre-treated with pertussis toxin (PTX) at 100 ng/ml for 16 h. For all other inhibitor experiments, cells were treated for 30 min before stimulation with appropriate drugs.

Data analysis

[^{125}I]cyanopindolol saturation binding isotherms were analyzed via nonlinear regression using GraphPad PRISM version 4.0 (GraphPad Software Inc., San Diego, CA) using a one-site mass action model to derive estimates of the radioligand dissociation constant (K_D) and maximal density of receptor binding sites (B_{max}). For functional assays, all results were expressed as a mean ± SEM of *n* experiments. Data were analysed using non-linear curve

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

fitting (GraphPad PRISM version 4.0). Concentration-response curves were analysed using the general equation for a sigmoid curve with a Hill slope of 1 (Equation 1).

$$Y = \text{Bottom} + (\text{Top}-\text{Bottom}) / (1 + 10^{\log EC_{50}-X}) \qquad \text{Equation 1}$$

where Y is the response, X is the log [ligand], Bottom is the Y response value for the bottom plateau, Top is the Y response value for the top plateau (E_{max}), and EC_{50} is the ligand concentration corresponding to the Y value half way between bottom and top. In functional experiments where L748337 was an antagonist of agonist-mediated cAMP responses, pK_B values were calculated according to the method of Furchgott (1972). Statistical significance was determined using 2-way ANOVA tests or Student's t-test. Probability values less than or equal to 0.05 were considered significant.

Drugs and reagents

RWJ67657 (4-[4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]-3-butyn-1-ol) was supplied by Dr John Siekierka, Johnson & Johnson, Raritan, NJ. L755507 (4-[[(Hexylamino)carbonyl]amino]-N-[4-[2-[[(2S)-2-hydroxy-3-(4-hydroxyphenoxy)propyl] amino]ethyl]phenyl]-benzenesulfonamide and L748337 ((*S*)-*N*-[4-[2-[[3-[3-(acetamidomethyl)phenoxy]-2-hydroxypropyl]ami-no]ethyl]phenyl]benzenesulfonamide) were supplied by Dr. Maria Luisa Candelore, Merck Research Laboratories, Rahway, NJ, U.S.A. Zinterol (N-[2-hydroxy-5-[1-hydroxy-2-[(2-methyl-1-phenyl-propan-2-yl)amino] ethyl] phenyl]methanesulfonamide hydrochloride) was a gift from Bristol-Myers Squibb, Noble Park, VIC, Australia. Drugs and reagents were purchased as follows: G418, LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), PD98059 (2′-amino-3′-methoxyflavone) (CalBiochem

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Corp, La Jolla, CA, U.S.A.); (-)-[^{125}I]-CYP (2200Ci mmol^{-1}, NEN Life Science Products, Boston, MA, U.S.A.); (-)-alprenolol, bacitracin, IBMX, polyethyleneimine, forskolin, 2',3'-Dideoxyadenosine (DDA), H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) (Sigma Chemical Company, St. Louis, MO, U.S.A.); aprotinin, leupeptin, pepstatin A (Valeant Pharmaceuticals, Costa Mesa, CA, U.S.A.). All cell culture media and supplements were obtained from Trace Biosciences (Castle Hill, NSW, Australia). Antibodies were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). All other drugs and reagents were of analytical grade.

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Radioligand binding characteristics of transfected CHO-K1 cells

Levels of expression in stably transfected CHOhβ_3 cells were determined by saturation binding experiments using [^{125}I] cyanopindolol. The pK_D and B_{max} values for the human β_3-AR clone used were 9.00 ± 0.34 and 203 ± 45 fmol/mg respectively. This expression level is similar to that which occurs physiologically in newborn human adipose tissue (Deng et al., 1996).

Effects of zinterol, L755507 and L748337 on cAMP accumulation

In CHOhβ_3 cells, both the selective β_3-AR agonist L755507 and the recognised β_2-AR agonist zinterol caused a concentration-dependent increase in cAMP, whereas the selective β_3-AR antagonist, L748337, was a weak partial agonist relative to zinterol (Table 1, Fig 3). L755507 is a highly potent agonist for cAMP accumulation, displaying a 25,000-fold amplification of response relative to its binding affinity, while zinterol shows a 30-fold amplification of response (Table 1). PTX pre-treatment did not significantly affect pEC_{50} values for cAMP accumulation in response to stimulation but did increase maximal responses. This could suggest that one or more conformations of the human β_3-AR couple to both Gs and Gi, or may reflect the removal of non-competitive basal effects of constitutively active Gi following PTX treatment. The latter seems unlikely, however, as we see no effect of PTX on the cAMP response to L748337 at the human receptor (Fig 3), or on CL316243 responses mediated by the mouse β_{3a}-AR expressed in the same cellular background (Sato et al, 2005).

The human β_3-AR antagonist L748337 blocked increases in cAMP levels stimulated by zinterol (50 nM) and L755507 (10 pM) with pK_B values of 9.20 ± 0.25 (IC_{50} 63 nM) and 9.46

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

± 0.14 (IC_{50} 35 nM) respectively (Fig 3). In comparison, Candelore et al. (1999) found that L748337 was a highly potent antagonist of isoproterenol (70 nM)-stimulated cAMP responses, with an IC_{50} value of 6 nM. Small differences in the behavior of L748337 may reflect the somewhat higher abundance of the β_3-AR in our study (203 fmol/mg protein) compared with the previous work (40-60 fmol/mg protein; Candelore et al., 1999). The higher β_3-AR expression level used in our studies is also consistent with the acquisition of weak partial agonist activity.

Effects of zinterol, L755507 and L748337 on extracellular acidification rate

To examine the net effect of receptor activation, we measured changes in extracellular acidification rate (ECAR). In contrast to cAMP signaling, L748337 caused a marked increase in ECAR with an efficacy similar to that observed for zinterol and L755507 but with significantly lower potency (Table 1, Fig 4). This suggested that a significant proportion of the ECAR response to L748337 was due to activation of pathways other than the Gs/adenylate cyclase/cAMP cascade. Untransfected CHO-K1 cells showed no response to zinterol, L755507 and L748338 (data not shown) suggesting that these ligands had no effect on other receptor or signaling components.

To investigate the mechanisms involved in agonist or antagonist-stimulated changes in ECAR, we tested the effect of pathway inhibitors on cells treated with concentrations of zinterol (100 nM), L755507 (10 nM) or L748337 (1 μM) that produced 80-90% of maximal responses. Responses to zinterol, L755507 or L748337 were unaffected by the adenylate cyclase inhibitor, DDA (50 μM), but were partially blocked by the PKA inhibitor, H-89 (10 μM) (Fig. 5). In addition, the ECAR response to zinterol was partially inhibited and that to L748337 markedly inhibited by the p38 MAPK inhibitor, RWJ67657 (10 μM), whereas

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

responses to L755507 were unaffected (Fig. 5). Responses to all three ligands were not significantly affected by the PI-3-kinase inhibitor LY294002 (10 μM), the Src inhibitor PP2 (10 μM) or the MEK inhibitor, PD98059 (10 μM).

It was surprising that H-89 reduced ECAR responses given that an upstream adenylate cyclase inhibitor (DDA) had no effect, so we tested whether H-89 might have other actions at the human β_3-AR. cAMP responses to zinterol and L755507 were inhibited to a similar extent by DDA (50 μM) and H-89 (10 μM) (Fig 6a). While the reduced cAMP in the presence of DDA reflects inhibition of adenylate cyclase, that in response to H-89 contrasts with previous studies where H-89 potentiated cAMP accumulation in CHOmβ_3 cells (Sato et al., 2007) or in mouse brown adipocytes endogenously expressing this receptor (Fredriksson et al., 2001). Since H-89 has been shown to be an antagonist at β_1- and β_2-ARs (Penn et al., 1999), we tested whether it binds to the human β_3-AR. As shown in Fig 6b, H-89 competed for binding of [^{125}I] cyanopindolol with a pK_i value of 5.00 ± 0.11 (4), equivalent to the concentration used for inhibition of PKA. Thus, in contrast to a lack of effect on the mouse β_3-AR, H-89 is clearly an antagonist at the human β_3-AR. The p38 MAPK inhibitor RWJ67657 also inhibited cAMP accumulation in response to zinterol and L755507, but did not compete for ICYP binding, suggesting that activation of p38 MAPK activity can potentiate cAMP signaling by the human β_3-AR. The other difference between H-89 and RWJ67657 is that H-89 caused similar inhibition of ECAR in response to zinterol, L755507 and L748337, consistent with an antagonist action, whereas RWJ67657 only caused a substantial reduction in the ECAR response to L748337.

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

L755507, zinterol and L748337 stimulate p38 MAP kinase phosphorylation

The experiments examining the effects of signaling pathway inhibitors on the ECAR response to ligands suggested that p38 MAPK was responsible for a greater proportion of the response to L748337 than for either L755507 or zinterol. This was further examined using Western blotting. We first determined the time course of p38 MAPK phosphorylation in response to concentrations of L755507 and L748337 known to give maximal ECAR responses. Exposure to L755507 (10 nM) increased the ratio of phospho/total p38 MAPK by 70%, whereas L748337 (1 μM) produced a 5-fold increase. In both cases the plateau was reached after 10 to 15 min (data not shown). We next determined full concentration-response curves for L755507, zinterol and L748337 at the 15 min time point (Fig 7). In our previous study using CHO-K1 cells expressing the mouse β_3-AR at 115 fmol/mg protein, the antagonist SR59230A had a 3.6-fold higher efficacy than the agonist CL316243 for p38 MAPK phosphorylation (Sato et al., 2007). Here, in CHO-K1 cells expressing the human β_3-AR at 203 fmol/mg protein, the efficacies of the antagonist L748337 and the agonist L755507 were similar, and were both higher than that of zinterol. L755507, L748337 and zinterol had similar potency, with pEC_{50} values of 5.5, 5.7 and 5.9 respectively (Table 1). It appears that the lesser contribution of p38 MAPK signaling to L755507-stimulated ECAR reflects a higher efficacy towards alternative signaling pathways rather than a reduced capacity to activate p38 MAPK phosphorylation, whereas zinterol acts as a partial agonist for this pathway.

Interaction between the cAMP and p38 kinase pathways

We also showed previously that cell-permeable cAMP analogues inhibit p38 MAPK phosphorylation (Sato et al., 2007). It seemed likely that the same interaction would occur in CHOhβ_3 cells, and we therefore examined the effect of the cAMP analogue 8-Br-cAMP (8-

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

bromoadenosine 3',5'-cAMP) on p38 MAPK phosphorylation stimulated by sorbitol, L755507 and L748337 (Fig. 8). 8-Br-cAMP did not affect basal p38 MAPK phosphorylation, but responses to sorbitol (500 mM), L755507 (10 μM) and L748337 (10 μM) were reduced in the presence of 8-Br-cAMP by 45%, 60% and 57% respectively. This inhibition by 8-Br-cAMP mirrors that seen in mouse CHOβ_3L cells, but does not seem consistent with the contrast between agonist and antagonist responses in cells expressing the mouse and human β_3-AR. We suggest that L755507 may have an inherently higher efficacy for p38 MAPK activation than L748337, but that this is masked by the inhibitory effect of high cAMP levels. In contrast, at the mouse β_3-AR, CL316243 may have a lower efficacy than SR59230A, and this is compounded by the inhibitory effect of cAMP.

The effect of inhibitors on p38 MAPK signaling

We next examined the effect of inhibitors on p38 MAPK phosphorylation elicited by L755507 and L748337 to determine whether different signaling pathways are utilized (Fig. 9). PTX (100 ng/mL) almost abolished p38 MAPK phosphorylation to L748337 (119% compared to basal) while there was no effect on the response to L755507. DDA (50 μM) slightly increased p38 MAPK phosphorylation to L755507 whilst there was a small inhibitory effect on the response to L748337. Surprisingly H-89 (10 μM) increased phosphorylation of p38 MAPK in response to both L755507 and L748337, in contrast to its inhibitory effect on both cAMP accumulation and changes in ECAR (Fig 5 & 6). In the case of L755507, H-89 may remove the inhibitory effect of the cAMP/PKA pathway on p38 MAPK phosphorylation, but this would be expected to be negligible with L748337 as little or no cAMP is generated. As expected, RWJ67657 (10 μM) completely abolished p38 MAPK phosphorylation to both L755507 and L748337.

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Stimulation of Erk1/2 phosphorylation by L755507 and L748337

In the light of differences between particular drugs acting at the mouse or human β_3-AR, we assessed Erk1/2 phosphorylation in response to L755507 and L748337 in CHOhβ_3 cells. In contrast to the mouse β_3-AR data, the level of Erk1/2 phosphorylation caused by activation of the human β_3-AR by L755507 and L748337 was similar. The maximal responses elicited by 15 min exposure of L755507 and L748337 expressed as phospho/total Erk1/2 ratio over basal were 552% and 499% respectively (Fig 10), and both compounds had similar high potency with pEC_{50} values of 11.7 and 11.6 (Table 1). Zinterol had a lower efficacy (416% basal) and potency (pEC_{50} 10.9) compared to L755507 or L748337. If we compare pEC_{50} values with binding affinities for the β_3-AR, L755507 and zinterol display a 6300-fold amplication of the Erk1/2 response relative to their pK_i values, while L748337 has a 1600-fold amplification (Table 1). Whereas 100 pM L755507 and L748337 produced maximal Erk1/2 responses in cells expressing the human β_3-AR (Fig 10), there was no detectable phosphorylation in untransfected CHO-K1 cells (Fig 2). Unlike p38 MAPK, the Erk1/2 phosphorylation response to 100 pM L755507 or L748337 was not significantly reduced in the presence of 1 mM 8-Br-cAMP (Fig 11).

The effect of inhibitors on Erk1/2 signaling

Several inhibitors were utilized to examine the signaling pathways that are involved in Erk1/2 signaling in response to 100 pM L755507 and L748337 (Fig. 12). DDA (50 μM) had a small inhibitory effect, but also reduced basal Erk1/2 phosphorylation. In contrast to the cAMP experiments, H-89 (10 μM) had no inhibitory effect on Erk1/2 responses. This is not consistent with its antagonist action at the human β_3-AR (Fig 6), suggesting that H-89 may selectively interfere with a β_3-AR conformation coupled to Gsα/adenylate cyclase/cAMP, but not that coupled to Erk1/2. Like p38 MAPK, PTX substantially reduced the Erk1/2 response

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

to L748337 (from 564% to 137% of basal), but in this case also somewhat inhibited the L755507 response (from 601% to 422%). Thus L748337 stimulation of Erk1/2 phosphorylation involves a major contribution by inhibitory G proteins, while the response to L755507 is partially mediated by Gi/o.

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

We show here that the human β_3-AR displays ligand-directed signaling (LDS) when expressed at physiological levels. L748337 acts as a competitive antagonist of cAMP accumulation, but has high potency and efficacy for Erk1/2 phosphorylation. The agonist zinterol, on the other hand, has high efficacy for cAMP accumulation, but lower efficacy than L748337 for Erk1/2 and p38 MAPK phosphorylation. Based on efficacy alone, these findings reproduce our previous demonstration of LDS by the agonist CL316243 and the antagonist SR59230A acting at the mouse β_3-AR (Sato et al., 2007). If we compare the potency of the drugs with their binding affinity for the β_3-AR, however, a different pattern emerges. At the mouse β_3-AR, the potencies of CL316243 and SR59230A for Erk1/2 phosphorylation tracked within an order of magnitude of the affinity values (Sato et al., 2007). We found a sizable amplification of response only for cAMP accumulation in high-expressing CHOmβ_3 cells stimulated with CL316243. It is therefore a unique feature of the human β_3-AR that three separate drugs, two of them agonists with differing structures (Fig 1), and the other an antagonist for cAMP, promote over 1000-fold amplification of Erk1/2 phosphorylation relative to their affinity values.

Although previous studies have shown β_3-AR mediated Erk1/2 signaling, we are the first to demonstrate the extraordinary responsiveness of the human receptor (Gerhardt et al., 1999; Soeder et al., 1999; Hutchinson et al., 2002). The difference between the mouse and human β_3-AR may correlate with observed differences in Gi/o signaling. Previous studies have demonstrated involvement of Gi/o in agonist-activated Erk1/2 phosphorylation in CHOhβ_3 cells, but not in CHOmβ_3 cells. Our data confirm that agonist activation of the human β_3-AR promotes Gi/o coupling, as pre-treatment of cells with PTX increases cAMP accumulation. PTX partially inhibits L755507-stimulated Erk1/2 phosphorylation, but almost completely

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

blocks responses to L748337. Thus L748337 recognises or induces a conformation of the β_3-AR that efficiently couples to Gi/o but not to Gs (Fig 13). This finding also highlights differences between the three human β-ARs, as antagonist-stimulated Erk1/2 phosphorylation at the β_1- and β_2-AR is PTX-insensitive (Wisler et al., 2007; Galandrin et al., 2008).

Unlike Erk1/2 signaling, L755507, L748337 and zinterol stimulate p38 MAPK phosphorylation with very low potency. Thus half-maximal responses occur at agonist or antagonist concentrations that should saturate the receptor population (Table 1). The confounding effect of cAMP generation on p38 MAPK phosphorylation may explain the low potency of the agonists, but would not explain the antagonist result. L748337 may have lower affinity for the conformation of the human β_3-AR coupled to p38 MAPK than that coupled to the Erk1/2 pathway. In fact, L748337 competition binding curves indicate a small population of low affinity β_3-ARs (Candelore et al., 1999). PTX pre-treatment completely blocks p38 MAPK phosphorylation in response to L748337, suggesting that both pathways involve Gi/o or Gβγ. However, since pEC_{50} values for L748337-stimulated Erk1/2 and p38 MAPK phosphorylation differ widely, Gi/o or Gβγ cannot represent a common intermediate. This suggests that separate conformations of the human β_3-AR activated or stabilized by L748337 may couple to different Gi/o subtypes, or to different signaling complexes incorporating common Gi/o proteins but additional distinct β_3-AR binding partners.

Zinterol had a significantly lower efficacy for p38 MAPK than L748337, whereas L748337 and L755507 had similar efficacy. L755507 may have a higher intrinsic efficacy for p38 MAPK than L748337, but this is masked by its ability to generate cAMP. In CHO-K1 cells expressing the human β_3-AR at 2.3 pmol/mg protein (Gerhardt et al., 1999), CGP12177A (10 μM) failed to stimulate p38 MAPK activation. We suggest that cAMP levels generated by

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

this concentration of CGP12177A in cells with high β_3-AR expression completely suppressed p38 MAPK activation. Differential signaling pathway activation in cells expressing high or low receptor levels has important implications for drug screening, which is usually done using high-expressing cells. However the low potency of all three drugs in our system with physiological expression levels indicates that activation of p38 MAPK via the human β_3-AR is unlikely to mediate side effects of drugs given at therapeutic doses.

We have demonstrated LDS at the human β_3-AR based on the reversal of efficacy between zinterol and L748337. In addition, our observations indicate that L755507 and L748337 each act on multiple receptor conformations. The coupling of receptors to multiple pathways indicates that (i) subsets of a homogeneous population of receptors couple to particular pathways due to stochastic effects or differential localisation and interaction with protein complexes, or (ii) ligands induce or stabilize multiple receptor conformations, creating a heterogeneous population of receptors and potentially giving rise to LDS. The large difference in pEC_{50} values for L748337 between p38 MAPK and Erk1/2 phosphorylation, and differential effects of H-89 on Erk1/2 phosphorylation versus cAMP accumulation stimulated by L755507 are consistent with the involvement of heterogeneous receptor subpopulations. The study by Galandrin et al. (2008) provides another example of two drugs that fail to show the hallmark reversal of efficacy but nonetheless display LDS. Bucindolol acts as a partial agonist for cAMP accumulation and Erk1/2 phosphorylation at the human β_1-AR, but the Erk1/2 response to bucindolol is PTX insensitive whereas isoproterenol-stimulated Erk1/2 phosphorylation is partially Gi/o dependent. In addition, direct biophysical measurement of BRET ratios indicates that isoproterenol and bucindolol promote distinct conformations of the β_1-AR.

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Multicenter, large-scale clinical trials show that β-AR antagonists represent an effective treatment for cardiac failure. Following myocardial damage, reduced cardiac output activates compensatory mechanisms involving the sympathetic nervous system (SNS) that initially maintain function, however chronic release of norepinephrine results in adverse ventricular remodelling. Both β_1- and β_2-ARs are associated with cardiac hypertrophy, oxidative stress and apoptosis. It was widely accepted that drugs with high β_1-AR selectivity and low partial agonist (or sympathomimetic) activity would produce optimal clinical outcomes, however this is not borne out in practice (Lopez-Sendo et al., 2004). For example, carvedilol is a beneficial β-AR antagonist despite having low β_1-AR selectivity and some partial agonist activity. Other effective β-AR antagonists include metoprolol, bisoprolol, nebivolol and atenolol, while drugs such as xamoterol and bucindolol are associated with adverse outcomes. The emerging evidence for agonist activity of β-AR antagonists independent of cAMP blockade may shed light on the variable clinical efficacy of these drugs. We have now shown that the human β_3-AR is strongly coupled to Erk1/2 activation in response to agonists and an antagonist. This in combination with demonstrated roles for the β_3-AR in the cardiovascular system indicates that, like the β_1- and β_2-AR, the β_3-AR should be regarded as a player in determining the potential therapeutic efficacy of β-AR antagonists.

In addition to well-characterised functions in adipose tissue, the gastrointestinal tract and the uterus, the β_3-AR is known to mediate vasodilation of human and animal vessels (Trochu et al., 1999; Dessy et al., 2004). Treatment of mice with the β_3-AR agonist CL316243 produces a significant and sustained drop in blood pressure (Rohrer et al., 1999). Most intriguing are data concerning nebivolol, an antagonist used clinically to treat heart failure, that displays β_1-AR selectivity with respect to blockade of cAMP. This drug relaxes human coronary arteries

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

via eNOS activation, NO production, and Ca^{2+} signaling mediated by β_3-ARs located on vascular endothelial cells, and also stimulates angiogenesis (Dessy et al., 2005; Rozec et al., 2006; Evangelista et al., 2007).

β_3-AR agonists decrease contractility in ventricular strips from human and dog myocardium. Unlike the vasodilatory actions of β_3-AR ligands across mammalian species, the negative inotropic effect of β_3-AR agonists is weak or absent in rat, mouse or ferret (Gauthier et al., 1999; Rohrer et al., 1999). This highlights again the idea of species differences in the expression or signaling properties of the β_3-AR. In particular, the capacity of the human β_3-AR to couple to Gi/o proteins is consistent with its negative inotropic activity (Gauthier et al., 1996). The presence of β_3-ARs in human vascular endothelial cells and in ventricular myocardium indicates that our observed stimulation of Erk1/2 phosphorylation at low antagonist concentrations is of considerable clinical interest. For example, the Erk1/2 signaling pathway is known to be cardioprotective, in part due to inhibition of the cardiomyocyte apoptosis that results from ischemia/reperfusion injury or oxidative stress (Yue et al., 2000; Lips et al., 2004).

In conclusion, although L748337 is a β_3-AR antagonist with respect to the cAMP pathway, it acts as an agonist for Erk1/2 and p38 MAPK phosphorylation. It is particularly striking that L748337 has exquisitely high potency in stimulating Erk1/2 phosphorylation, similar to L755507 and zinterol. We suggest that the β_3-AR agonist L755507 couples to both Gs and Gi to activate adenylate cyclase and Erk1/2 whereas the β_3-AR antagonist L748337 couples predominantly to Gi to activate Erk1/2. This activation of a MAPK pathway by a human β_3-AR antagonist highlights the importance of screening new drugs developed as antagonists to determine whether they have agonist properties for alternative signaling pathways, including

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

those involved in cell proliferation, differentiation and survival, that could mediate unwanted side-effects or perhaps desirable therapeutic outcomes.

ACKNOWLEDGEMENTS:

We are grateful to Prof Arthur Christopoulos for critical reading of the manuscript.

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

References

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M and Pineyro G (2003) β-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proc Natl Acad Sci U S A* **100**:11406-11411.

Baker JG, Hall IP and Hill SJ (2003) Agonist and inverse agonist actions of β-blockers at the human β_2-adrenoceptor provide evidence for agonist-directed signaling. *Mol Pharmacol* **64**:1357-1369.

Candelore MR, Deng L, Tota L, Guan XM, Amend A, Liu Y, Newbold R, Cascieri MA and Weber AE (1999) Potent and selective human β_3-adrenergic receptor antagonists. *J Pharmacol Exp Ther* **290**:649-655.

Deng C, Paoloni-Giacobino A, Kuehne F, Boss O, Revelli JP, Moinat M, Cawthorne MA, Muzzin P and Giacobino JP (1996) Respective degree of expression of β_1-, β_2- and β_3-adrenoceptors in human brown and white adipose tissues. *Br J Pharmacol* **118**:929-934.

Dessy C, Moniotte S, Ghisdal P, Havaux X, Noirhomme P and Balligand JL (2004) Endothelial β_3-adrenoreceptors mediate vasorelaxation of human coronary microarteries through nitric oxide and endothelium-dependent hyperpolarization. *Circulation* **110**:948-954.

Dessy C, Saliez J, Ghisdal P, Daneau G, Lobysheva, II, Frerart F, Belge C, Jnaoui K, Noirhomme P, Feron O and Balligand JL (2005) Endothelial β_3-adrenoreceptors mediate nitric oxide-dependent vasorelaxation of coronary microvessels in response to the third-generation β-blocker nebivolol. *Circulation* **112**:1198-1205.

Evangelista S, Garbin U, Pasini AF, Stranieri C, Boccioletti V and Cominacini L (2007) Effect of DL-nebivolol, its enantiomers and metabolites on the intracellular production of superoxide and nitric oxide in human endothelial cells. *Pharmacol Res* **55**:303-309.

Fisher MH, Amend AM, Bach TJ, Barker JM, Brady EJ, Candelore MR, Carroll D, Cascieri MA, Chiu SH, Deng L, Forrest MJ, Hegarty-Friscino B, Guan XM, Hom GJ, Hutchins JE, Kelly LJ, Mathvink RJ, Metzger JM, Miller RR, Ok HO, Parmee ER, Saperstein R, Strader CD, Stearns RA and MacIntyre DE (1998) A selective human β_3 adrenergic receptor agonist increases metabolic rate in rhesus monkeys. *J Clin Invest* **101**:2387-2393

Fredriksson JM, Thonberg H, Ohlson KB, Ohba K, Cannon B and Nedergaard J (2001) Analysis of inhibition by H89 of UCP1 gene expression and thermogenesis indicates protein kinase A mediation of β_3-adrenergic signaling rather than β_3-adrenoceptor antagonism by H89. *Biochim Biophys Acta* **1538**:206-217.

Furchgott, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbook of Experimental Pharmacology*. pp. 283-335. Berlin: Springer Verlag.

Galandrin S and Bouvier M (2006) Distinct signaling profiles of β_1 and β_2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol* **70**:1575-1584.

Galandrin S, Oligny-Longpré G, Bonin H, Ogawa K, Galés C and Bouvier M (2008) Conformational rearrangements and signaling cascades involved in ligand-biased mitogen-activated protein kinase signaling through the β_1 adrenergic receptor. *Mol Pharmacol* **74**:162-172.

Gauthier C, Tavernier G, Charpentier F, Langin D and Le Marec H (1996) Functional β_3-adrenoceptor in the human heart. *J Clin Invest* **98**:556-562.

Gauthier C, Tavernier G, Trochu JN, Leblais V, Laurent K, Langin D, Escande D and Le Marec H (1999) Interspecies differences in the cardiac negative inotropic effects of β_3-adrenoceptor agonists. *J Pharmacol Exp Ther* **290**:687-693.

Gerhardt CC, Gros J, Strosberg AD and Issad T (1999) Stimulation of the extracellular signal-regulated kinase 1/2 pathway by human β_3 adrenergic receptor: new pharmacological profile and mechanism of activation. *Mol Pharmacol* **55**:255-262.

Halls ML, Bathgate RA and Summers RJ (2006) Relaxin family peptide receptors RXFP1 and RXFP2 modulate cAMP signaling by distinct mechanisms. *Mol Pharmacol* **70**(1):214-226.

Hu B, Ellingboe J, Han S, Largis E, Mulvey R, Oliphant A, Sum FW and Tillett J (2001) (4-Piperidin-1-yl)phenyl amides: potent and selective human β_3 agonists. *J Med Chem* **44**(9):1456-1466.

Hutchinson DS, Bengtsson T, Evans BA and Summers RJ (2002) Mouse β_{3a}- and β_{3b}-adrenoceptors expressed in Chinese hamster ovary cells display identical pharmacology but utilize distinct signaling pathways. *Br J Pharmacol* **135**:1903-1914.

Hutchinson DS, Chernogubova E, Sato M, Summers RJ and Bengtsson T (2006) Agonist effects of zinterol at the mouse and human β_3-adrenoceptor. *Naunyn Schmiedebergs Arch Pharmacol* **373**:158-168.

Hutchinson DS, Sato M, Evans BA, Christopoulos A and Summers RJ (2005) Evidence for pleiotropic signaling at the mouse β_3-adrenoceptor revealed by SR59230A [3-(2-Ethylphenoxy)-1-[(1,S)-1,2,3,4-tetrahydronapth-1-ylamino]-2S-2-propanol oxalate]. *J Pharmacol Exp Ther* **312**:1064-1074.

Lips DJ, Bueno OF, Wilkins BJ, Purcell NH, Kaiser RA, Lorenz JN, Voisin L, Saba-El-Leil MK, Meloche S, Pouyssegur J, Pages G, De Windt LJ, Doevendans PA and Molkentin JD (2004) MEK1-ERK2 signaling pathway protects myocardium from ischemic injury in vivo. *Circulation* **109**:1938-1941.

Lopez-Sendo J et al., (2004) Expert consensus document on β-adrenergic receptor blockers: The Task Force on β-Blockers of the European Society of Cardiology. *Eur Heart J*, **25**:1341-1362.

Neubig RR (2007) Missing links: mechanisms of protean agonism. *Mol Pharmacol* **71**:1200-1202.

Parmee ER, Ok HO, Candelore MR, Tota L, Deng L, Strader CD, Wyvratt MJ, Fisher MH and Weber AE (1998) Discovery of L-755,507: a subnanomolar human β_3 adrenergic receptor agonist. *Bioorg Med Chem Lett* **8**:1107-1112.

Penn RB, Parent JL, Pronin AN, Panettieri RA, Jr. and Benovic JL (1999) Pharmacological inhibition of protein kinases in intact cells: antagonism of β-adrenergic receptor ligand binding by H-89 reveals limitations of usefulness. *J Pharmacol Exp Ther* **288**:428-437.

Rohrer DK, Chruscinski A, Schauble EH, Bernstein D and Kobilka BK (1999) Cardiovascular and metabolic alterations in mice lacking both β_1- and β_2-adrenergic receptors. *J Biol Chem* **274**:16701-16708.

Rozec B, Quang TT, Noireaud J and Gauthier C (2006) Mixed β_3-adrenoceptor agonist and α_1-adrenoceptor antagonist properties of nebivolol in rat thoracic aorta. *Br J Pharmacol* **147**:699-706.

Sato M, Hutchinson DS, Bengtsson T, Floren A, Langel U, Horinouchi T, Evans BA and Summers RJ (2005) Functional domains of the mouse β_3-adrenoceptor associated with differential G protein coupling. *J Pharmacol Exp Ther* **315**:1354-61.

Sato M, Horinouchi T, Hutchinson DS, Evans BA and Summers RJ (2007) Ligand-directed signaling at the β_3-adrenoceptor produced by SR59230A relative to receptor agonists. *Mol Pharmacol* **72**:1359-1368.

Soeder KJ, Snedden SK, Cao W, Della Rocca GJ, Daniel KW, Luttrell LM and Collins S (1999) The β_3-adrenergic receptor activates mitogen-activated protein kinase in adipocytes through a Gi-dependent mechanism. *J Biol Chem* **274**:12017-12022.

Trochu JN, Leblais V, Rautureau Y, Bévérelli F, Le Marec H, Berdeaux A and Gauthier C (1999) Beta 3-adrenoceptor stimulation induces vasorelaxation mediated essentially by endothelium-derived nitric oxide in rat thoracic aorta. *Br J Pharmacol* **128**:69-76.

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M and Mailman RB (2007) Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320**:1-13.

Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, Shenoy SK, Lefkowitz RJ. (2007) A unique mechanism of β-blocker action: carvedilol stimulates β-arrestin signaling. *Proc Natl Acad Sci U S A*. **104**:16657-16662.

Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC, Feuerstein GZ, Thomas H, Maleeff B and Ohlstein EH (2000) Inhibition of extracellular signal-regulated kinase enhances Ischemia/Reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. *Circulation Res* **86**:607-609.

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This work was supported by National Health & Medical Research Council (NH&MRC) of Australia Project Grants #236884 and #491190 (RJS and BAE). DSH is an NH&MRC CJ Martin Fellow, MS is a Monash University Research Scholar.

Reprint requests to:

Prof R.J. Summers, Department of Pharmacology, PO Box 13E, Monash University, Victoria 3800, Australia

Email: Roger.summers@med.monash.edu.au

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1: Structures of the human β_3-AR agonist L755507, antagonist L748337, and the β_2-/β_3-AR agonist zinterol.

Figure 2: Lack of Erk 1/2 and p38 MAPK phosphorylation in untransfected CHO-K1 cells. p38MAPK phosphorylation was examined following administration of sorbitol (500 mM), L755507 (10 μM), or L748337 (10 μM). Erk1/2 phosphorylation was examined following administration of FBS (10% v/v), L755507 (100 pM), or L748337 (100 pM). L755507 and L748337 did not stimulate either p38 MAPK or Erk1/2 phosphorylation in untransfected CHO-K1 cells.

Figure 3: Concentration-response curves for cAMP accumulation in response to zinterol, L755507, or L748337 in cells expressing the human β_3-AR. (a) The β_2-/ β_3-AR agonist zinterol and the β_3-AR agonist L755507 produced concentration-dependent increases in cAMP accumulation. The β_3-AR antagonist L748337 weakly increased cAMP accumulation. Pertussis toxin (PTX) treatment (100 ng/mL for 16h) significantly increased responses to zinterol (P<0.05) and L755507 (P<0.05). **(b)** Concentration-dependent antagonism of increases in cAMP accumulation to zinterol (50 nM) or L755507 (10 pM) by L748337 in cells expressing the human β_3-AR. All results are expressed as a % of the forskolin (100 μM) response. Values represent mean ± S.E.M. from four individual experiments, with each point the average of duplicate determinations.

Figure 4: Concentration-response curves for extracellular acidification rate (ECAR) in response to zinterol, L755507, or L748337 in cells expressing the human β_3-AR. The results are expressed as a % of the maximum response to L755507. Each point shows mean ±

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

S.E.M. (n=4). Note that zinterol, L755507, and L748337 produce equivalent responses for ECAR, albeit with differing potency (Table 1).

Figure 5: The effect of inhibitors on extracellular acidification rate (ECAR) responses to zinterol (hatched bars), L755507 (black bars) and L748337 (open bars) in CHO-K1 cells expressing human β_3-AR. The results are expressed as the % increase from control induced by the agonist over basal ECAR. Each point represents the mean ± S.E.M. (*n*=4-7). The inhibitors, DDA (adenylate cyclase, 50 μM), LY294002 (PI3K, 10 μM), PP2 (Src, 10 μM), or PD98059 (MEK, 10 μM) had no effect on ECAR responses to zinterol (100 nM), L755507 (10 nM) or L748337 (1 μM). H89 (PKA, 10 μM) partially inhibited ECAR responses to zinterol, L755507, or L748337 (*** P<0.001). RWJ67657 (p38 MAPK, 10 μM) weakly inhibited responses to zinterol (*** P<0.001) and effectively inhibited responses to L748337 (*** P<0.001).

Figure 6: (a) The effect of inhibitors of adenylate cyclase (DDA), PKA (H-89) and p38 MAPK (RWJ67657) on cAMP accumulation in response to zinterol, L755507 and L748337 in CHO-K1 cells expressing the human β_3-AR. The results are expressed as a % of the forskolin (100 μM) response. Each point shows mean ± S.E.M. (*n*=4). DDA (50 μM), H-89 (10 μM), and RWJ67657 (10 μM) all significantly reduced cAMP accumulation in response to zinterol (50nM) and L755507 (10pM). None of the inhibitors significantly affected cAMP levels in the presence of L748337 (50nM) **(b) Competition by bupranolol, H-89, or RWJ67657 for [^{125}I]CYP binding to the human β_3-AR expressed in CHO-K1 cells.** Incubations were for 60 min at room temperature and non-specific binding was defined by (-)-alprenolol (1 mM). Competition for ICYP binding was demonstrated for the β-AR antagonist bupranolol (pKi 8.65 ± 0.12) and H89 (pKi 5.00 ± 0.11) but not RWJ67657. The

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

results are expressed as a % of the maximum specific binding for ICYP in each individual experiment. Points show mean ± S.E.M. (*n*=4).

Figure 7: p38 MAPK phosphorylation in response to L755507, L748337 and zinterol in CHO-K1 cells expressing the human β_3-AR. Concentration-response curves for p38 MAPK phosphorylation in response to 15 min exposure to L755507, L748337 or zinterol in cells expressing the human β_3-AR, with below, representative immunoblots from 4-6 experiments performed in duplicate (P-p38 MAPK, phosphorylated p38 MAPK; T-p38 MAPK, total p38 MAPK). Each point represents the mean ± S.E.M. The maximum responses of phospho/total p38 MAPK elicited by L755507, L748337, and zinterol over basal were 662 ± 67%, 939 ± 87%, and 394 ± 18% respectively. The response to zinterol is significantly lower than those to both L755507 ($P<0.01$) and L748337 ($P<0.001$).

Figure 8: Interaction between cAMP and p38 MAPK signaling in CHO-K1 cells expressing the human β_3-AR. p38 MAPK phosphorylation was examined in response to sorbitol (500 mM), L755507 (10 μM), or L748337 (10 μM) in the presence or absence of 8-Br-cAMP (8-Bromoadenosine 3',5'-cAMP) treatment (1 mM, 30 min). Values represent means ± S.E.M. (*n* = 4, performed in duplicate, ** $P<0.01$; * $P<0.05$). 8-Br-cAMP did not affect basal p38 MAPK phosphorylation (90 ± 11%). Sorbitol, L755507, and L748337 all increased p38 MAPK phosphorylation (by 1245 ± 168%, 506 ± 76%, and 501 ± 91% respectively), and the responses were significantly inhibited (to 682 ± 63%, 204 ± 26%, and 215 ± 43% respectively) in the presence of 8-Br-cAMP.

Figure 9: The effect of inhibitors on p38 MAPK signaling in CHO-K1 cells expressing the human β_3-AR. The results are expressed as the % increase from control induced by the

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

agonist over basal, with below, a representative immunoblot. Each histogram represents the mean ± S.E.M. (*n*=4). L755507 (10μM) and L748337 (10μM) increased p38 MAPK phosphorylation (535 ± 48%, and 585 ± 28% respectively). PTX (Gi, 100ng/mL) almost completely abolished p38 MAPK phosphorylation in response to L748337 (119 ± 4%, *** P<0.001) while there was no significant effect on the response to L755507 (554 ± 41%). DDA (adenylate cyclase, 50 μM) had no effect on p38 MAPK phosphorylation to L755507 (657 ± 42%) but there was some inhibition of the response to L748337 (461 ± 42%, * P<0.05). H89 (PKA, 10 μM) slightly increased phosphorylation of p38 MAPK in response to both L755507 and L748337 (769 ± 62%, and 838 ± 39% respectively, * P<0.05). RWJ67657 (p38 MAPK, 10 μM) completely abolished p38 MAPK phosphorylation to L755507 and L748337 (110 ± 22%, and 99 ± 10% respectively, *** P<0.001).

Figure 10: Erk1/2 phosphorylation in response to L755507, L748337 and zinterol in CHO-K1 cells expressing the human β_3-AR. Concentration-response curves for Erk1/2 phosphorylation in response to 15 min exposure to L755507, L748337 or zinterol in cells expressing the human β_3-AR are shown at the top, with, underneath, representative immunoblots (P-Erk1/2, phosphorylated Erk1/2; T-Erk1/2, total Erk1/2). Each point represents the mean ± S.E.M. (*n* = 5-6, performed in duplicate). The maximum responses expressed as phospho/total Erk1/2 ratio elicited by L755507, L748337, and zinterol over basal were 553 ± 36%, 499 ± 33%, and 417 ± 21% respectively. The response to zinterol was significantly lower than those to L755507 (P<0.01) and L748337 (P<0.05).

Figure 11: Effect of treatment with 8-Br-cAMP on Erk1/2 signaling in CHO-K1 cells expressing the human β_3-AR. Erk1/2 phosphorylation was examined in response to L755507 (100 pM), or L748337 (100 pM) in the presence or absence of 8-Br-cAMP (8-

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Bromoadenosine 3',5'-cAMP) treatment (1 mM, 30 min), with below, a representative immunoblot. Values represent means ± S.E.M. (*n* = 4, performed in duplicate). Erk1/2 phosphorylation was increased by 8-Br-cAMP (292 ± 15%), L755507, and L748337 (444 ± 62%, and 457 ± 112% respectively), but the latter two responses were not significantly altered (368 ± 33%, and 345 ± 46% respectively) in the presence of 8-Br-cAMP.

Figure 12: The effect of inhibitors on Erk1/2 signaling in CHO-K1 cells expressing the human β_3-AR. The results are expressed as the % increase from control induced by the agonist over basal, with below, a representative immunoblot. Each histogram represents the mean ± S.E.M. (*n*=4). L755507 (100 pM) and L748337 (100 pM) increased Erk1/2 phosphorylation (601 ± 70%, and 564 ± 56% respectively). PTX (Gi, 100 ng/mL) almost completely abolished Erk1/2 phosphorylation to L748337 (138 ± 28%, *** P<0.001) and there was also some inhibitory effect on the response to L755507 (422 ± 44%, * P<0.05). DDA (adenylate cyclase, 50 μM) had no significant effect on Erk1/2 phosphorylation to L755507 (452 ± 56%) whilst there was some inhibition of the response to L748337 (308 ± 43%, * P<0.05). H89 (PKA, 10 μM) had no effect on Erk1/2 phosphorylation to either L755507 or L748337 (523 ± 61%, and 576 ± 55% respectively) as did RWJ67657 (p38 MAPK, 10 μM) (520 ± 60%, and 522 ± 57% respectively).

Figure 13: Proposed signaling pathways for L755507 and L748337 in cells expressing the human β_3-AR. a) L755507 stimulates Gs to activate adenylate cyclase (AC) leading to cAMP production that inhibits p38 MAPK activation, but has little effect on Erk1/2 phosphorylation. b) L748337 produces little cAMP that has a weak if any effect on either p38 MAPK or Erk1/2. L748337 strongly activates p38 MAPK and Erk1/2 predominantly via Gi.

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1: Comparison of pEC_{50} values (top) and E_{max} values (bottom) relative to L755507 for responses in functional bioassays in CHO-K1 cells expressing human β_3-adrenoceptors (CHOhβ_3). Note the relatively low E_{max} value for L748337 for cAMP accumulation but that the same compound has high E_{max} values for ECAR, p38MAPK and Erk1/2 activation. Agonist potency values are mean ± S.E.M. (*n*).

Ligand	pEC_{50}(*n*) (E_{max} relative to L755507)[a]				pK_i Binding
	cAMP	ECAR	p38 MAPK	Erk1/2	
Zinterol	8.55±0.09(*4*) (1.02)	8.14±0.05(*4*) (0.96)	5.93±0.19(*6*) (0.60)	10.86±0.18(*5*) (0.75)	7.1[b]
L755507	12.28±0.03(*4*) (1.00)	8.64±0.08(*4*) (1.00)	5.54±0.34(*4*) (1.00)	11.72±0.31(*6*) (1.00)	7.9[c]
L748337	9.12±0.82(*4*) (0.11)	7.15±0.06(*4*) (0.95)	5.66±0.29(*4*) (1.42)	11.58±0.33(*6*) (0.90)	8.4[d]

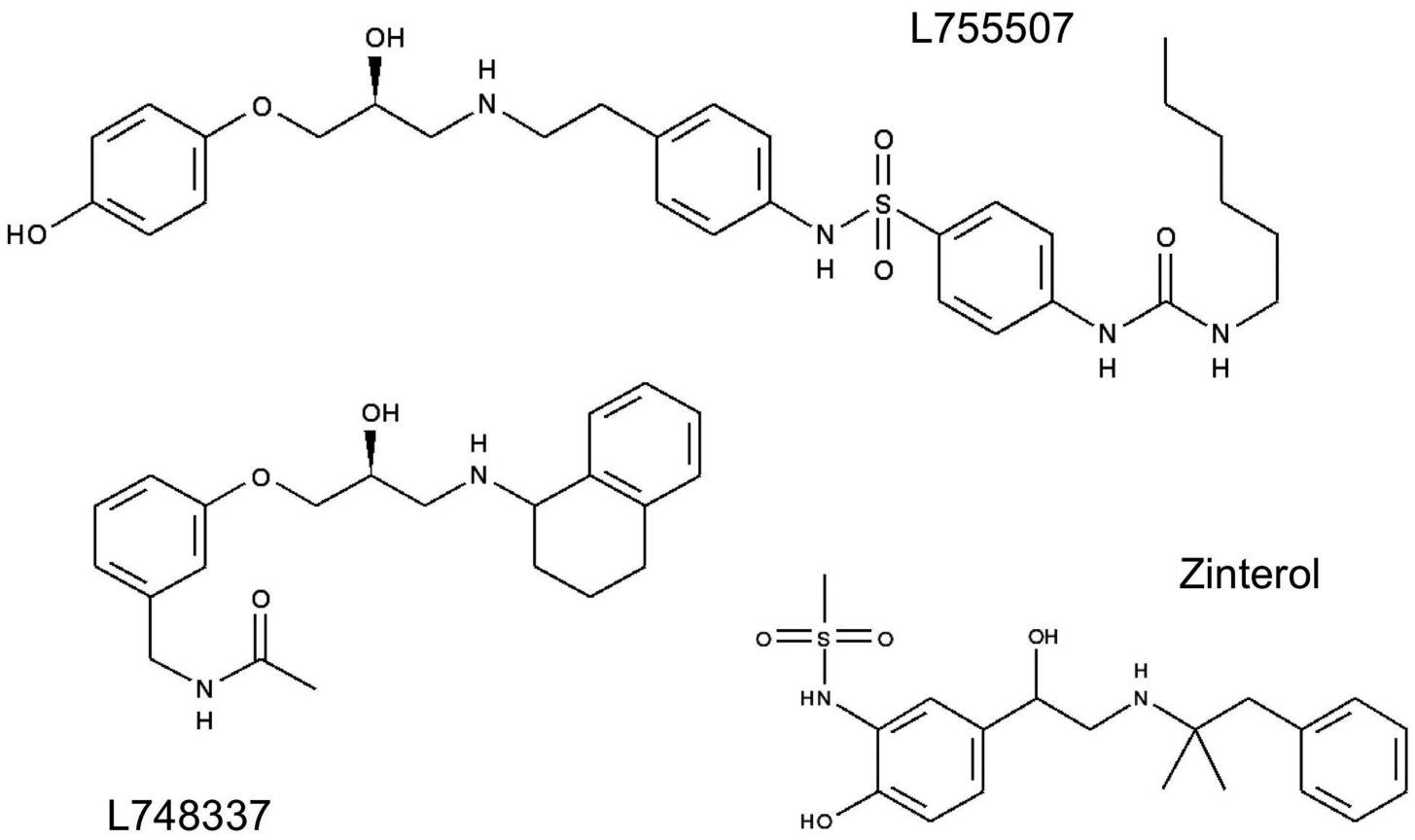
[a] Defined as a fraction of the absolute maximal response to L755507 in each individual experiment

[b] Data from Hutchinson et al., (2006)

[c] Data from Fischer et al., (1998)

[d] Data from Candelore et al., (1999)

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.



L748337

Figure 1:

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

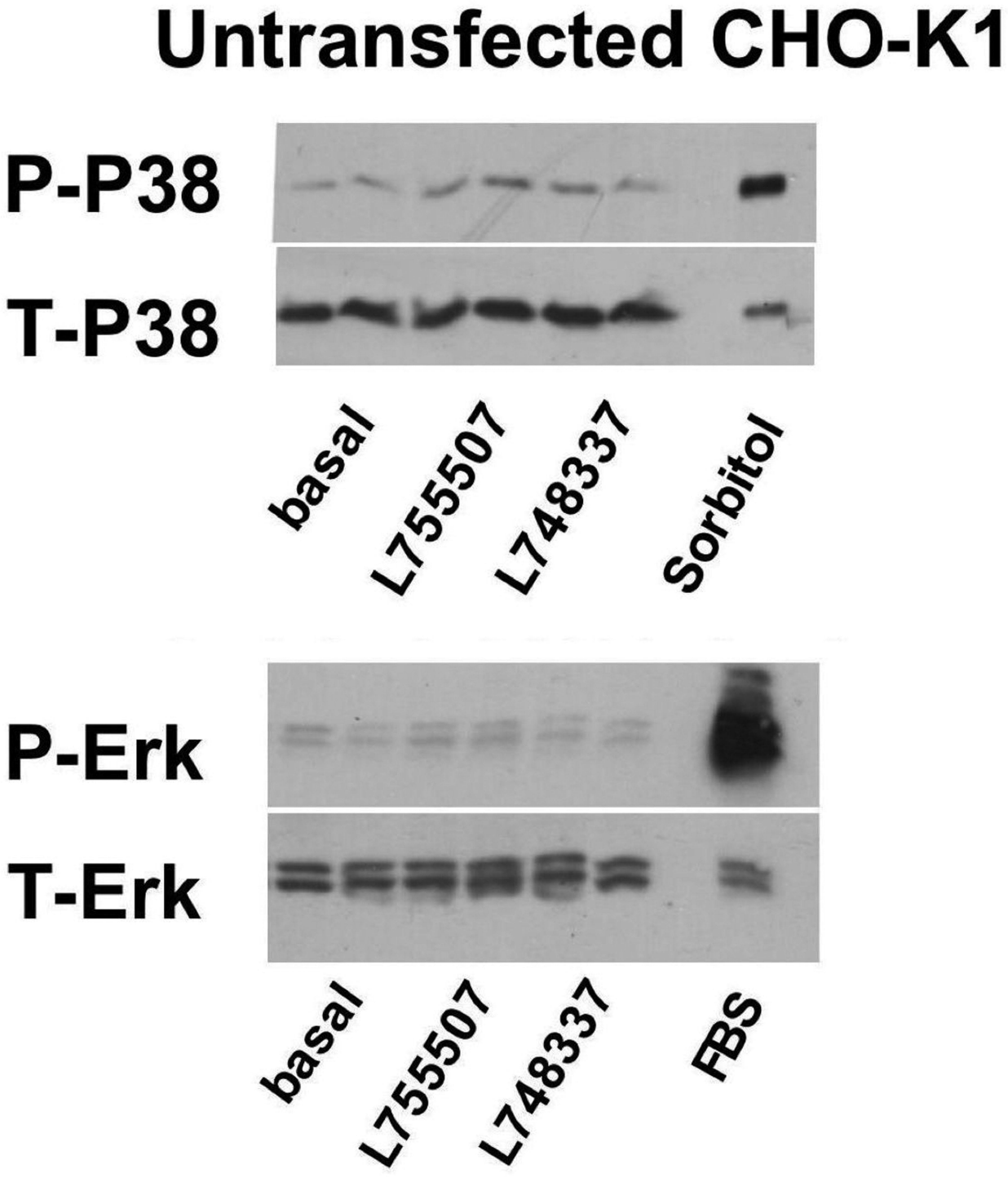


Figure 2:

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

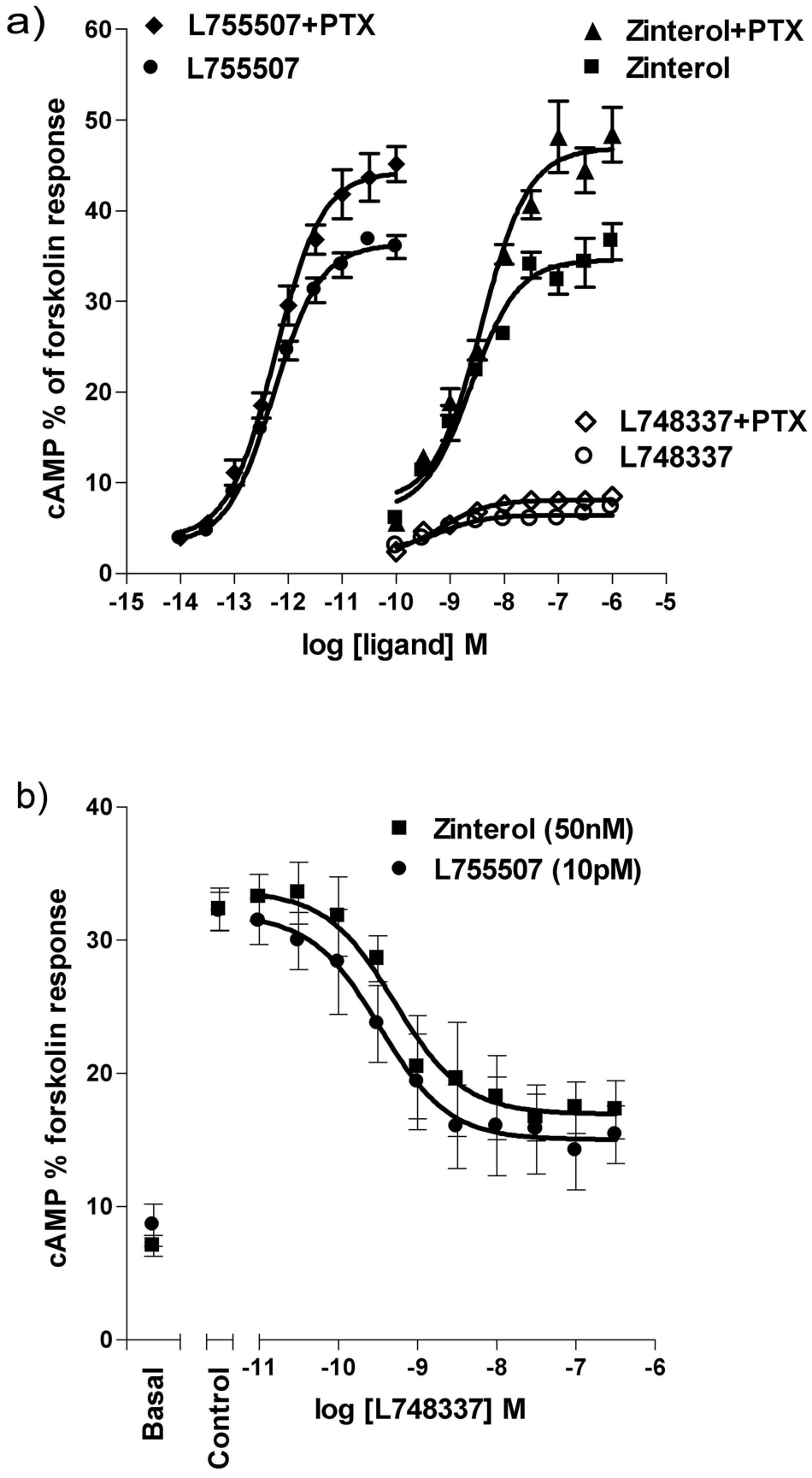


Figure 3:

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

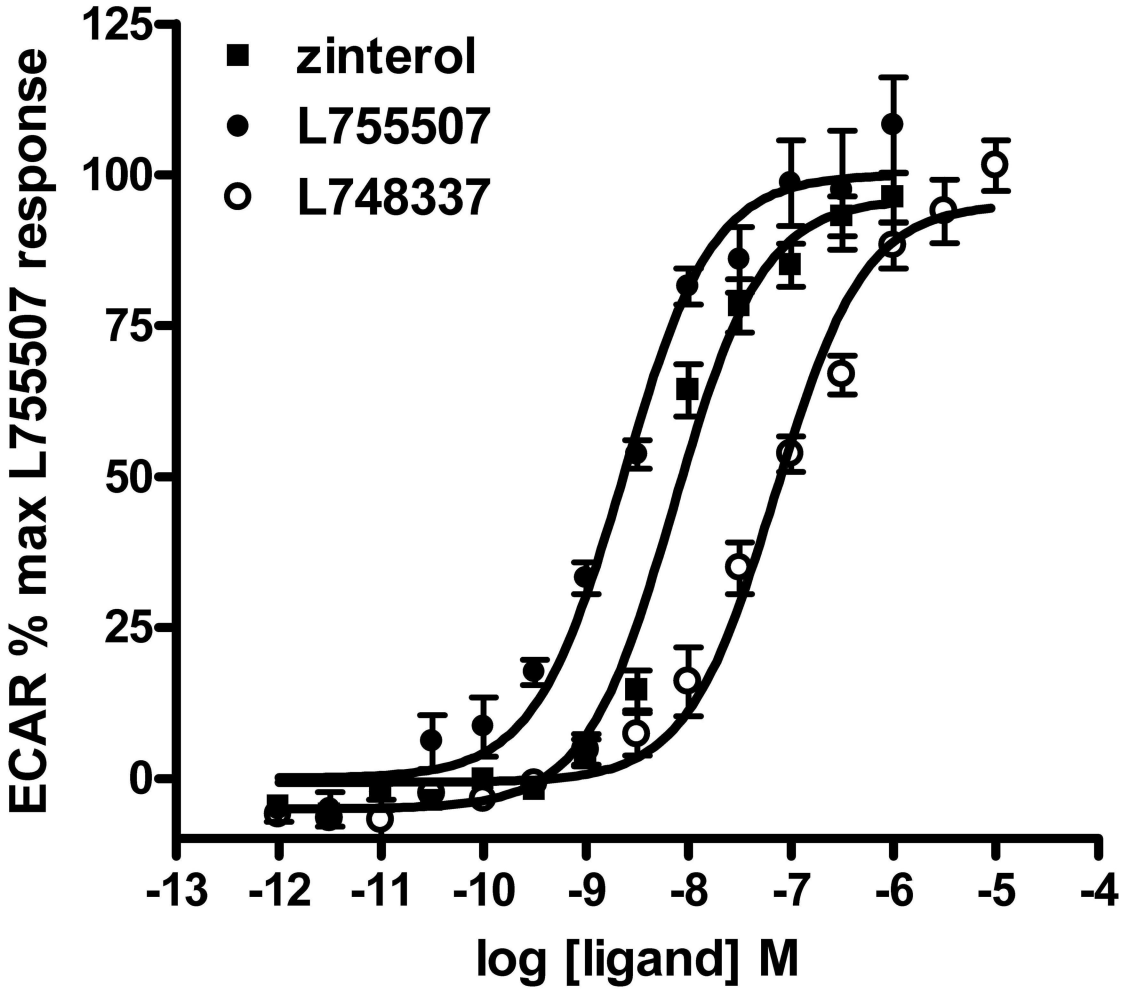


Figure 4:

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

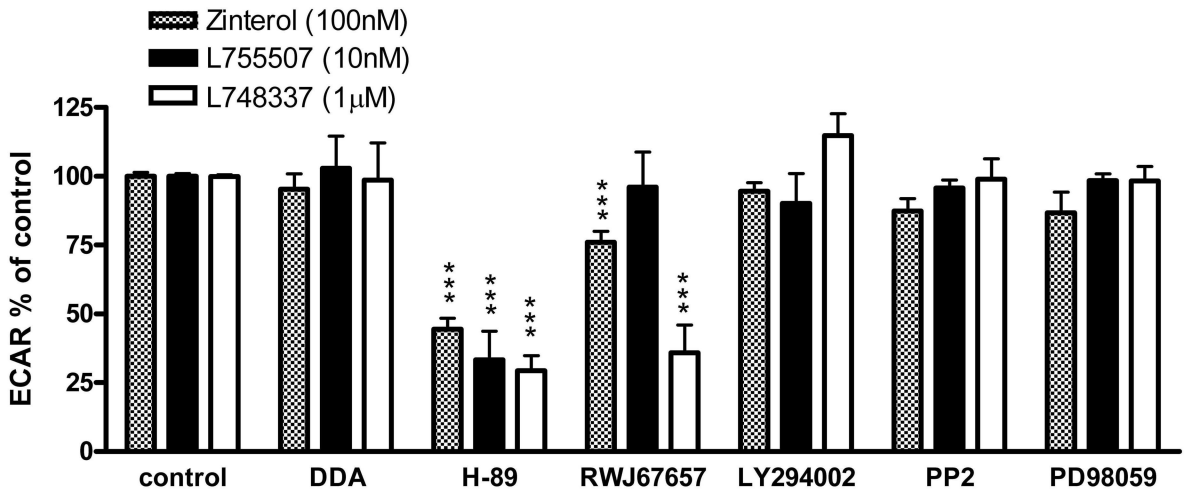


Figure 5:

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

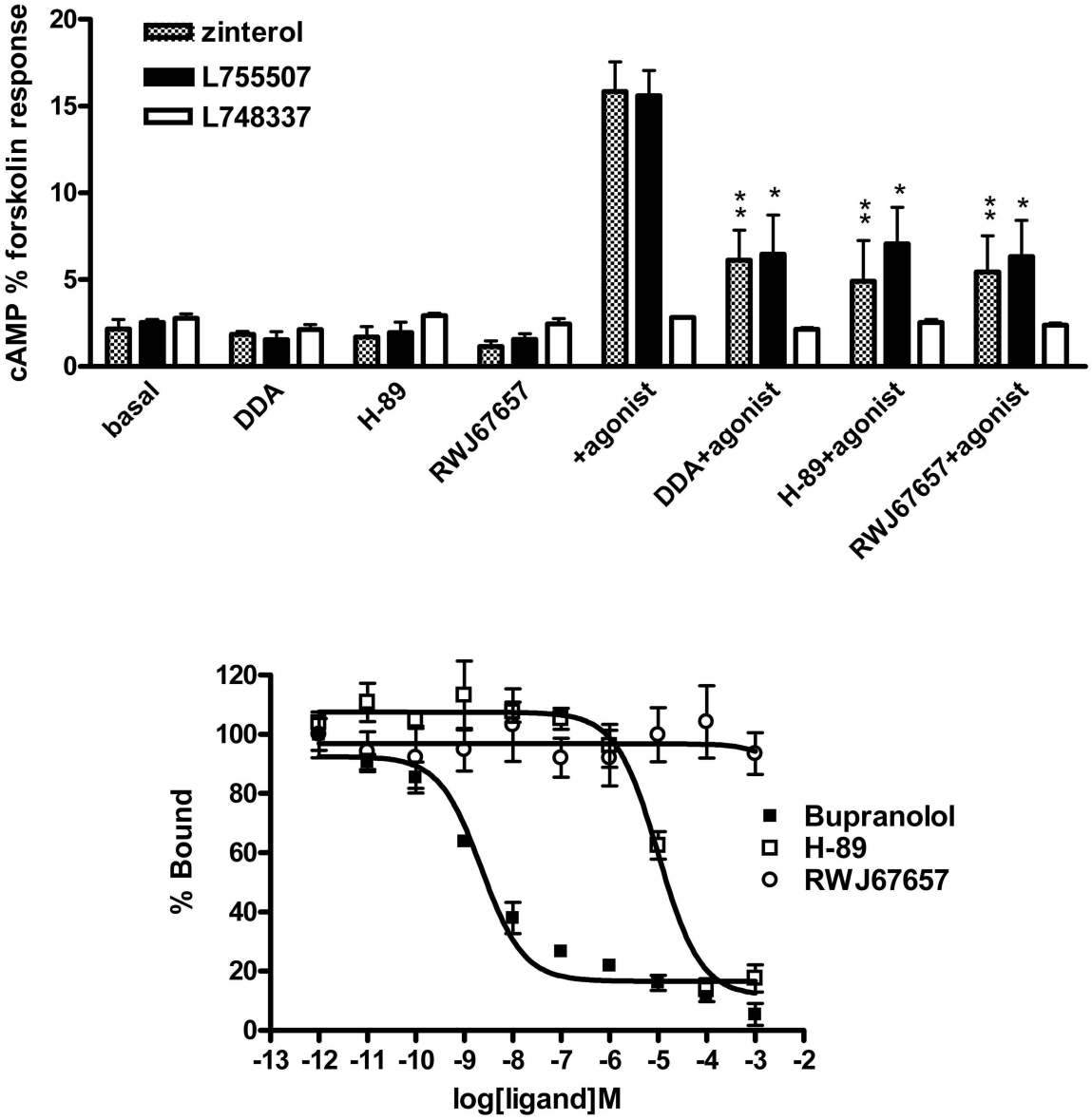
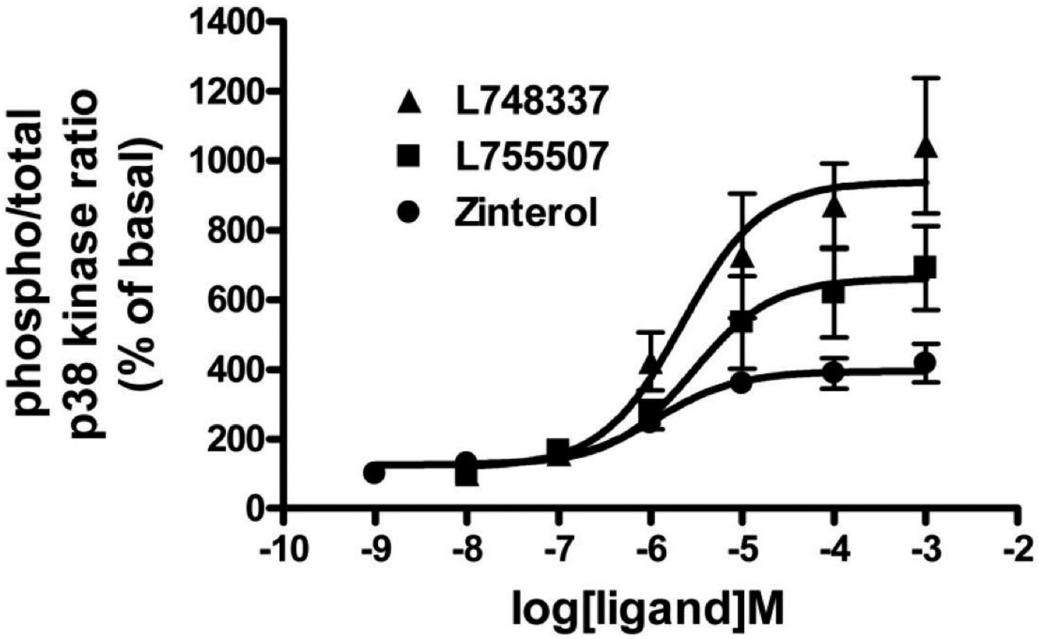


Figure 6:

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.



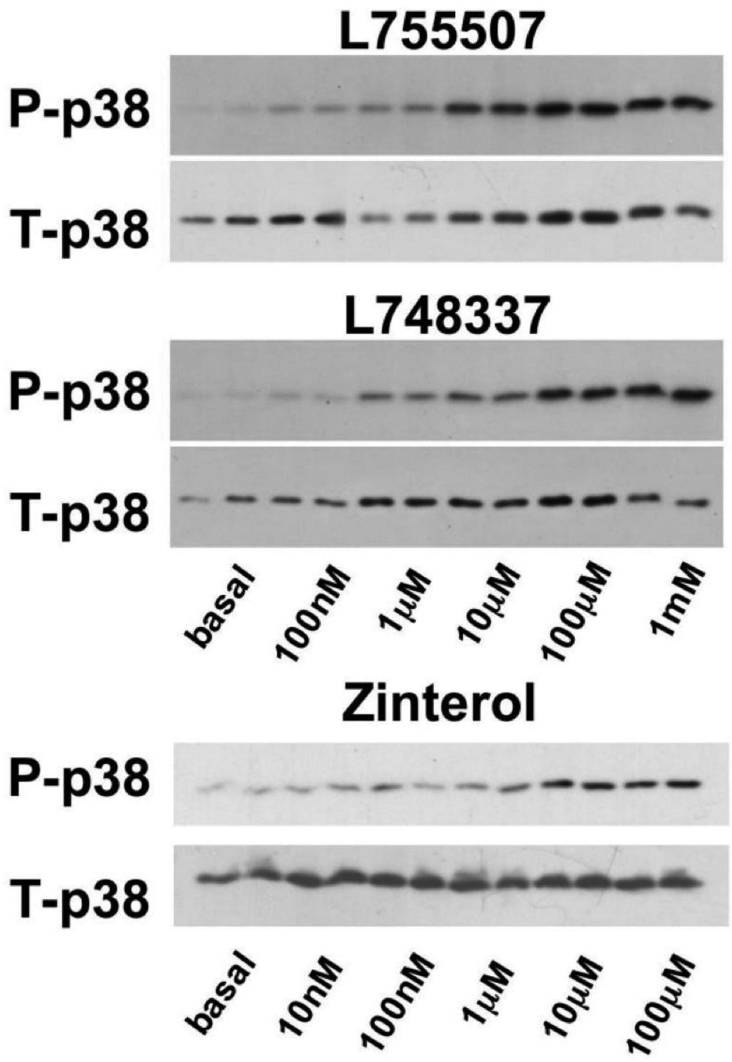


Figure 7:

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

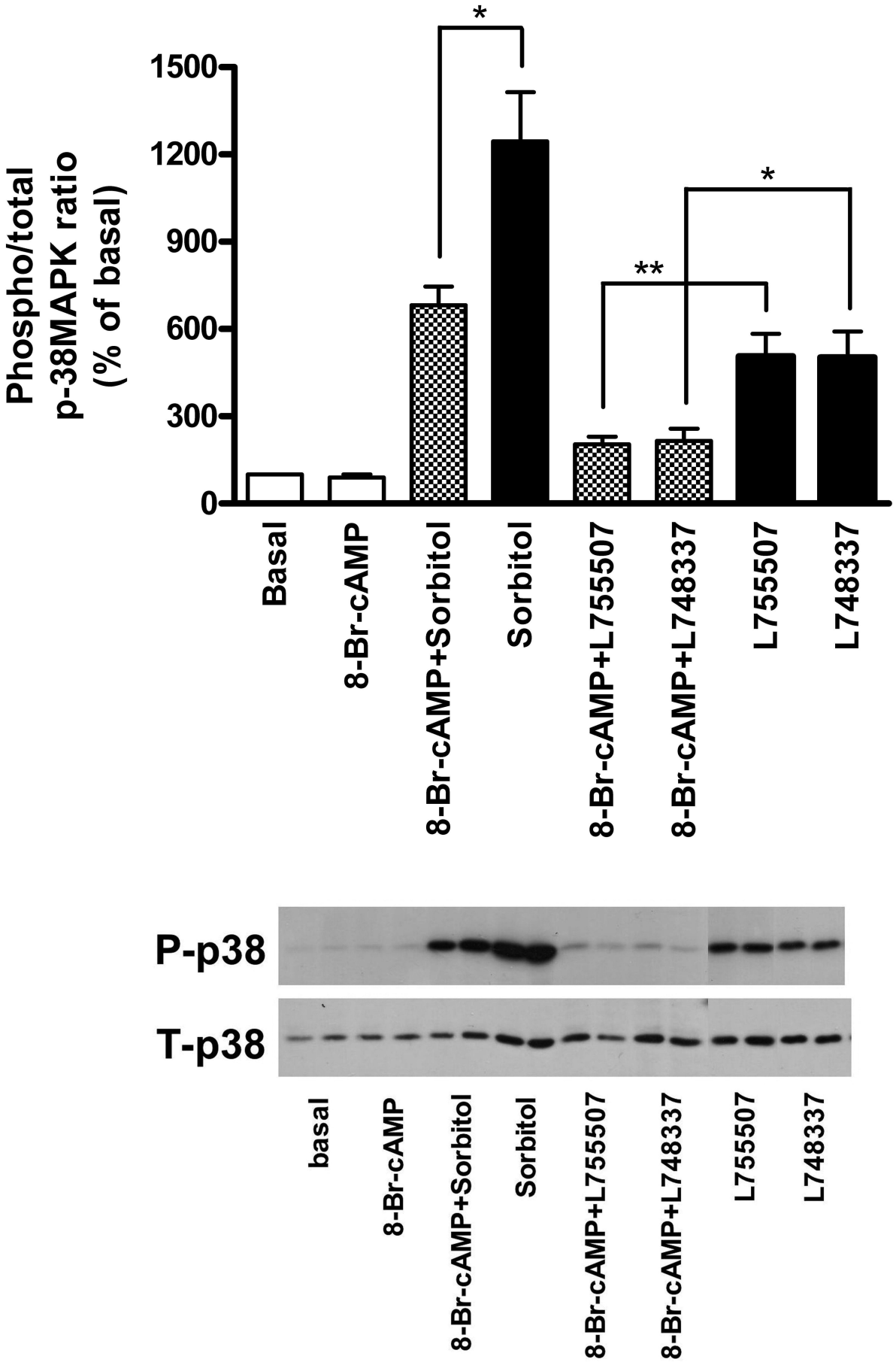
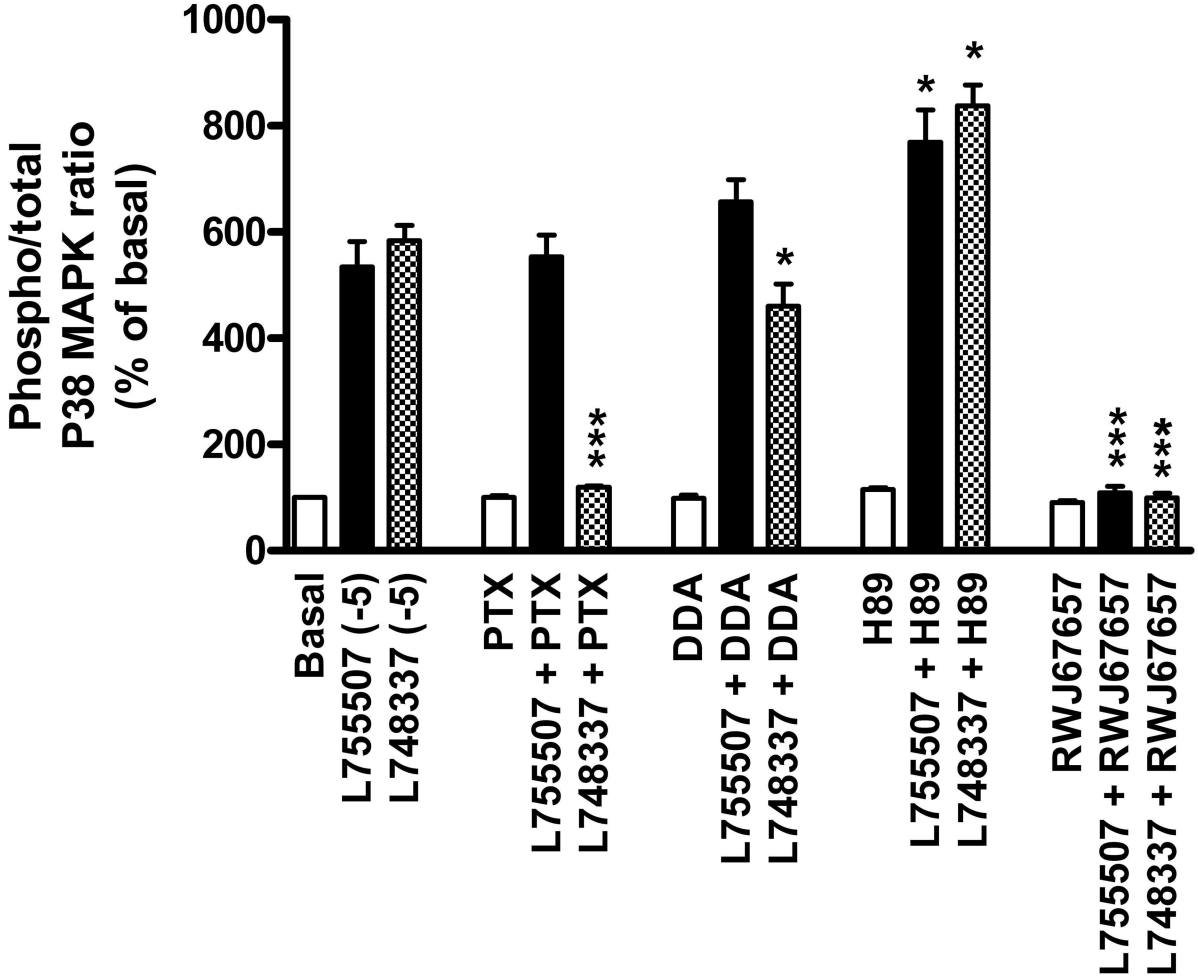


Figure 8:

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.



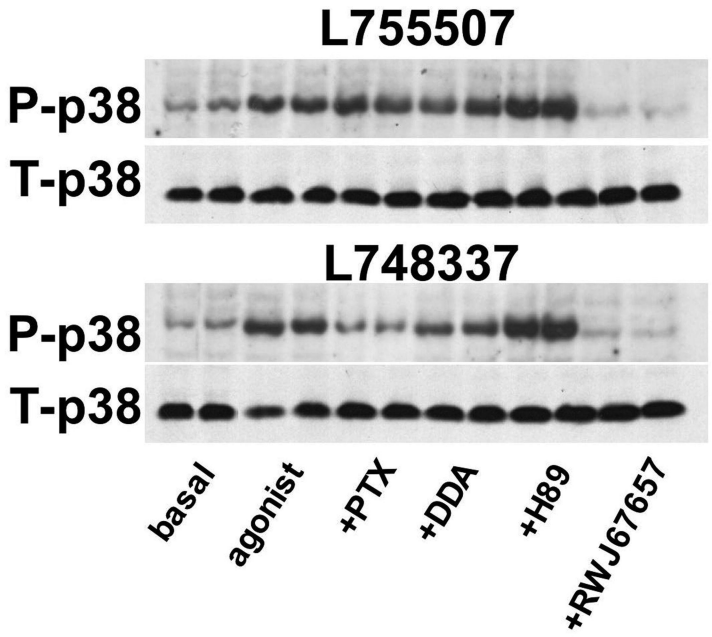


Figure 9 :

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

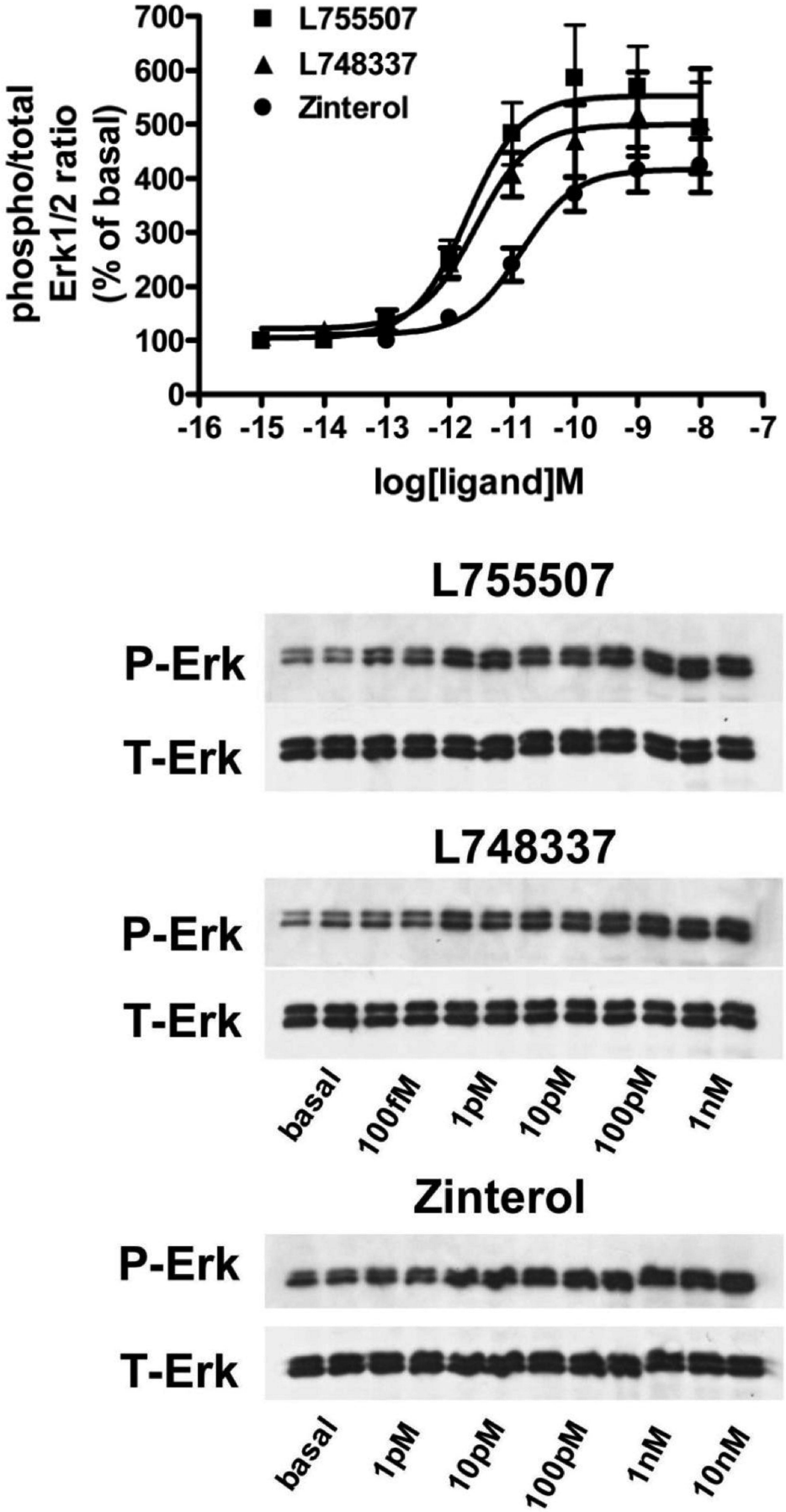


Figure 10:

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

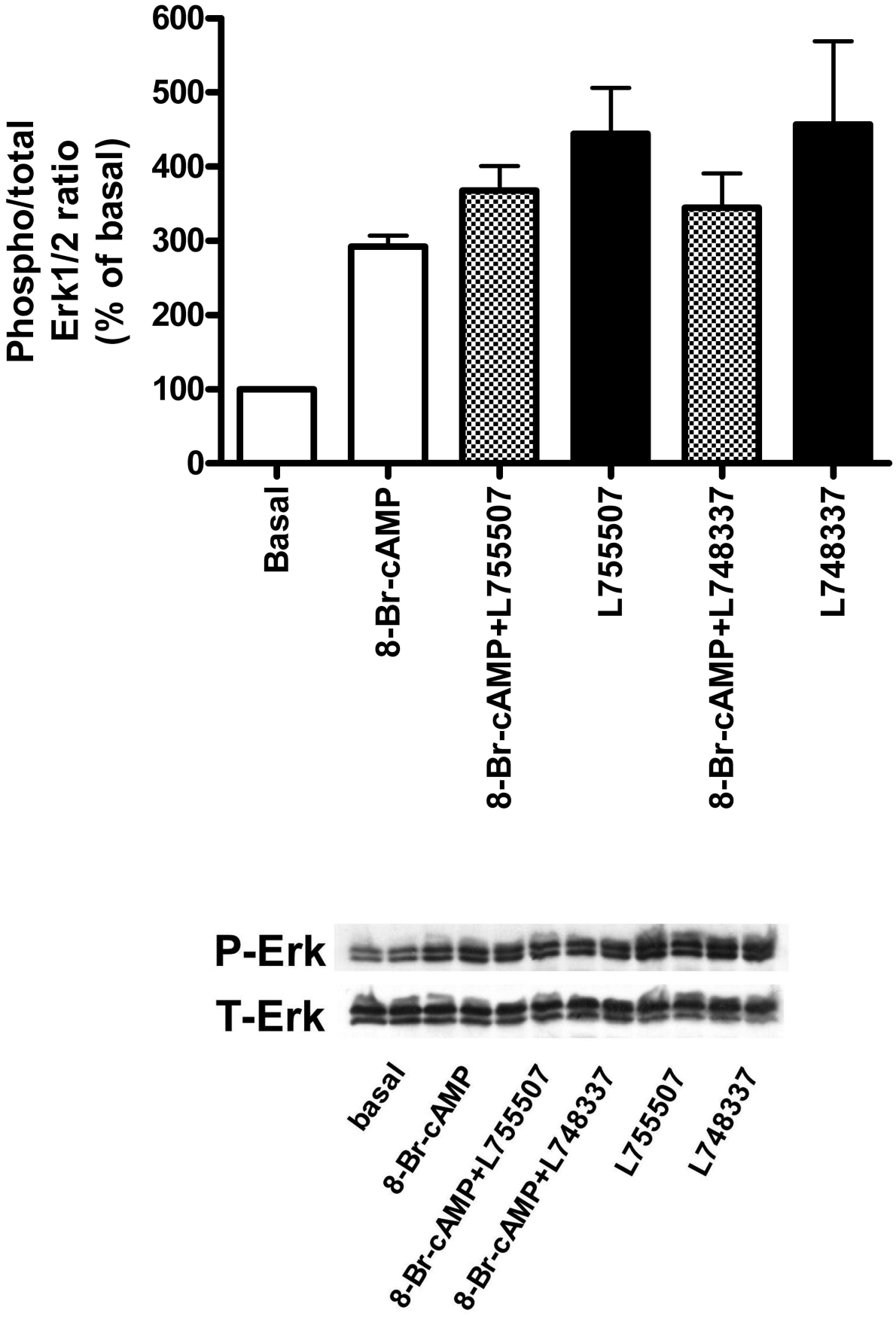
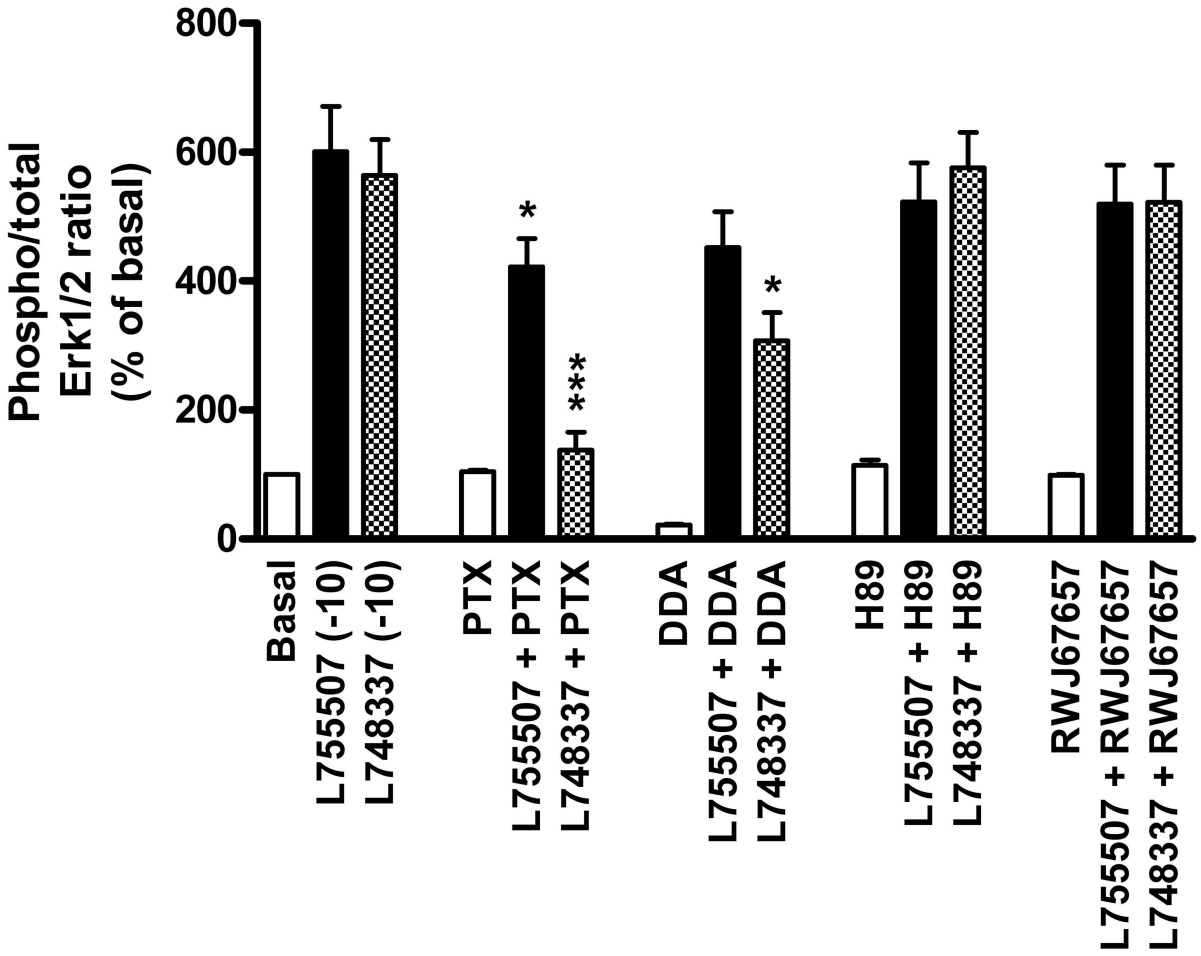


Figure 11:

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.



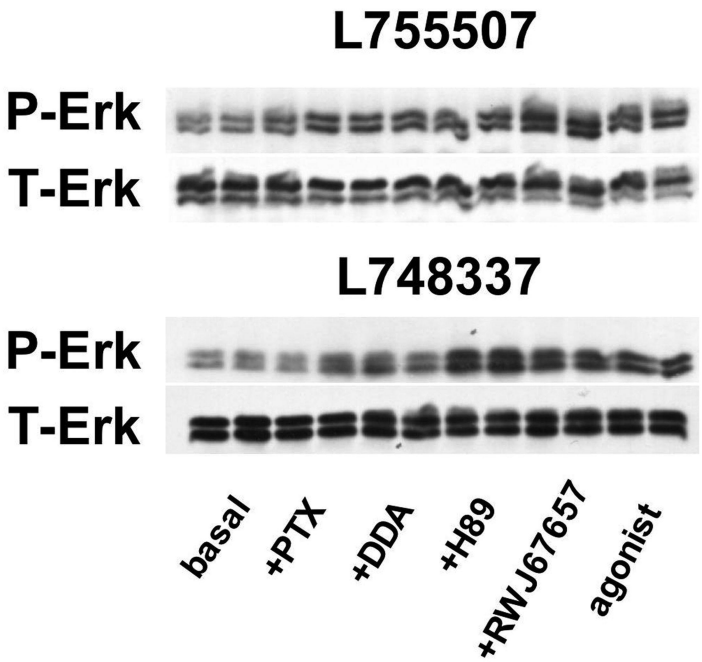


Figure 12:

Molecular Pharmacology Fast Forward. Published on August 6, 2008 as DOI: 10.1124/mol.108.046979
This article has not been copyedited and formatted. The final version may differ from this version.

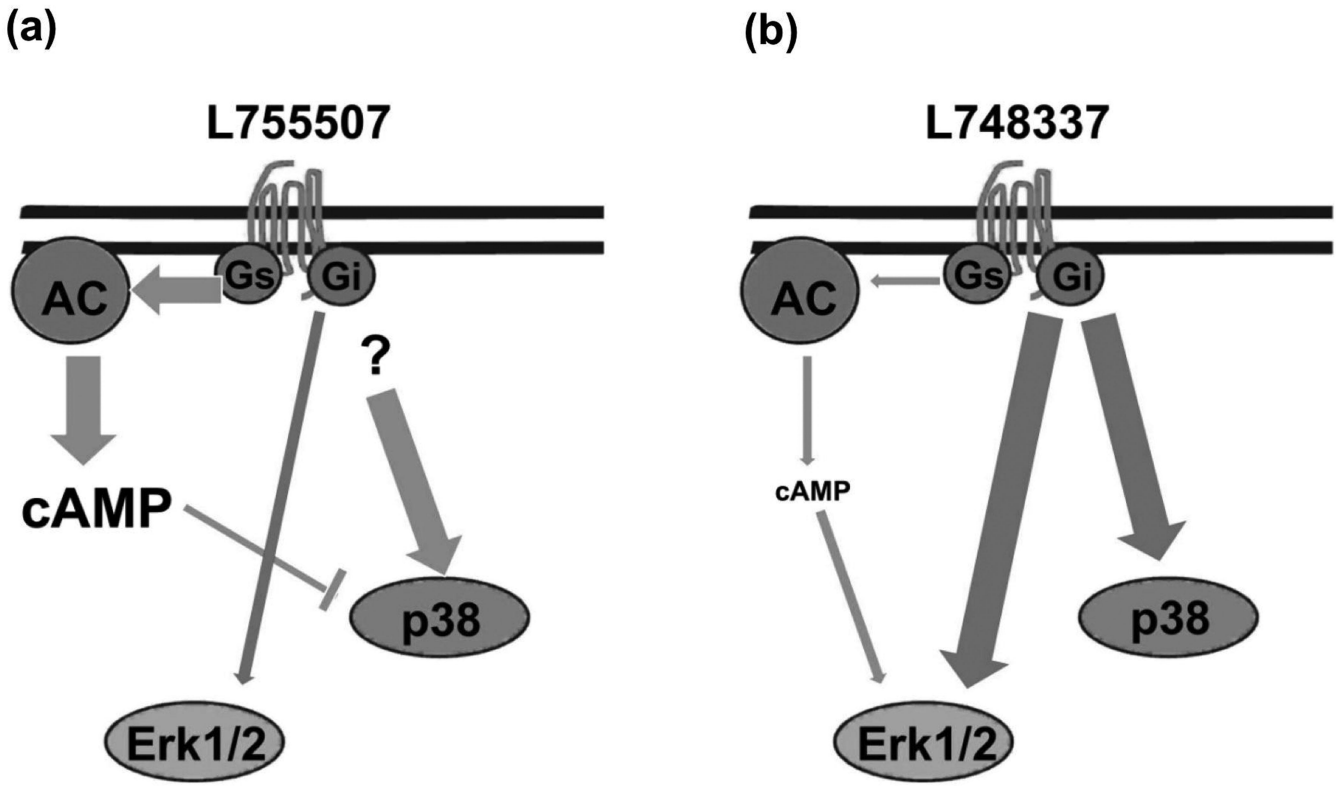


Figure 13: