Cannabinoid Receptor Activation Correlates with the Proapoptotic Action of the β_2 -Adrenergic Agonist (*R,R'*)-4-Methoxy-1-Naphthylfenoterol in HepG2 Hepatocarcinoma Cells^[S]

Rajib K. Paul, Anuradha Ramamoorthy, Jade Scheers, Robert P. Wersto, Lawrence Toll, Lucita Jimenez, Michel Bernier, and Irving W. Wainer

Laboratory of Clinical Investigation (R.K.P., A.R., M.B., I.W.W.) and Flow Cytometry Unit, Research Resources Branch (J.S., R.P.W.), National Institute on Aging, National Institutes of Health, Baltimore, Maryland; Torrey Pines Institute for Molecular Studies, Port St. Lucie, Florida (L.T.); and SRI International, Menlo Park, California (L.J.)

Received April 4, 2012; accepted July 6, 2012

ABSTRACT

Inhibition of cell proliferation by fenoterol and fenoterol derivatives in 1321N1 astrocytoma cells is consistent with β_2 -adrenergic receptor (β_2 -AR) stimulation. However, the events that result in fenoterol-mediated control of cell proliferation in other cell types are not clear. Here, we compare the effect of the β_2 -AR agonists (*R,R'*)-fenoterol (Fen) and (*R,R'*)-4-methoxy-1-naphthylfenoterol (MNF) on signaling and cell proliferation in HepG2 hepatocarcinoma cells by using Western blotting and [³H]thymidine incorporation assays. Despite the expression of β_2 -AR, no cAMP accumulation was observed when cells were stimulated with isoproterenol or Fen, although the treatment elicited both mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt activation. Unexpectedly, isoproterenol and Fen promoted HepG2 cell growth, but MNF reduced proliferation together with increased apoptosis. The mitogenic responses of Fen were attenuated by 3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy]butan-2-ol (ICI 118,551),

a β_2 -AR antagonist, whereas those of MNF were unaffected. Because of the coexpression of β_2 -AR and cannabinoid receptors (CBRs) and their impact on HepG2 cell proliferation, these $G_{\alpha_i}/G_{\alpha_o}$ -linked receptors may be implicated in MNF signaling. Cell treatment with (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (WIN 55,212-2), a synthetic agonist of CB₁R and CB₂R, led to growth inhibition, whereas inverse agonists of these receptors blocked MNF mitogenic responses without affecting Fen signaling. MNF responses were sensitive to pertussis toxin. The β_2 -AR-deficient U87MG cells were refractory to Fen, but responsive to the antiproliferative actions of MNF and WIN 55,212-2. The data indicate that the presence of the naphthyl moiety in MNF results in functional coupling to the CBR pathway, providing one of the first examples of a dually acting β_2 -AR-CBR ligand.

Introduction

(*R,R'*)-fenoterol (Fen) is a potent and selective agonist of the β_2 -adrenergic receptor (β_2 -AR), with an EC_{50cAMP} value

of 0.3 nM for the stimulation of cAMP accumulation in human embryonic kidney cells expressing human β_2 -AR (Jozwiak et al., 2010). We had reported the synthesis and characterization of a number of Fen analogs and stereoisomers with a range of β_2 -AR selectivity and potency (Jozwiak et al., 2007, 2010). One of these analogs, (*R,R'*)-4-methoxy-1-naphthylfenoterol (MNF), has a β_2 -AR/ β_1 -AR selectivity of 573 with an EC_{50cAMP} value of 3.90 nM.

β_2 -ARs associate with heterotrimeric G proteins (e.g., G_s, G_i), ion channels, and cytosolic scaffold proteins, including β -arrestin, to initiate various signaling pathways and mod-

This research was supported in part by the Intramural Research Program of the National Institutes of Health and the National Institutes of Health National Institute on Aging under Contract N01AG-3-1009.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

<http://dx.doi.org/10.1124/jpet.112.195206>.

[S] The online version of this article (available at <http://jpet.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: Fen, (*R,R'*)-fenoterol; β_2 -AR, β_2 -adrenergic receptor; MNF, (*R,R'*)-4-methoxy-1-naphthylfenoterol; WIN 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide; AM630, 1-[2-(morpholin-4-yl)ethyl]-2-methyl-3-(4-methoxybenzoyl)-6-iodoindole; ICI 118,551, 3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy]butan-2-ol; CBR, cannabinoid receptor; ERK, extracellular-regulated kinase; GPCR, G protein-coupled receptor; NF, naphthylfenoterol; IBMX, 3-isobutyl-1-methylxanthine; MRP, multidrug resistance protein; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PCR, polymerase chain reaction; siRNA, short interfering RNA; PI, propidium iodide; Iso, isoproterenol.

ulate the activity of intracellular effectors such as adenylyl cyclase and mitogen-activated protein kinases (Audet and Bouvier, 2008; Kahsai et al., 2011). The difference in the G protein and β -arrestin signaling by β_2 -AR agonists has been attributed to interaction with ligand-specific GPCR conformations and functional selectivity, which is based on the assumption that the β_2 -AR exists in an inactive (R) state and one or more ligand-specific active conformations (R^{*n}) (Seifert and Dove, 2009). The basis for the ligand-specific differences in pharmacological outcome lies in the interplay between the molecular structure of the agonist (Kahsai et al., 2011) and the cellular environment of the receptor. In the first instance, we have shown that the G_s/G_i selectivity of Fen is a function of molecular structure and stereochemistry, because Fen preferentially activated G_s signaling in a cardiomyocyte contractility model, whereas (S,S')-fenoterol and MNF activated both G_s and G_i proteins (Woo et al., 2009; Jozwiak et al., 2010).

We have also demonstrated that β_2 -AR agonists such as Fen and isoproterenol exert antiproliferative effects in the human-derived 1321N1 astrocytoma cell line specifically through the cAMP-dependent pathway (Toll et al., 2011), whereas Yuan et al. (2010) reported that isoproterenol dose-dependently induced the growth of the human-derived HepG2 hepatocellular carcinoma cell line. This cell type-specific divergence on cell proliferation by β_2 -AR agonists raises questions about whether there is cross-talk between β_2 -AR and other receptor-linked signaling cascades. A link between β_2 -AR and other GPCRs, including α_2 -AR, bradykinin, oxytocin, and cannabinoid receptors (CBRs), has been described previously (Uberty et al., 2005; Haack et al., 2010; Hudson et al., 2010; Wrzal et al., 2012). Of significance, CBRs have been reported to modulate β_2 -AR activity (Gardiner et al., 2005). The two known cannabinoid receptors, CB₁R and CB₂R, are coupled to $G_{\alpha_{i/o}}$ proteins and consequently inhibit adenylyl cyclase activity upon agonist binding (Pertwee, 2006). It is interesting that the endogenous endocannabinoid anandamide induces cell death in hepatic stellate cells (Yang et al., 2010) and promotes necrosis of HepG2 cells via activation of CB₁R and CB₂R (Wu et al., 2010). Treatment with selective pharmacological CB₂R agonists promotes neural progenitor cell proliferation and survival both in vitro and in vivo (Palazuelos et al., 2006). However, despite the fact that β_2 -AR and CBRs are coexpressed and can affect similar cellular processes in a variety of tissues, no studies have examined the possible role that CBRs may have in influencing cell fate decision mediated by β_2 -agonists.

The current study was designed to investigate the effect of the molecular structure and stereochemistry of β_2 -AR agonists on [³H]thymidine incorporation in HepG2 cells and compare these results to similar studies conducted with the 1321N1 and human-derived U87MG glioblastoma cell lines. Here, we report that proliferative responses that occur with Fen and isoproterenol were opposite to those induced by MNF, even though the latter fenoterol analog is also a full and potent β_2 -AR agonist. Unexpectedly, we show that the CBR pathway is intimately involved in the cell type-dependent control of cell proliferation by MNF. These data suggest an additional level of molecular regulation between fenoterol analogs.

Materials and Methods

Materials. (R,R')-, (R,S')-, (S,R')-, and (S,S')-fenoterol and the fenoterol analogs (R,R')-ethylfenoterol, (R,R')-4'-aminofenoterol, (R,R')-1-naphthylfenoterol, and (R,R')- and (R,S')-4-methoxy-1-naphthylfenoterol were synthesized as described previously (Jozwiak et al., 2007, 2010). [³H]thymidine (70–90 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Eagle's minimum essential medium, trypsin solution, phosphate-buffered saline (PBS), fetal bovine serum (FBS), and 100× solutions of sodium pyruvate (100 mM), L-glutamine (200 mM), and penicillin/streptomycin (a mixture of 10,000 units/ml penicillin and 10,000 µg/ml streptomycin) were obtained from Quality Biological (Gaithersburg, MD). (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (WIN 55,212-2), 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide (AM251), and 1-[2-(morpholin-4-yl)ethyl]-2-methyl-3-(4-methoxybenzoyl)-6-iodoindole (AM630) were purchased from Cayman Chemical (Ann Arbor, MI). 3-(Isopropylamino)-1-[(7-methyl-4-indanyl)oxy]butan-2-ol (ICI 118,551) hydrochloride, (R)-isoproterenol, pertussis toxin, 3-isobutyl-1-methylxanthine (IBMX), and probenecid were obtained from Sigma-Aldrich (St. Louis, MO). The primary antibodies for β_2 -AR were obtained from Enzo Life Sciences, Inc. (Farmingdale, NY) and Abcam Inc. (Cambridge, MA). Rabbit antiphospho-Akt (Ser-473), phospho-ERK1/2, total Akt, and total ERK2 were from Cell Signaling Technology (Danvers, MA), and anti- β -actin was purchased from Abcam Inc. Rat anti-multidrug resistance protein (MRP) 4 and goat anti-MRP5 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Maintenance and Treatment of Cell Lines. Human HepG2 hepatocarcinoma cells and human U87MG glioma cells (American Type Culture Collection, Manassas, VA) were maintained in Eagle's minimum essential medium supplemented with 1% L-glutamine, 1% sodium pyruvate, 1% penicillin/streptomycin, and 10% Hyclone FBS (Thermo Fisher Scientific, Waltham, MA). The human 1321N1 astrocytoma cells (European Collection of Cell Cultures, Porton Down, Wiltshire, UK and Sigma-Aldrich) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and penicillin/streptomycin. All cell lines were cultured at 37°C in 5% CO₂, and the medium was replaced every 2 to 3 days. The passage numbers of the cell lines used ranged from 15 to 30.

Unless otherwise indicated, cells at 70 to 80% confluence were depleted of serum for 3 h, followed by the addition of ICI 118551, AM251, AM630 or WIN 55,212-2 for 1 h before treatment with vehicle, Fen, MNF, and other fenoterol derivatives at the indicated concentrations.

[³H]Thymidine Incorporation Assay. Cells were seeded in 12-well plates at approximately 50,000 cells/well and incubated at 37°C. After 24 h, the wells were rinsed with PBS and replaced with serum-free medium containing the appropriate concentration of the test compounds. After another 24-h incubation at 37°C, 1 µCi of [³H]thymidine was added to each well and incubated at 37°C for 16 h. [³H]thymidine incorporation into DNA was monitored after the cells were washed twice with PBS and then lysed in 600 µl of 0.1 N NaOH for 30 min with shaking. The lysate was then mixed with 3 ml of liquid scintillation cocktail (Beckman Coulter, Fullerton, CA), and radioactivity was measured by liquid scintillation counting using a Beckman Coulter LS6000IC scintillation counter. Data are shown as cpm incorporated compared with the control cells.

cAMP Accumulation. HepG2 cells were seeded in 96-well plates and grown to confluence. Cells were rinsed in Krebs-HEPES buffer, pH 7.4, preincubated for 20 min with the buffer supplemented with 50 µM IBMX, a phosphodiesterase inhibitor, and then 1 µM isoproterenol, Fen, or MNF was added for an additional 10 min. In some experiments, the MRP inhibitor probenecid (2.5 mM) was included in the preincubation buffer. The levels of cAMP accumulated in cells

were determined and normalized to the amount of protein per well as described previously (Toll et al., 2011).

RNA Interference. HepG2 and 1321N1 cells were plated at a density of 3×10^5 cells/well in six-well cell culture plates and incubated for 48 h. Transfection of the cells was carried out in Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA) with 50 nM each of a combination of three siRNAs (Applied Biosystems, Foster City, CA and Santa Cruz Biotechnology, Inc.) targeted against human β_2 -AR or a nonsilencing siRNA control (Santa Cruz Biotechnology, Inc.). Forty-eight hours later, cell lysates were prepared and immunoblotted for β_2 -AR by Western blotting.

RNA Extraction, cDNA Synthesis, and Reverse Transcription-PCR Analysis. Total RNA was isolated from HepG2, 1321N1, and U87MG cells by using the RNeasy Mini kit (QIAGEN, Valencia, CA). The RNA preparation included a DNase digestion step. RNA concentration and quality was measured by using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). To obtain cDNA, 1 μ g of total RNA was reverse-transcribed by using the Promega reverse transcription kit (Promega, Madison, WI). PCRs were performed to determine the expression of CB₁R, CB₂R, and β_2 -AR mRNAs by using glyceraldehyde-3-phosphate dehydrogenase as an internal control. The PCR primers and conditions are found in Supplemental Table 1.

Cell Cycle Analysis. Cell cycle distributions were performed by flow cytometry on propidium iodide (PI)-stained nuclei prepared by the NIM technique (Kopp and Wersto, 1992). DNA histograms of at least 10,000 cells acquired on a BD FACScanto II (BD Biosciences, San Jose, CA) were deconvoluted by using the Multicycle program (Phoenix Flow Systems, San Diego, CA) for estimates of the percentage of cells in the G₀/1, S, and G₂+M phases of the cell cycle. Debris and doublets were removed from the analysis by software algorithms.

Apoptosis Assay. The degree of apoptosis induced by drug treatment was assayed by flow cytometry using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen) following the standard manufacturer's protocol. In brief, HepG2 cells (5×10^5) were grown on 100-mm dishes for 24 h followed by treatment with vehicle, Fen, or MNF, all in serum-free medium. Cells were subsequently harvested after 24-h incubation, washed in cold PBS, and resuspended in 100 μ l of 1 $\times$ annexin-binding buffer to maintain a density of $\sim 1 \times 10^6$ cells/ml. Five microliters of Alexa Fluor 488 Annexin V and 1 μ l of 100 μ g/ml propidium iodide were added to the cell suspensions. Cells were then incubated at room temperature for 15 min, and 400 μ l of 1 $\times$ annexin-binding buffer was added followed by gentle mixing. Stained cells were analyzed on a BD FACScanto II flow cytometer.

Western Blotting. Cells were lysed with radioimmunoprecipitation assay buffer containing EGTA and EDTA (Boston BioProducts, Ashland, MA). The lysis buffer was mixed with a protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were measured by using the bicinchoninic acid reagent (Thermo Fisher Scientific). Proteins (20 μ g/well) were separated on 4 to 12% precast gels (Invitrogen) using SDS-polyacrylamide gel electrophoresis under reducing conditions and electrophoretically transferred onto polyvinylidene fluoride membrane (Invitrogen). Western blots were performed according to standard methods, and the antibodies were used at a dilution recommended by the manufacturer. The visualization of immunoreactive bands was performed by using the ECL Plus Western Blotting Detection System (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), and their quantification was done by volume densitometry using ImageJ software (National Institutes of Health, Bethesda, MD) and normalization to β -actin.

Statistical Analysis. Results were expressed as relative to the control value. Experiments were performed in at least two to three different culture preparations, and two to three dishes for each experimental condition were plated in each preparation. Results are expressed as means $\pm$ S.E. Statistical comparisons between groups were made by *t* test. Analyses were performed by using SigmaPlot software (Systat Software, Inc. San Jose, CA), Prism 4 (GraphPad

Software, Inc., La Jolla, CA), and Excel (Microsoft, Redmond, WA), with *p* values ≤ 0.05 considered significant.

Results

Expression of β_2 -AR in the HepG2 and 1321N1 Cell Lines. The mRNA and protein levels of the β_2 -AR were determined in HepG2 hepatocarcinoma cells and 1321N1 astrocytoma cells. Semiquantitative PCR analysis indicated higher expression of β_2 -AR mRNA in 1321N1 cells compared with HepG2 cells (Supplemental Fig. 1A). Accordingly, 1321N1 cells expressed more β_2 -AR protein than HepG2 cells, when three commercial antibodies were tested by using a Western blot technique (Supplemental Fig. 1B). As indicated in Fig. 1A, the knockdown of β_2 -AR expression by siRNA-based approach led to $\sim 70\%$ reduction in the level of β_2 -AR protein, validating the specificity of these antibodies. U87MG cells were found previously to be devoid of β_2 -AR (Toll et al., 2011).

Effect of β -AR Agonists on cAMP Accumulation and Phosphorylation of Akt and ERK1/2 in HepG2 Cells. Isoproterenol, Fen, or MNF at 1.0 μ M did not elicit an increase in cAMP production in HepG2 cells, whereas cell treatment with the adenylyl cyclase activator forskolin in-

Fig. 1. Responses of HepG2 cells to β -agonist stimulation. A, HepG2 and 1321N1 cells were transfected with the negative control siRNA (-) or β_2 -AR siRNA (+) for 48 h. Cell lysates were immunoblotted with a specific anti- β_2 -AR antibody (ab40834), using Hsp90 as a loading control. B, increase in cAMP accumulation in HepG2 cells was observed with forskolin (10 μ M), but not with 1 μ M of either isoproterenol (Iso), Fen, or MNF. Data shown are from a single experiment conducted in quadruplicate. Error bars indicate mean $\pm$ S.D. C, serum-starved HepG2 cells were incubated in the presence of Iso (1 μ M) or Fen (1 μ M) for 5, 10, and 30 min. Cell lysates were immunoblotted with antibodies against phosphorylated Akt (p-Akt; Ser473) and total Akt, as well as phosphorylated ERK1/2 (p-ERK1/2) and total ERK2. The experiments shown in B and C were repeated twice with comparable results. The positions of molecular mass markers (in kilodaltons) are shown to the left of the immunoblots.

duced significant accumulation of cAMP (Fig. 1B). Because of the ability of cyclic nucleotides to move across the cell membrane via the drug efflux pump MRP4 (ABCC4) and MRP5 (ABCC5) (Wielinga et al., 2003; Cheng et al., 2010), we investigated whether the active export of cAMP accounted for the apparent lack of effect of isoproterenol and fenoterol compounds on intracellular cAMP accumulation. HepG2 cells were pretreated with the MRP inhibitor probenecid (Copsel et al., 2011), followed by agonist stimulation. The presence of probenecid did not produce an increase in the intracellular cAMP levels under conditions where phosphodiesterase-mediated cAMP hydrolysis was inhibited by IBMX and the cells stimulated either with β_2 -AR agonists or forskolin (data not shown). Moreover, Western blotting of total cell extracts revealed that the MRP4/5 protein expression levels were below the limit of detection (data not shown). These data indicate that the export of cAMP plays a little role, if any, in the apparent lack of effect of β_2 -AR agonists on cAMP accumulation in HepG2 cells. Rather, this behavior may indicate uncoupling between agonist-stimulated β_2 -AR and G α s protein. Alternatively, because of the low number of β_2 -ARs in HepG2 cells, agonist-stimulated adenylyl cyclase activity may be below detectable levels.

Previous studies have demonstrated that β_2 -AR can signal to the mitogen-activated protein kinases ERK1 and ERK2 (Ahn et al., 1999; Fan et al., 2001) independently of a functional adenylyl cyclase coupling (Agarwal and Glasel, 1999). The effect of isoproterenol and Fen on Akt and ERK1/2 activation was assessed by immunoblotting using selective antibodies to phosphorylated peptides that correspond to the active forms of Akt and ERK1/2. We observed that treatment of HepG2 cells with these β -agonists induced a time-dependent increase in Akt and ERK activation (Fig. 1C), which was blocked by ICI 118551, a β_2 -AR inhibitor (data not shown).

Stimulation with MNF had no effect on the activation of Akt and ERK (data not shown). These results indicate that different types of agonist-stimulated β_2 -AR signaling events can occur, probably through the coupling to different G α proteins and/or other G protein-independent pathways (Shenoy et al., 2006).

The Effects of Isoproterenol and Fen Analogs on the Proliferation of HepG2 Cells. The effect of isoproterenol, Fen, and selected fenoterol analogs on cell proliferation was determined in HepG2 cells. Both isoproterenol and Fen produced a significant increase in cell proliferation, as assessed by [3 H]thymidine incorporation, with EC₅₀ values of 0.40 ± 0.08 and $1.17 \pm 0.37 \mu\text{M}$, respectively (Table 1; Fig. 2A). Yuan et al. (2010) reported similar potency of isoproterenol toward HepG2 cell proliferation, where saturation was reached at $10 \mu\text{M}$ of the β -AR agonist. The EC₅₀ value reported here for isoproterenol was 400-fold lower than that reported in U118 cells (Toll et al., 2011). The lower potency of isoproterenol in HepG2 cells raised the concern that this cell model may not contain spare β_2 -AR receptors that would increase the efficacy of isoproterenol as an agonist (Nicolas et al., 1991).

Fen has two chiral centers and four possible stereoisomeric forms: (*R,R'*), (*R,S'*), (*S,R'*), and (*S,S'*). The effect of the stereochemistry on the proliferative effect of Fen was determined by using a concentration of $1 \mu\text{M}$ of each isomer. The data indicate that all of the isomers induced an increase in [3 H]thymidine incorporation, and stereochemistry had only a quantitative effect on this process, with Fen producing the greatest increase (51.3%) and (*S,S'*)-fenoterol producing the lowest (9.7%) (Table 1). This result was consistent with the previously reported inhibitory effect of Fen stereoisomers on mitogenesis in 1321N1 cells in which the inhibitory potency was (*R,R'*) > (*R,S'*) $\approx$ (*S,R'*) $\gg$ (*S,S'*) (Toll et al., 2011).

Fig. 2. The effects of isoproterenol, Fen, and fenoterol derivatives on cell growth are cell type specific. A and B, serum-starved HepG2 cells were incubated with vehicle or the indicated concentrations of Iso, Fen, or (*R,R'*)-aminofenoterol (NH₂-fen) (A) or MNF (B) for 24 h, and the levels of [3 H]thymidine incorporation were measured; see *Materials and Methods* for experimental details. Representative concentration-response curves are shown. C, HepG2 cells in serum-depleted medium and 1321N1 cells in complete medium were treated with compounds at $1 \mu\text{M}$ for 24 h. D, HepG2 and 1321N1 cells were incubated without (SFM) or with serum (CM) in the presence of the indicated concentrations of Iso or Fen. Quantification of percentage change in [3 H]thymidine incorporation versus control is expressed as means $\pm$ S.E. and represents results from two to six independent experiments, each performed in triplicate dishes. In most instances, error bars are smaller than the symbols.

TABLE 1

Structures, percentage change in thymidine incorporation, and IC_{50}/EC_{50} of fenoterol and analogs that were used for this study

Compound	R1	R2	IC_{50}/EC_{50}	Change in HepG2 cells	Mitogenesis Inhibition in 1321N1 Cells*
			μM	%	IC_{50} nM
Fenoterol	CH ₃		1.17 ± 0.37 ($n = 6$)	(<i>R,R</i>): 51.3 (<i>R,S</i>): 19.1 (<i>S,R</i>): 28.7 (<i>S,S</i>): 9.7	0.14 ± 0.07 6.09 ± 1.93 6.74 ± 2.18 184.2 ± 26.1
Ethylfenoterol	CH ₃ -CH ₂		N.D.	(<i>R,R</i>): 50.90 at 10 μM	1.44 ± 0.27
Aminofenoterol	CH ₃		0.47 ± 0.09 ($n = 3$)	(<i>R,R</i>): 54.37	N.D.
1-Naphthyl fenoterol	CH ₃		0.21 ± 0.07 ($n = 2$)	(<i>R,R</i>): -67.52	1.57 ± 0.34
4'-Methoxy-1-naphthylfenoterol	CH ₃		0.39 ± 0.09 ($n = 6$)	(<i>R,R</i>): -59.4 (<i>R,S</i>): -68.1	3.98 ± 0.28 4.37 ± 0.70

N.D., not determined.

* From Table 2 in Toll et al. (2011).

(see Table 1). The effect of the change of the *N*-alkyl methyl group to an ethyl moiety {(*R,R'*)-ethylfenoterol} and the substitution of an 4'-amino group for the 4'-hydroxyl group {(*R,R'*)-aminofenoterol} were also investigated. Neither alteration changed the direction of the effect on [³H]thymidine incorporation, and (*R,R'*)-aminofenoterol seemed to be 3-fold more active than Fen with an EC_{50} value of 0.47 ± 0.09 μM (Table 1; Fig. 2A).

In a previous study, we reported that the incorporation of a naphthyl moiety into the Fen molecule reduced the potency of the resulting compound, but not the inhibitory effect on mitogenesis in 1321N1 cells (Toll et al., 2011). In this study, the opposite effect was observed as MNF and 1-naphthylfenoterol inhibited [³H]thymidine incorporation with IC_{50} values of 0.39 ± 0.09 and 0.21 ± 0.07 μM , respectively (Table 1; Fig. 2B). The change in the stereochemistry of the chiral center on the *N*-alkyl portion of the MNF molecule had no effect on the antiproliferative response because 1 μM concentrations of MNF and (*R,S'*)-4-methoxy-1-naphthylfenoterol

produced equivalent decreases in [³H]thymidine incorporation of -59.4 and -68.1%, respectively (Table 1).

MNF is a full and potent β_2 -AR agonist in respect to the stimulation of cAMP expression in human embryonic kidney cells stably transfected with β_2 -AR and 1321N1 cells, with EC_{50} values of 3.9 and 68.9 nM, respectively (Jozwiak et al., 2010; Toll et al., 2011). Because HepG2 cells displayed substantial sensitivity to (*R,R'*)-aminofenoterol ($EC_{50} = 0.47 \pm 0.09$ μM) and MNF ($IC_{50} = 0.39 \pm 0.09$ μM) with regard to [³H]thymidine incorporation, the responsiveness of 1321N1 cells to the two compounds was determined and found to be markedly lower (Fig. 2C). The specificity of the observed β_2 -AR response to Fen and MNF in the HepG2 and 1321N1 cells was tested by using the U87MG cells, which have been shown to be devoid of β_2 -AR binding activity (Toll et al., 2011). In this cell line, MNF produced a potent inhibition of cellular proliferation, whereas Fen had no effect (Supplemental Fig. 2).

In the previous study of the effect of isoproterenol and Fen on mitogenesis in 1321N1 cells, the experiments were con-

ducted by using complete medium. To determine whether the presence of serum or its absence significantly influenced the extent of mitogenesis in response to isoproterenol and Fen, the experiments were repeated using both experimental protocols. The results indicated that HepG2 cells exhibited a better sensitivity in serum-depleted medium, whereas the sensitivity was greater in 1321N1 cells maintained in complete medium (Fig. 2D). These data suggest that there are contrasting mitogenic responses to β_2 -AR agonists in HepG2 and 1321N1 cells.

β_2 -AR Antagonism Does Not Inhibit the Antiproliferative Action of MNF While Preventing the Growth-Promoting Effects of Fen in HepG2 Cells. The divergent actions mediated by Fen and MNF are consistent with activation of distinct signaling pathways with opposite effects on cell proliferation. To test this hypothesis, HepG2 cells were pretreated with ICI 118,551, followed by incubation in the presence of Fen or MNF for 24 h. Although ICI 118,551 alone showed a modest, but significant, concentration-dependent increase in cell proliferation, up to $\sim 16\%$ at $1 \mu\text{M}$ (Fig. 3A), its addition markedly blocked Fen-stimulated mitogenesis (Fig. 3, B and C). However, the antiproliferative effect of MNF was refractory to ICI 118,551 pretreatment (Fig. 3, B and D).

We then tested the possibility that the action of Fen could be hampered by the coaddition of MNF. The results showed clearly a mitogenic response in HepG2 cells that was intermediate between Fen and MNF alone, and the pretreatment with ICI 118,551 partially restored the antiproliferative ef-

fects of MNF (Supplemental Fig. 2, top). However, characteristics of the cell proliferation profile elicited by Fen in 1321N1 cells and MNF in U87MG cells were maintained by the cotreatment with Fen and MNF (Supplemental Fig. 2, middle and bottom). As expected, pretreatment with ICI 118,551 blocked Fen signaling in 1321N1 cells while being inactive against the antiproliferative action of MNF in U87MG cells (Supplemental Fig. 2). These results indicate that the effects of Fen and MNF on cell proliferation are cell type-specific and may require the activation of distinct receptors.

MNF Induces Apoptosis in HepG2 Cells. The proliferation of HepG2 cells was assessed by flow cytometry analysis using propidium iodide staining to examine the cell cycle. Fen produced no significant alterations of the cell cycle, but MNF caused a temporal decrease in the G_2/M - and S-phase cell populations (G_2/M , $13.8 \pm 1.1\%$ in control versus $10.2 \pm 0.9\%$ after 6 h, $14.6 \pm 1.8\%$ after 12 h, and $8.9 \pm 1.6\%$ after 24 h; S, $34.7 \pm 0.3\%$ in control versus $34.1 \pm 0.9\%$ after 6 h, $13.7 \pm 1.2\%$ after 12 h, and $24.6 \pm 4.2\%$ after 24 h) in HepG2 cells treated with $1 \mu\text{M}$ MNF (Fig. 4). The treatment with MNF also yielded a time-dependent increase in the number of sub- G_1 events, reaching a maximum of $21.5 \pm 0.7\%$ by 12 h (Fig. 4, bottom right). No significant increase in sub- G_1 events was observed when cells were treated with Fen ($1 \mu\text{M}$) for up to 24 h.

Sub- G_1 events occur when cells have proceeded to the late stage of apoptosis or are already dead. To directly measure apoptosis, flow cytometry analysis with Annexin V/PI staining was carried out in HepG2 cells. The percentage of apo-

Fig. 3. β_2 -AR antagonist does not inhibit the antiproliferative action of MNF in HepG2 cells. A and B, serum-depleted HepG2 cells were incubated with the indicated concentrations of the β_2 -AR antagonist ICI-118,551 (ICI) for 1 h followed by the addition of vehicle (A), Fen (B, left), or MNF (B, right) for 24 h, and levels of [^3H]thymidine incorporation were measured. *, $p < 0.05$. Representative concentration-response curves for Fen and MNF are shown in B. C and D, quantification of percentage change in [^3H]thymidine incorporation versus control is expressed as means $\pm$ S.E. and represents results from three independent experiments, each performed in triplicate dishes. C, Fen. D, MNF.

Fig. 4. MNF increases the number of sub- G_1 events in HepG2 cells. Serum-depleted HepG2 cells were harvested after 6-, 12-, and 24-h treatment with vehicle (top left), Fen (1 μ M) (top right), or MNF (1 μ M) (bottom left). Cells were fixed, stained and then analyzed for DNA content by using flow cytometry. Representative DNA content analysis in various phases of the cell cycle after 24-h treatment with vehicle, Fen, or MNF is shown. The number of sub- G_1 events, which displays cells in late-stage apoptosis or already dead, in function of treatment duration was quantified and represents results from two independent experiments, each performed in duplicate dishes (bottom right). Data are expressed as means $\pm$ S.E. ($n = 4$).

ptotic cells induced by a 24-h treatment with MNF (1 μ M) was increased 5.7-fold compared with control ($p < 0.01$). However, Fen treatment reduced apoptosis compared with control untreated cells (Fig. 5).

Role of Cannabinoid Receptors in the Control of Cell Proliferation of MNF and Fen. Because of the coexpression of β_2 -AR and CBRs and their impact on HepG2 cell proliferation (Wu et al., 2010; Yuan et al., 2010), we assessed whether the regulation of mitogenesis in response to Fen and MNF could occur through CBR signaling mechanisms.

The mRNA levels of CB₁R and CB₂R were determined by reverse transcription-PCR in HepG2, 1321N1, and U87MG cells (Fig. 6A). The results indicated that HepG2 and U87MG cells expressed CB₁R and CB₂R, whereas 1321N1 cells had no detectable levels of CBR mRNAs. Therefore, one should expect cell type-specific differences in responsiveness to CBR ligands. Indeed, potent regulatory effects of synthetic cannabinoid compounds were observed in cells treated with Fen and MNF compared with controls. Similar to MNF, treatment of HepG2 cells with the cannabinoid receptor agonist WIN 55,212-2 (1 μ M) reduced cell proliferation and canceled out the growth-promoting action of Fen (Fig. 6B). AM251 and AM630 are synthetic inverse agonists for CB₁R and CB₂R, respectively (Pertwee, 2005). We observed that cell pretreatment with AM251 or AM630 had no impact on the mitogenic responses of Fen (Fig. 6C), indicating that basal-level activity of these two CBRs does not play a major role in Fen's proliferative action. However, preincubation with AM251 or AM630 completely inhibited the antiproliferative effects of MNF in HepG2 cells (Fig. 6C), which is consistent with the

Fig. 5. MNF induces apoptosis in HepG2 cells. Serum-depleted HepG2 cells were treated with vehicle (top left), Fen (1 μ M) (top right), or MNF (1 μ M) (bottom left) for 24 h, stained with Annexin V and PI, and then analyzed by flow cytometry. Representative profiles are shown. The fraction of annexin V-positive HepG2 cells that were apoptotic was quantitated and represents results from two independent experiments, each performed in duplicate dishes (lower right). Data are expressed as means $\pm$ S.E. ($n = 4$).

involvement of CBRs in MNF signaling. In support of this hypothesis, we found that 1321N1 cells, which were unresponsive to MNF, were refractory to CBR ligands, when added alone or combined with Fen (Supplemental Fig. 3, A and B). However, the antiproliferative effects of MNF were partially blocked by AM251 and AM630 in the β_2 -AR-deficient U87MG cells (Supplemental Fig. 3, C and D).

Coupling of MNF to cell proliferation seemed to depend on $G_{\alpha_{i/o}}$ proteins, because the MNF and WIN 55,212-2 antiproliferative responses were inhibited by an 18-h pretreatment with pertussis toxin (50 ng/ml) in HepG2 cells (Fig. 6D). Under these conditions, pertussis toxin had no effect on Fen-induced cell proliferation.

Discussion

β -AR agonists and antagonists influence cell growth and function (Evans et al., 2010), which can lead to inhibition or induction of malignant diseases. The effect is cell specific because both β_2 -AR antagonists and agonists have been shown to attenuate cell growth (Carie and Sebt, 2007; Zhang et al., 2010; Toll et al., 2011). In the case of β_2 -AR agonists, the effect seems to be through cAMP-dependent pathways and is subject to antagonism by ICI 118,551 (Carie and Sebt, 2007; Toll et al., 2011). However, β_2 -AR agonism has also been shown to elicit growth and survival of several different cancer cell types (Sastry et al., 2007; Yuan et al., 2010), such as the isoproterenol-induced increase in HepG2 cell proliferation (Yuan et al., 2010).

To explore the intercellular differences in the prosurvival and antisurvival pathways activated by β_2 -AR agonists, we examined the effect of Fen and its analogs on the proliferation of HepG2 cells, with the objective of comparing the data with the compounds' previously observed attenuation of 1321N1 cellular growth (Toll et al., 2011). In this study, Fen

Fig. 6. Role of CBR activation in the antiproliferative action of MNF in HepG2 cells. **A**, total RNA was extracted from HepG2, 1321N1, and U87MG cells, and then analyzed semiquantitatively by PCR. A nontemplate control (NTC) has been included (lane 1). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **B** and **C**, serum-depleted HepG2 cells were incubated with the CBR agonist WIN 55,212-2 (Win; 1 μ M) (**B**) or antagonists AM251 (1 μ M) or AM630 (0.5 μ M) (**C**) for 1 h followed by the addition of vehicle, Fen (0.5 μ M), or MNF (0.25 μ M) for 24 h. **D**, serum-depleted HepG2 cells were pretreated without or with pertussis toxin (PTX; 50 ng/ml) for 16 h followed by the addition of vehicle, Fen (0.5 μ M), MNF (0.5 μ M), or WIN 55,212-2 (0.5 μ M) for 24 h. In **B** to **D**, the levels of [3 H]thymidine incorporation were measured. Quantification of percentage change in [3 H]thymidine incorporation versus control is expressed as means $\pm$ S.D. and represents results from three (**B** and **C**) or two (**D**) independent experiments, each performed in triplicate dishes. *, $p < 0.05$.

increased HepG2 cell proliferation, consistent with the previously reported effect of isoproterenol (Yuan et al., 2010), and the fact that ICI 118,551 blocked this response indicates the involvement of β_2 -ARs. However, neither Fen nor isoproterenol induced cAMP accumulation in HepG2 cells, although treatment with forskolin demonstrated that the cells express functional adenylyl cyclase. The lack of effect of β_2 -AR agonists on cAMP accumulation may be caused by the low level of β_2 -AR protein expression in these cells. It is also possible that the intracellular accumulation of cAMP may be reduced by MRP4/5-mediated export (Cheng et al., 2010). In this study, the presence of the MRP inhibitor probenecid had no effect on isoproterenol-mediated increase in cAMP accumulation in HepG2 cells, and the overall expression levels for MRP4/5 proteins in these cells were found to be below the detection limit. Another possibility is that although the β_2 -ARs are poorly coupled to the stimulatory $G\alpha$ protein, they may exhibit functional interactions with other signaling intermediates that promote cell growth. Our results demonstrate that Fen activated the phosphatidylinositol 3-kinase/Akt and ERK pathways in HepG2 cells. In support of our findings, the nontyrosine kinase c-Src has been implicated in the switching of signaling of β_2 -AR from adenylyl cyclase coupling to the mitogen-activated protein kinase/ERK pathway (Ahn et al., 1999; Luttrell et al., 1999). It should also be noted that scaffold proteins dock c-Src to the membrane-bound β_2 -AR and lead to β_2 -AR-mediated cell proliferation through the activation of ERK1/2 (Tao and Malbon, 2008; Zhang et al., 2011).

Our previous studies have examined the effect of Fen stereochemistry and structure on β_2 -AR stimulation of cAMP accumulation and inhibition of mitogenesis in 1321N1 cells. The data demonstrated that changes in the molecule's two chiral centers produce only quantitative changes in β_2 -AR

agonism (Jozwiak et al., 2010; Toll et al., 2011). A similar effect was observed in the HepG2 cells because all of the Fen stereoisomers produced an increase in [3 H]thymidine incorporation (reported as percentage change) with $(R,R') \gg (S,R') \approx (R,S') \gg (S,S')$ (Table 1). The same result was produced by the Fen analogs (R,R') -ethylfenoterol and (R,R') -aminofenoterol (Table 1; Fig. 2A). The results indicate that when the Fen molecule contains a 4'-substituted phenyl ring the compound stimulates [3 H]thymidine incorporation in HepG2 cells and the stereochemistry of the molecule influences this effect, but does not qualitatively change it. A full structure-activity relationship study has been initiated, and the results will be reported elsewhere.

Previous studies have also demonstrated that the substitution of a naphthyl moiety for the phenyl ring on the *N*-alkyl portion of Fen, to generate naphthylfenoterol (NF) analogs, does not affect the β_2 -AR agonist activity with respect to the stimulation of cAMP accumulation, inhibition of mitogenesis in 1321N1 cells, or cardiomyocyte contractility (Jozwiak et al., 2010; Toll et al., 2011). Thus, the incubation of HepG2 cells with (R,R') -1-naphthylfenoterol and MNF was expected to produce a stimulation of [3 H]thymidine incorporation. However, a qualitative difference was observed as MNF and (R,R') -1-naphthylfenoterol inhibited [3 H]thymidine incorporation (Table 1; Fig. 2B). In addition, unlike Fen, a change in the stereochemistry of the chiral center on the *N*-alkyl portion of the MNF molecule had no effect on the antiproliferative response (Table 1).

Because the Fen and NF analogs used in this study are potent and selective β_2 -AR agonists, a potential explanation for the effect produced by replacing a phenyl ring with a naphthyl ring is "ligand-directed signaling" or "biased agonism" (Seifert and Dove, 2009). It has been demonstrated that β_2 -AR binds ligands in multiple conformations and bind-

ing to different receptor conformations can lead to differences in signal transduction (Wisler et al., 2007; Seifert and Dove, 2009). In respect to the Fen and NF molecules, initial comparative molecular field analysis studies of the interaction of the Fen analogs with the β_2 -AR have indicated that the naphthyl substituent of the NF molecules can interact with the β_2 -AR through a series of π - π and π -hydrogen bond interactions unavailable to the phenyl moiety on the Fen molecule (Jozwiak et al., 2007, 2010). It is doubtful that the binding of NF analogs to the β_2 -AR is the primary mechanism responsible for the decrease in [3 H]thymidine incorporation, because the MNF response was insensitive to ICI 118,551 (Fig. 3). Moreover, MNF treatment of U87MG cells, which lack β_2 -AR binding activity, reduced cell growth, whereas Fen had no effect (Supplemental Fig. 2). These data do not eliminate the possibility that NFs bind to and stabilize a conformation of the β_2 -ARs expressed endogenously in HepG2 cells that is distinct from the conformation stabilized by Fen. However, such conformation may not be critical for the initiation of cell proliferation.

The data suggest that other GPCRs may be involved in the antiproliferative effects of MNF. One potential target is the CBRs because previous studies have suggested that CBRs modulate β_2 -AR activity (Gardiner et al., 2005). Thus, although the effect of MNF on CBR function was never anticipated, the molecular structure of MNF does share some similarities with recently reported CBR ligands. In particular, binding and molecular modeling studies indicate that the presence of a naphthyl moiety increases binding to the CB₂R through π - π and hydrophobic interactions (Osman et al., 2010). It is interesting to note that the CB₂R model used in the study by Osman et al. (2010) was constructed based on the structure of the β_2 -AR. Therefore, it is likely that the extent of β_2 -AR and CBR expression within a given cell line may influence the overall growth-promoting properties and survival function of Fen versus MNF.

We now report pharmacological evidence to suggest that MNF mediates its antiproliferative effects through activation of the CBRs. First, WIN 55,212-2 mimicked this MNF response, and the combination of MNF plus WIN 55,212-2 showed a lack of additive effect. Moreover, selective inhibition of the CB₁R and CB₂R showed suppression of MNF signaling. The inability of WIN 55,212-2 to modulate cell proliferation in 1321N1 cells may be best explained by their substantially lower CBR expression levels compared with HepG2 and U87MG cell lines (Biswas et al., 2003; Aguado et al., 2007; Curran et al., 2005; Wu et al., 2010) (Fig. 6A). Moreover, our data demonstrate that the Fen-mediated increase in HepG2 cell proliferation is neutralized by WIN 55,212-2; the most plausible mechanism accounting for this observation is that stimulation of G α_i -linked CBRs in response to WIN 55,212-2 inhibits cell growth primarily by negatively targeting the phosphatidylinositol 3-kinase/Akt and/or ERK pathways (Wang et al., 1999). This observation is supported by the inhibition of the MNF and WIN 55,212-2 responses in HepG2 cells pretreated with pertussis toxin. Taken together, our findings indicate a complex cell type-specific involvement of CBRs in the antimetogenic and proapoptotic activities of the β_2 -AR agonist MNF through a mechanism that does not require β_2 -AR activation.

In the last several years, development of new CBR ligands has become an intense area in cancer research because of the

role of the endocannabinoid system in the regulation of cell proliferation and apoptosis (Oesch and Gertsch, 2009; Guindon and Hohmann, 2011; Hermanson and Marnett, 2011). In considering the overall influence of CBRs on MNF signaling, it is clear that CBR-directed signaling has the potential to be much more complex than initially thought, because it depends not only on which CBRs are present (CB₁R versus CB₂R), but also on the multiplicity of active and intermediate states of each receptor. Further complicating the issue is our demonstration, using selective inverse agonists, that the inhibition of either CB₁R or CB₂R antagonizes MNF response, which probably results from the ability of these receptors to form functional heteromers (Calln et al., 2012). The coexpression of β_2 -AR with CBRs in many tissues and cancer cell lines and their propensity to heterodimerize (Milligan, 2009; Hudson et al., 2010) suggest an even more complex picture, whereby MNF and other potential dually acting β_2 -AR-CBR ligands may yield a new class of compounds capable of having an unique affinity and/or selectivity profile. The therapeutic potential for synthetic small bivalent ligands holds great promise as new lead compounds in a wide range of disparate diseases (for review, see Valant et al., 2012).

Acknowledgments

We thank Sutapa Kole for expert technical assistance.

Authorship Contributions

Participated in research design: Paul, Wersto, Toll, Bernier, and Wainer.

Conducted experiments: Paul, Ramamoorthy, Scheers, and Jimenez.

Contributed new reagents or analytic tools: Wersto.

Performed data analysis: Paul, Ramamoorthy, Wersto, Bernier, and Wainer.

Wrote or contributed to the writing of the manuscript: Paul, Wersto, Bernier and Wainer.

References

- Agarwal D and Glaser JA (1999) Differential effects of opioid and adrenergic agonists on proliferation in a cultured cell line. *Cell Prolif* **32**:215–229.
- Aguado T, Carracedo A, Julien B, Velasco G, Milman G, Mechoulam R, Alvarez L, Guzmn M, and Galve-Roperh I (2007) Cannabinoids induce glioma stem-like cell differentiation and inhibit gliomagenesis. *J Biol Chem* **282**:6854–6862.
- Ahn S, Maudsley S, Luttrell LM, Lefkowitz RJ, and Daaka Y (1999) Src-mediated tyrosine phosphorylation of dynamin is required for β_2 -adrenergic receptor internalization and mitogen-activated protein kinase signaling. *J Biol Chem* **274**:1185–1188.
- Audet M and Bouvier M (2008) Insights into signaling from the β_2 -adrenergic receptor structure. *Nat Chem Biol* **4**:397–403.
- Biswas KK, Sarker KP, Abejama K, Kawahara K, Iino S, Otsubo Y, Saigo K, Izumi H, Hashiguchi T, Yamakuchi M, et al. (2003) Membrane cholesterol but not putative receptors mediates anandamide-induced hepatocyte apoptosis. *Hepatology* **38**:1167–1177.
- Calln L, Moreno E, Barroso-Chinea P, Moreno-Delgado D, Corts A, Mallol J, Casad V, Lanciego JL, Franco R, Lluis C, et al. (2012) Cannabinoid receptors CB1 and CB2 form functional heteromers in brain. *J Biol Chem* **287**:20851–20865.
- Carie AE and Sebt SM (2007) A chemical biology approach identifies a β_2 -adrenergic receptor agonist that causes human tumor regression by blocking the Raf-1/Mek-1/Erk1/2 pathway. *Oncogene* **26**:3777–3788.
- Cheng D, Ren J, and Jackson EK (2010) Multidrug resistance protein 4 mediates cAMP efflux from rat preglomerular vascular smooth muscle cells. *Clin Exp Pharmacol Physiol* **37**:205–207.
- Copel S, Garcia C, Diez F, Vermeulen M, Baldi A, Bianciotti LG, Russel FG, Shayo C, and Davio C (2011) Multidrug resistance protein 4 (MRP4/ABCC4) regulates cAMP cellular levels and controls human leukemia cell proliferation and differentiation. *J Biol Chem* **286**:6979–6988.
- Curran NM, Griffin BD, O'Toole D, Brady KJ, Fitzgerald SN, and Moynagh PN (2005) The synthetic cannabinoid R(+)-WIN 55,212-2 inhibits the interleukin-1 signaling pathway in human astrocytes in a cannabinoid receptor-independent manner. *J Biol Chem* **280**:35797–35806.
- Evans BA, Sato M, Sarwar M, Hutchinson DS, and Summers RJ (2010) Ligand-directed signalling at β -adrenoceptors. *Br J Pharmacol* **159**:1022–1038.
- Fan G, Shumay E, Malbon CC, and Wang H (2001) c-Src tyrosine kinase binds the β_2 -adrenergic receptor via phospho-Tyr-350, phosphorylates G-protein-linked receptor kinase 2, and mediates agonist-induced receptor desensitization. *J Biol Chem* **276**:13240–13247.

- Gardiner SM, March JE, Kemp PA, and Bennett T (2005) Involvement of CB1-receptors and β -adrenoceptors in the regional hemodynamic responses to lipopoly-saccharide infusion in conscious rats. *Am J Physiol Heart Circ Physiol* **288**: H2280–H2288.
- Guindon J and Hohmann AG (2011) The endocannabinoid system and cancer: therapeutic implication. *Br J Pharmacol* **163**:1447–1463.
- Haack KK, Tougas MR, Jones KT, El-Dahr SS, Radhakrishna H, and McCarty NA (2010) A novel bioassay for detecting GPCR heterodimerization: transactivation of β 2 adrenergic receptor by bradykinin receptor. *J Biomol Screen* **15**:251–260.
- Hermanson DJ and Marnett LJ (2011) Cannabinoids, endocannabinoids, and cancer. *Cancer Metastasis Rev* **30**:599–612.
- Hudson BD, Hébert TE, and Kelly ME (2010) Physical and functional interaction between CB1 cannabinoid receptors and β 2-adrenoceptors. *Br J Pharmacol* **160**: 627–642.
- Jozwiak K, Khalid C, Tanga MJ, Berzetei-Gurske I, Jimenez L, Kozocas JA, Woo A, Zhu W, Xiao RP, Abernethy DR, et al. (2007) Comparative molecular field analysis of the binding of the stereoisomers of fenoterol and fenoterol derivatives to the β 2 adrenergic receptor. *J Med Chem* **50**:2903–2915.
- Jozwiak K, Woo AY, Tanga MJ, Toll L, Jimenez L, Kozocas JA, Plazinska A, Xiao RP, and Wainer IW (2010) Comparative molecular field analysis of fenoterol derivatives: a platform towards highly selective and effective β 2-adrenergic receptor agonists. *Bioorg Med Chem* **18**:728–736.
- Kahsai AW, Xiao K, Rajagopal S, Ahn S, Shukla AK, Sun J, Oas TG, and Lefkowitz RJ (2011) Multiple ligand-specific conformations of the β 2-adrenergic receptor. *Nat Chem Biol* **7**:692–700.
- Kopp WK and Wersto RP (1992) Flow cytometry in the monitoring of therapeutic trials, in *Manual of Clinical Laboratory Immunology*, 4th ed (Rose NR, DeMacario EC, Fahey JL, Friedman H, and Penn GM, eds) pp 933–941, ASM Press, Washington, DC.
- Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, et al. (1999) β -Arrestin-dependent formation of β 2 adrenergic receptor-Src protein kinase complexes. *Science* **283**:655–661.
- Milligan G (2009) G protein-coupled receptor hetero-dimerization: contribution to pharmacology and function. *Br J Pharmacol* **158**:5–14.
- Nicolas C, Lacasa D, Giudicelli Y, Demarne Y, Agli B, Lecourtier MJ, and Lhuillery C (1991) Dietary (n-6) polyunsaturated fatty acids affect β -adrenergic receptor binding and adenylate cyclase activity in pig adipocyte plasma membrane. *J Nutr* **121**:1179–1186.
- Oesch S and Gertsch J (2009) Cannabinoid receptor ligands as potential anticancer agents—high hopes for new therapies? *J Pharm Pharmacol* **61**:839–853.
- Osman NA, Mahmoud AH, Allarà M, Niess R, Abouzid KA, Di Marzo V, and Abadi AH (2010) Synthesis, binding studies and molecular modeling of novel cannabinoid receptor ligands. *Bioorg Med Chem* **18**:8463–8477.
- Palazuelos J, Aguado T, Egia A, Mechoulam R, Guzmán M, and Galve-Roperh I (2006) Non-psychoactive CB2 cannabinoid agonists stimulate neural progenitor proliferation. *FASEB J* **20**:2405–2407.
- Pertwee RG (2005) Inverse agonism and neutral antagonism at cannabinoid CB1 receptors. *Life Sci* **76**:1307–1324.
- Pertwee RG (2006) The pharmacology of cannabinoid receptors and their ligands: an overview. *Int J Obes (Lond)* **30** (Suppl 1):S13–S18.
- Sastry KS, Karpova Y, Prokopovich S, Smith AJ, Essau B, Gersappe A, Carson JP, Weber MJ, Register TC, Chen YQ, et al. (2007) Epinephrine protects cancer cells from apoptosis via activation of cAMP-dependent protein kinase and BAD phosphorylation. *J Biol Chem* **282**:14094–14100.
- Seifert R and Dove S (2009) Functional selectivity of GPCR ligand stereoisomers: new pharmacological opportunities. *Mol Pharmacol* **75**:13–18.
- Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O, and Lefkowitz RJ (2006) β -Arrestin-dependent, G protein-independent ERK1/2 activation by the β 2 adrenergic receptor. *J Biol Chem* **281**:1261–1273.
- Tao J and Malbon CC (2008) G-protein-coupled receptor-associated A-kinase anchoring proteins AKAP5 and AKAP12: differential signaling to MAPK and GPCR recycling. *J Mol Signal* **3**:19.
- Toll L, Jimenez L, Waleh N, Jozwiak K, Woo AY, Xiao RP, Bernier M, and Wainer IW (2011) β 2-Adrenergic receptor agonists inhibit the proliferation of 1321N1 astrocytoma cells. *J Pharmacol Exp Ther* **336**:524–532.
- Uberti MA, Hague C, Oller H, Minneman KP, and Hall RA (2005) Heterodimerization with β 2-adrenergic receptors promotes surface expression and functional activity of α 1D-adrenergic receptors. *J Pharmacol Exp Ther* **313**:16–23.
- Valant C, Robert Lane J, Sexton PM, and Christopoulos A (2012) The best of both worlds? Bitopic orthosteric/allosteric ligands of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **52**:153–178.
- Wang XQ, Lindberg FP, and Frazier WA (1999) Integrin-associated protein stimulates 21-dependent chemotaxis via Gi-mediated inhibition of adenylate cyclase and extracellular-regulated kinases. *J Cell Biol* **147**:389–400.
- Wielinga PR, van der Heijden I, Reid G, Beijnen JH, Wijnholds J, and Borst P (2003) Characterization of the MRP4- and MRP5-mediated transport of cyclic nucleotides from intact cells. *J Biol Chem* **278**:17664–17671.
- Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, Shenoy SK, and 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.
- Woo AY, Wang TB, Zeng X, Zhu W, Abernethy DR, Wainer IW, and Xiao RP (2009) Stereochemistry of an agonist determines coupling preference of β 2-adrenoceptor to different G proteins in cardiomyocytes. *Mol Pharmacol* **75**:158–165.
- Wrzal PK, Devost D, Pétrin D, Goupil E, Iorio-Morin C, Laporte SA, Zingg HH, and Hébert TE (2012) Allosteric interactions between the oxytocin receptor and the β 2-adrenergic receptor in the modulation of ERK1/2 activation are mediated by heterodimerization. *Cell Signal* **24**:342–350.
- Wu WJ, Yang Q, Cao QF, Zhang YW, Xia YJ, Hu XW, and Tang WX (2010) [Membrane cholesterol mediates the endocannabinoids-anandamide affection on HepG2 cells.] *Zhonghua Gan Zang Bing Za Zhi* **18**:204–208.
- Yang Q, Liu HY, Zhang YW, Wu WJ, and Tang WX (2010) Anandamide induces cell death through lipid rafts in hepatic stellate cells. *J Gastroenterol Hepatol* **25**:991–1001.
- Yuan A, Li Z, Li X, Yi S, Wang S, Cai Y, and Cao H (2010) The mitogenic effectors of isoproterenol in human hepatocellular carcinoma cells. *Oncol Rep* **23**:151–157.
- Zhang D, Ma QY, Hu HT, and Zhang M (2010) β 2-Adrenergic antagonists suppress pancreatic cancer cell invasion by inhibiting CREB, NF κ B and AP-1. *Cancer Biol Ther* **10**:19–29.
- Zhang P, He X, Tan J, Zhou X, and Zou L (2011) β -Arrestin2 mediates β -2 adrenergic receptor signaling inducing prostate cancer cell progression. *Oncol Rep* **26**:1471–1477.

Address correspondence to: Michel Bernier, Laboratory of Clinical Investigation, National Institute on Aging, National Institutes of Health, 251 Bayview Boulevard, Suite 100, Baltimore, MD 21224-6825. E-mail: bernierm@mail.nih.gov