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Cannabinoid receptor activation correlates with the pro-apoptotic action of the β2-adrenergic agonist, (R,R')-4-methoxy-1-naphthylfenoterol, in HepG2 hepatocarcinoma cells

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Running Title: CB receptor activation by 4-methoxy-1-naphthylfenoterol

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List of nonstandard abbreviations: Fen, (R,R')-fenoterol; β_2-AR, $\beta_{\square}$-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-napthalenylmethanone; 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.

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Abstract

Inhibition of cell proliferation by fenoterol and fenoterol derivatives in 1321N1 astrocytoma cells is consistent with beta2-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 using Western blotting and [^{3}H]-thymidine incorporation assays. Despite expression of β_2-AR, no cAMP accumulation was observed when cells were stimulated with isoproterenol or Fen, although the treatment elicited both MAPK and PI3K/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 ICI118,551, a β2-AR antagonist, while those of MNF were unaffected. Because of the co-expression of β_2-AR and cannabinoid receptors (CBRs) and their impact on HepG2 cell proliferation, these Gαi/o-linked receptors may be implicated in MNF signaling. Cell treatment with WIN 55,212-2, a synthetic agonist of the CB_1R and CB_2R, 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 anti-proliferative actions of MNF and WIN 55,212-2. The data indicates 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 β2-AR–CBR ligand.

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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 HEK cells expressing human β_2-AR (Jozwiak et al., 2010). We have recently 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; Jozwiak et al., 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} 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 modulate 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 upon 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 recently shown that the G_s/G_i selectivity of Fen is a function of molecular structure and stereochemistry as Fen preferentially activated G_S signaling in a cardiomyocyte contractility model while (S,R')-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 anti-proliferative effects in the human-derived 1321N1 astrocytoma cell line specifically through the cAMP-dependent pathway (Toll et al., 2011) while Yuan and colleagues reported that isoproterenol dose-dependently induced the growth of the human-derived HepG2 hepatocellular carcinoma cell line (Yuan et al., 2010). This cell type-specific divergence on cell proliferation by β_2-AR agonists raises questions about whether

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there is crosstalk 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 (Uberti et al., 2005; Haack et al., 2010; Wrzal et al., 2012; Hudson et al., 2010). Of significance, CBRs have been reported to modulate β_2-AR activity (Gardiner et al., 2005). The two known cannabinoid receptors, CB_1R and CB_2R, are coupled to Gαi/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 CB_1R and CB_2R (Wu et al., 2010). Treatment with selective pharmacological CB_2R 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 co-expressed and can impact 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 [^{3}H]-thymidine incorporation in HepG2 cells and to compare these results to similar studies conducted using the 1321N1 and human-derived U87MG glioblastoma cells lines. Here we report that proliferative responses that occur with Fen and isoproterenol were opposite to that induced by MNF, even though the latter fenoterol analog is also a full and potent β_2-AR agonist. Unexpectedly, we showed 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 previously described (Jozwiak et al., 2007; Jozwiak et al., 2010). [^{3}H]-Thymidine (70-90 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Eagle's Minimum Essential Medium (E-MEM), trypsin solution, phosphate-buffered saline (PBS), fetal bovine serum (FBS), 100X 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). WIN 55,212-2, AM251, and AM630 were purchased from Cayman Chemical (Ann Arbor, MI). 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. (Cat. No. ADI-905-742-100, Farmingdale, NY) and Abcam (Cat. No. ab69598 and ab40834, Cambridge, MA). Rabbit anti-phospho-Akt (Ser-473), phospho-ERK1/2, total Akt and total ERK2 were from Cell Signaling Technology (Beverly, MA), while anti-β-actin was purchased from Abcam. Rat anti-MRP4 (sc-59614) and goat anti-MRP5 (sc-5781) 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 (ATCC, Manassas, VA) were maintained in EMEM medium supplemented with 1% L-glutamine, 1% sodium pyruvate, 1% penicillin/streptomycin, and 10% FBS (Hyclone, Logan, UT). The human 1321N1 astrocytoma cells (European Collection of Cell Cultures, 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 ºC in 5% CO_2, and the medium was replaced every 2-3 days. The passage numbers of the cell lines employed ranged from 15-30.

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Unless otherwise indicated, cells at 70-80% confluency 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.

[^{3}H]-Thymidine Incorporation Assay. Cells were seeded in 12-well plates at approximately 50,000 cells/well and incubated at 37 ºC. 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 ºC, 1 µCi of [^{3}H]-thymidine was added to each well and incubated at 37 ºC for 16 h. [^{3}H] 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, Inc., Brea, CA), and radioactivity was measured by liquid scintillation counting using Beckman Coulter LS6000IC Scintillation Counter. Data are shown as cpm incorporated compared to the control cells.

cAMP Accumulation. HepG2 cells were seeded in 96-well plates and grown to confluency. Cells were rinsed in Krebs-HEPES buffer, pH 7.4, pre-incubated for 20 min with the buffer supplemented with 50 µM of IBMX, a phosphodiesterase inhibitor, and then 1 µM isoproterenol, Fen or MNF was added for an additional 10 min. In some experiments, the multidrug resistance protein (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 previously described (Toll et al., 2011).

RNA Interference. HepG2 and 1321N1 cells were plated at a density of 3 x 10^5 cells/well in 6-well cell culture plates and incubate for 48 h. Transfection of the cells was carried out in Lipofectamine™ RNAiMAX reagent (Life Technologies, Invitrogen) with 50 nM each of a combination of three siRNAs (s1121 and s1122, cat. No. 4392420 from Applied Biosystems, and sc-39866 from Santa Cruz) targeted against human β_2-AR or a non-silencing siRNA control (sc-37007, Santa Cruz). Forty-eight h later, cell lysates were prepared and immunoblotted for β_2-AR by Western blotting.

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RNA Extraction, cDNA Synthesis, and RT-PCR Analysis. Total RNA was isolated from HepG2, 1321N1 and U87MG cells using the RNeasy Mini kit (Qiagen, Valencia, CA). The RNA preparation included a DNAse digestion step. RNA concentration and quality was measured using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). To obtain cDNA, 1 μg total RNA was reverse-transcribed using the Promega reverse transcription kit (Promega Corp., Madison, WI). PCR reactions were performed to determine the expression of CB_1R, CB_2R, and β_2-AR mRNAs using GAPDH as 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-stained nuclei prepared by the NIM technique (Kopp and Wersto, 1992). DNA histograms of at least 10,000 cells acquired on a Becton-Dickinson FACScanto II (BD Biosciences, San Jose, CA) were deconvoluted using the Multicycle program (Phoenix Flow Systems) 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. Briefly, HepG2 cells (5×10^5) were grown on 100mm 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 μL of 1X annexin-binding buffer to maintain a density ~1×10^6 cells/mL. 5 μL Alexa Fluor® 488 annexin V and 1 μL 100 μg/mL propidium iodide were added to the cell suspensions. Cells were then incubated at room temperature for 15 min and 400 μL 1X annexin-binding buffer was added followed by gentle mixing. Stained cells were analyzed on a BDFACSCanto II flow cytometer.

Western Blotting. Cells were lysed with RIPA 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 using the bicinchoninic acid reagent (Thermo-Pierce Biotechnology, Inc., Rockford, IL). Proteins (20 μg/well) were separated on 4 to 12% precast gels

(Invitrogen, Carlsbad, CA) using SDS-polyacrylamide gel electrophoresis under reducing conditions and were 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 using the ECL Plus Western Blotting Detection System (GE Healthcare, NJ) and their quantification was done by volume densitometry using ImageJ software (NIH, 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 ± S.E. Statistical comparisons between groups were made by *t* test. Analyses were performed using the SigmaPlot Software (Systat Software, Inc. San Jose, CA), Graphpad Prism 4 (GraphPad Software, Inc., La Jolla, CA) and Microsoft® Office Excel, 2003 (Microsoft Corp., Redmond, WA), with p values ≤ 0.05 considered significant.

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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. Semi-quantitative PCR analysis indicated higher expression of β_2-AR mRNA in 1321N1 cells when compared to HepG2 cells (Supplemental Fig. 1A). Accordingly, 1321N1 cells expressed more β_2-AR protein than HepG2 cells, when three commercial antibodies were tested using Western blot technique (Supplemental Fig. 1B). As indicated in Figure 1A, the knockdown of β_2-AR expression by siRNA-based approach led to ~70% reduction in the level of β_2-AR protein, validating the specificity of these antibodies. U87MG cells were previously found 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. Neither isoproterenol, Fen nor MNF at 1.0 μM elicited an increase in cAMP production in HepG2 cells, whereas cell treatment with the adenylyl cyclase activator, forskolin, induced 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 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 export of cAMP plays little role, if any, in the apparent lack of effect of β2-AR agonists on cAMP accumulation in HepG2 cells. Rather, this behavior may indicate uncoupling between

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agonist-stimulated β_2-AR and Gαs protein. Alternatively, due to the low number of β_2-AR 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) independent 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 imunoblotting 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, likely through the coupling to different Gα 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 EC_{50} of 0.40 ± 0.08 μM and 1.17 ± 0.37 μM, respectively (Table 1; Fig. 2A). Yuan et al. (2010) reported similar potency of isoproterenol toward HepG2 cell proliferation, where saturation was reached at 10 μM of the β-AR agonist. The EC_{50} value reported here for isoproterenol was 400-fold lower than 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 has 4 possible steroisomeric forms, (R,R'), (R,S'), (S,R') and (S,S'). The effect of the stereochemistry on the proliferative effect of Fen was determined using a concentration of 1 μM of each isomer. The data indicate that all of the isomers induced an increase in [^{3}H]-thymidine incorporation and that stereochemistry had only a quantitative effect on this process, with Fen producing

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the greatest increase (51.3%) and (S,S')-fenoterol 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') ≈ (S,R') >> (S,S') (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 [^{3}H]-thymidine incorporation and (R,R')-aminofenoterol appeared to be 3-fold more active than Fen with an EC_{50} = 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 [^{3}H]-thymidine incorporation with IC_{50} values of 0.39 ± 0.09 μM 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 anti-proliferative response as 1 μM concentrations of MNF and (R,S')-4-methoxy-1-naphythylfenoterol produced equivalent decreases in [^{3}H]-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 HEK cells stably transfected with β_2-AR and in 1321N1 cells, with EC_{50} of 3.9 nM and 68.9 nM, respectively (Jozwiack et al., 2010; Toll et al., 2011). Since HepG2 cells displayed substantial sensitivity to (R,R')-aminofenoterol (EC_{50} = 0.47 ± 0.09 μM) and MNF (IC_{50} = 0.39 ± 0.09 μM) with regard to [^{3}H]-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 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 while Fen had no effect (Supplemental Fig. 2).

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In the previous study of the effect of isoproterenol and Fen on mitogenesis in 1321N1 cells, the experiments were conducted using complete medium. In order to determine if 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 anti-proliferative 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. While ICI 118,551 alone showed a modest, but significant concentration-dependent increase in cell proliferation, up to ~16% at 1 μM (Fig. 3A), its addition markedly blocked Fen-stimulated mitogenesis (Fig. 3B and 3C). However, the anti-proliferative effect of MNF was refractory to ICI 118,551 pretreatment (Fig. 3B and 3D).

We then tested the possibility that the action of Fen could be hampered by the co-addition 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 anti-proliferative effects of MNF (Supplemental Fig. 2, upper panel). However, characteristics of the cell proliferation profile elicited by Fen in 1321N1 cells and MNF in U87MG cells were maintained by the co-treatment with Fen and MNF (Supplemental Fig. 2, middle and lower panels). As expected, pretreatment with ICI 118,551 blocked Fen signaling in 1321N1 cells while being inactive against the anti-proliferative 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 activation of distinct receptors.

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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 (G2/M: 13.8 ± 1.1% in control *versus* 10.2 ± 0.9% after 6 h, 14.6 ± 1.8% after 12 h and 8.9 ± 1.6% after 24 h; S: 34.7 ± 0.3% in control *versus* 34.1 ± 0.9% after 6 h, 13.7 ± 1.2% after 12 h and 24.6 ± 4.2% after 24 h) in HepG2 cells treated with 1 μM 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 ± 0.7% by 12 h (Fig. 4, bottom, right panel). No significant increase in sub-G_1 events was observed when cells were treated with Fen (1 μM) 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 apoptotic cells induced by a 24-h treatment with MNF (1 μM) was increased 5.7-fold as compared to control ($P < 0.01$). However, Fen treatment reduced apoptosis when compared to control untreated cells (Fig. 5).

Role of cannabinoid receptors in the control of cell proliferation of MNF and Fen. Because of the co-expression 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 CB_1R and CB_2R were determined by RT-PCR in HepG2, 1321N1 and U87MG cells (Fig. 6A). The results indicated that HepG2 and U87MG cells expressed CB_1R and CB_2R, 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 as compared with controls. Similar to MNF, treatment of HepG2 cells with the cannabinoid receptor agonist, WIN55,212-2 (1μM), reduced cell proliferation and canceled out the growth-promoting action of Fen (Fig. 6B). AM251 and AM630 are synthetic inverse agonists for CB_1R and CB_2R, respectively (Pertwee, 2005). We observed that cell

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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 anti-proliferative effects of MNF in HepG2 cells (Fig. 6C), which is consistent with the 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. 3A and 3B). However, the anti-proliferative effects of MNF were partially blocked by AM251 and AM630 in the β_2-AR-deficient U87MG cells (Supplemental Fig. 3C and 3D).

Coupling of MNF to cell proliferation seemed to be dependent on Gαi/o proteins, because the MNF and WIN55,212-2 anti-proliferative 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.

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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 as both β_2-AR antagonists and agonists have been shown to attenuate cell growth (Zhang et al., 2010; Carie and Sebti, 2007; Toll et al., 2011). In the case of β_2-AR agonists, the effect appears to be through cAMP-dependent pathways and is subject to antagonism by ICI 118,551 (Carie and Sebti, 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).

In order to explore the inter-cellular differences in the pro– and anti–survival 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 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 due to 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 while the β_2-ARs are poorly coupled to the stimulatory Gα protein, they may exhibit functional interactions with other signaling intermediates that promote cell growth. Our results demonstrate that Fen activated the PI3-kinase/Akt and ERK

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pathways in HepG2 cells. In support to 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 MAPK/ERK pathway (Luttrell et al., 1999; Ahn 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 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 produces 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 as all of the Fen stereoisomers produced an increase in [^{3}H]-thymidine incorporation (reported as % change) with (R,R') >> (S,R') ≈ (R,S') >> (S,S') (Table 1). The same result was produced by the Fen analogs (R,R')-ethylfenoterol and (R,R')-aminofenoterol (Table 1 and 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 that 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 and 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 anti-proliferative response (Table 1).

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Since 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 that binding 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 (CoMFA) studies of the interaction of the Fen analogs with the β_2-AR have indicated that the naphthyl substituent of the NFs 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; Jozwiak et al., 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, as 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 in cell growth while Fen had no effect (Supplemental Fig. 2). This data does 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 suggests that other GPCRs may be involved in the anti-proliferative effects of MNF. One potential target is the CBRs as previous studies have suggested that CBRs modulate β_2-AR activity (Gardiner et al., 2005). Thus, while 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 CB_2R through π-π and hydrophobic interactions (Osman et al., 2010). It is interesting to note that the CB_2R model used in the study by Osman et al. (2010) was constructed based upon the structure of the β_2-AR. Therefore, it is likely that the extent of β_2-AR and CBR expression

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within a given cell line may influence the overall growth-promoting properties and survival function of Fen vs. MNF.

We now report pharmacological evidence to suggest that MNF mediates its anti-proliferative effects through activation of the CBRs. First, WIN 55,212-2 mimicked this MNF response and the combination, MNF plus WIN 55,212-2, showed lack of additive effect. Moreover, selective inhibition of the CB_1R and CB_2R 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 when compared to HepG2 and U87MG cell lines (Biswas et al., 2003; Wu et al., 2010; Aguado et al., 2007 Curran et al., 2005) and (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 $G\alpha_i$-linked CBRs in response to WIN 55,212-2 inhibits cell growth primarily by negatively targeting the PI3-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 anti-mitogenic 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 (Guindon and Hohmann, 2011; Hermanson and Marnett, 2011; Oesch and Gertsch, 2009). 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 (CB_1R vs. CB_2R), 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 inhibition of either CB_1R or CB_2R antagonizes MNF response, which probably results from the ability of these receptors to form functional heteromers (Callén et al., 2012). The co-expression of β2-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 β2-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).

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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

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Footnotes

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Figure Legends

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 μM), but not with 1 μM of either isoproterenol (Iso), Fen, or MNF. Data shown are from a single experiment conducted in quadruplicate. Error bars indicate mean ± S.D. C, Serum-starved HepG2 cells were incubated in the presence of isoproterenol (Iso; 1 μM) or Fen (1 μM) for 5, 10 and 30 min. Cell lysates were immunoblotted with antibodies against phosphorylated (Ser473) and total Akt, as well as phosphorylated 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.

Fig. 2. The effects of isoproterenol, Fen and fenoterol derivatives on cell growth are cell-type specific. A-B, Serum-starved HepG2 cells were incubated with vehicle or the indicated concentrations of isoproterenol (Iso), Fen, (R,R')-aminofenoterol (NH_2-fen) or MNF for 24 h, and levels of [^{3}H]-thymidine incorporation was 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 μM 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 percent change in [^{3}H]-thymidine incorporation *versus* control are expressed as means ± SE and represent results from 2-6 independent experiments, each performed in triplicate dishes. In most instances, error bars are smaller than the symbols.

Fig. 3. β_2-AR antagonist does not inhibit the anti-proliferative action of MNF in HepG2 cells. Serum-

depleted HepG2 cells were incubated with the indicated concentrations of the β-AR antagonist, ICI-118,551 (ICI), for 1 h followed by the addition of vehicle (A), Fen (B, left panel), or MNF (B, right panel) for 24 h, and levels of [^{3}H]-thymidine incorporation was measured. * $P < 0.05$. Representative concentration-response curves for Fen and MNF are shown (B). C and D, Quantification of percent change in [^{3}H]-thymidine incorporation *vs.* control are expressed as means ± SE and represent results from 3 independent experiments, each performed in triplicate dishes.

Fig. 4. MNF increases the number of sub-G1 events in HepG2 cells. Serum-depleted HepG2 cells were harvested after 6 h, 12 h and 24 h treatment with vehicle, Fen (1 μM) or MNF (1 μM). Cells were fixed, stained and then analyzed for DNA content using flow cytometry. Representative DNA content analysis in various phases of the cell cycle after 24 h treatment with vehicle, Fen, or MNF are shown. The number of sub-G1 events, which displays cells in late stage apoptosis or already dead, in function of treatment duration was quantified and represents results from 2 independent experiments, each performed in duplicate dishes (lower right panel). Data are expressed as means ± SE (n=4).

Fig. 5. MNF induces apoptosis in HepG2 cells. Serum-depleted HepG2 cells were treated with vehicle, Fen (1 μM), or MNF (1 μM) for 24 h, stained with Annexin V and propidium iodide (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 2 independent experiments, each performed in duplicate dishes (lower right panel). Data are expressed as means ± SE (n=4).

Fig. 6. Role of CBR activation in the anti-proliferative action of MNF in HepG2 cells. A, Total RNA was extracted from HepG2, 1321N1 and U87MG cells, and then analyzed semi-quantitatively by PCR. A non-template control (NTC) has been included (lane 1). B and C, Serum-depleted HepG2 cells were incubated with the CBR agonist, WIN 55,212-2 (Win; 1 μM), (B) or antagonists, AM251 (1 μM) or AM630 (0.5 μM), (C) for 1 h followed by the addition of vehicle, Fen (0.5 μM), or MNF (0.25 μM) for 24 h. D,

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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 μM), MNF (0.5 μM) or WIN 55,212-2 (0.5 μM) for 24 h. B-D, Levels of [^{3}H]-thymidine incorporation was measured. Quantification of percent change in [^{3}H]-thymidine incorporation *vs.* control are expressed as means ± SD and represent results from 3 (B, C) or 2 (D) independent experiments, each performed in triplicate dishes. * $P < 0.05$.

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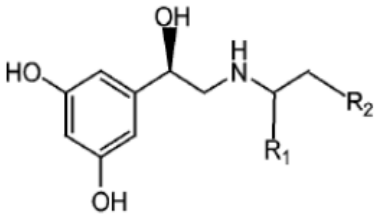
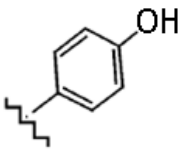
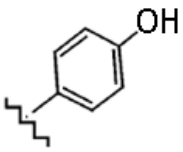
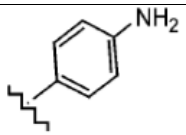
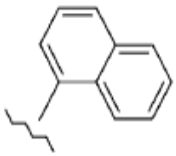
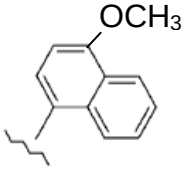
Table 1. Structures, percent change in thymidine incorporation and IC_{50}/EC_{50} of fenoterol and analogs that were used for this study



Compounds	R1	R2	IC_{50}/EC_{50} (μM)	% Change in HepG2 cells	Mitogenesis Inhibition in 1321N1 cells (IC_{50} nM)*
fenoterol	CH_3		1.17±0.37 (n=6)	(R,R): 51.3 (R,S): 19.1 (S,R): 28.7 (S,S): 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.	(R,R): 50.90 at 10 μM	1.44±0.27
aminofenoterol	CH_3		0.47±0.09 (n=3)	(R,R): 54.37	n.d.
1-naphthyl fenoterol	CH_3		0.21±0.07 (n=2)	(R,R): -67.52	1.57±0.34
4'-methoxy-1-naphthylfenoterol	CH_3		0.39±0.09 (n=6)	(R,R): -59.4 (R,S): -68.1	3.98±0.28 4.37±0.70

n.d.: not determined; *, from Table 2 in Toll et al. (2011).

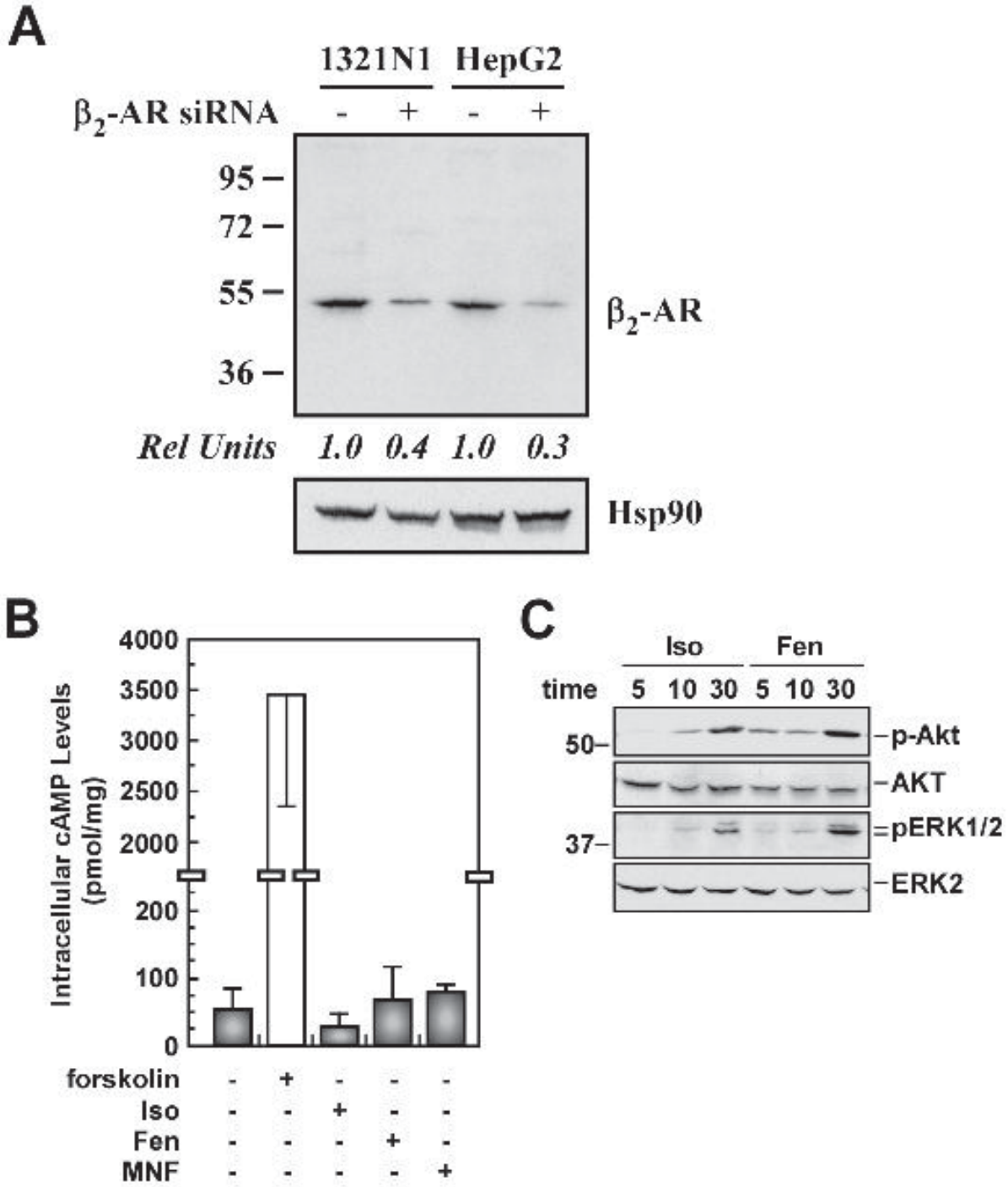


Fig. 1

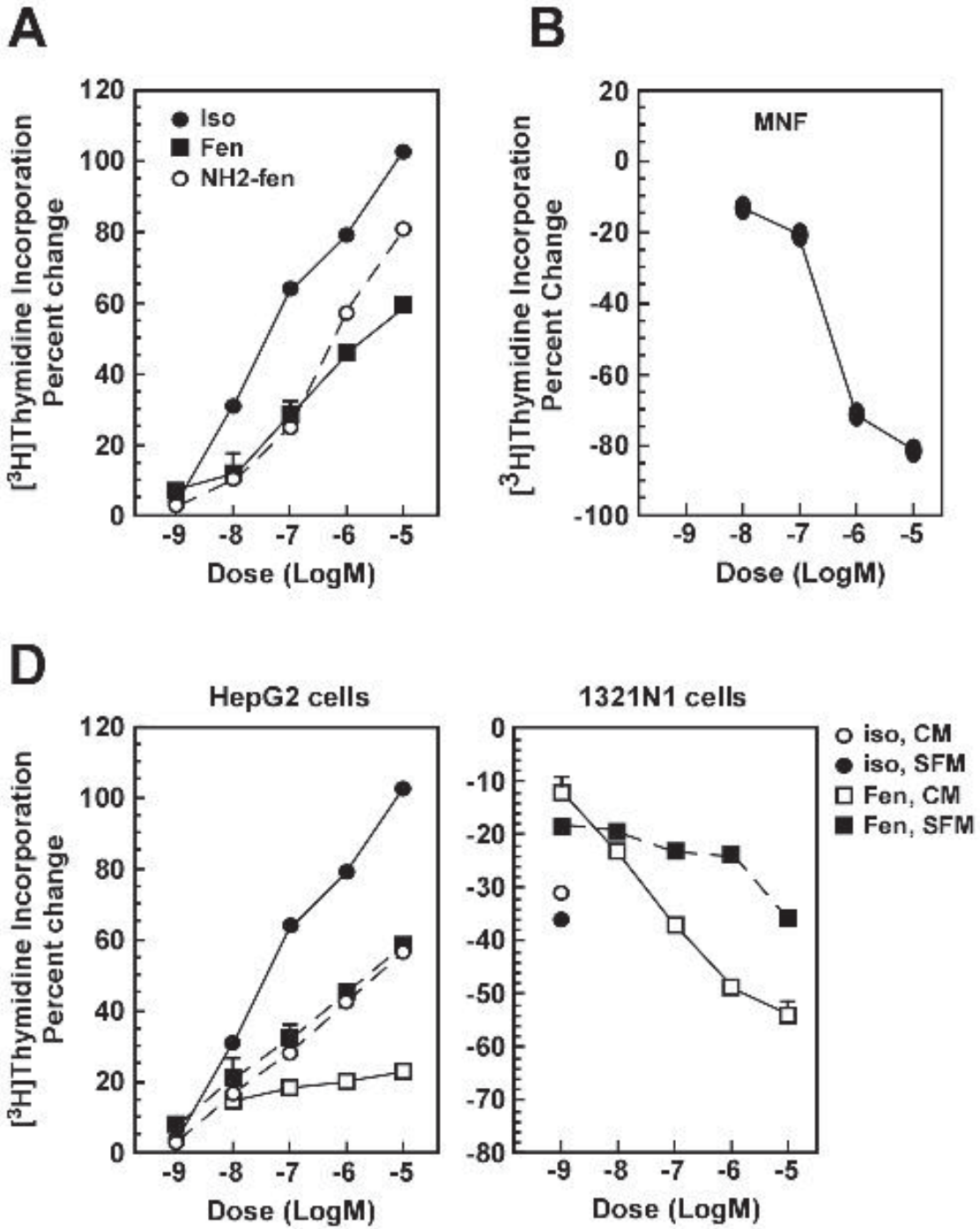


Fig. 2

Fig. 3

Fig. 4

Fig. 5

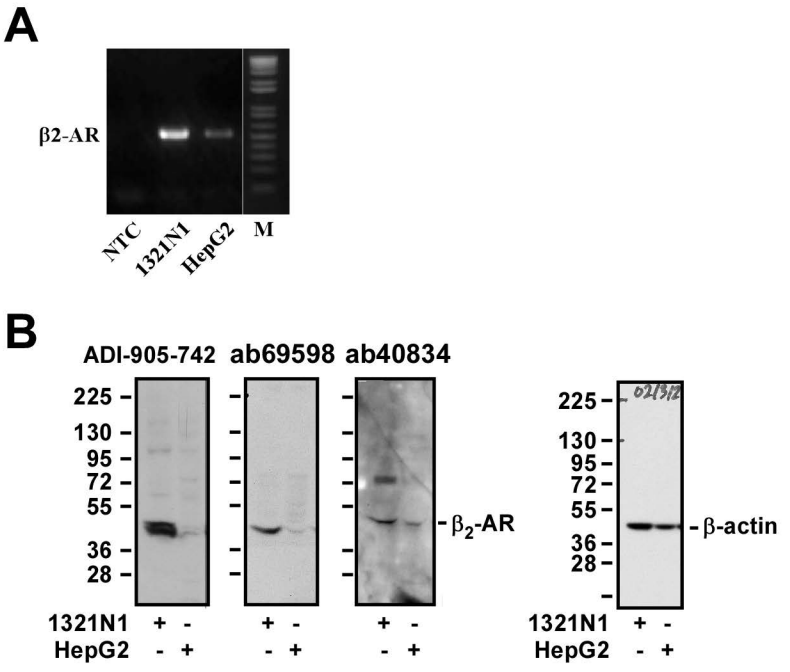
Fig. 6

Supplemental Information for manuscript # JPET/2012/195206

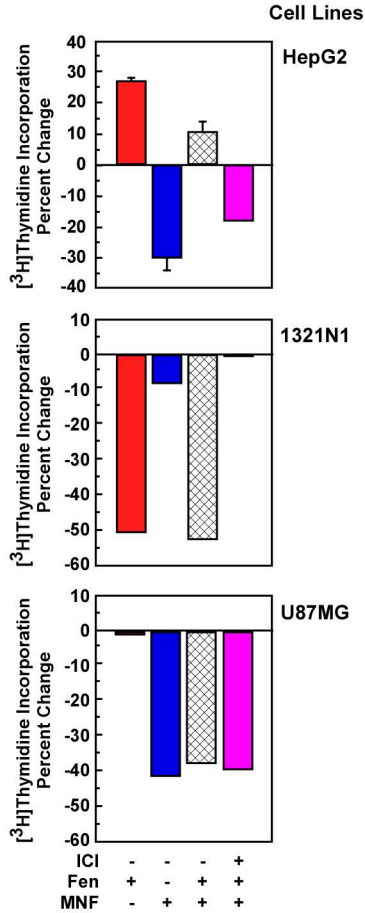
Title: Cannabinoid receptor activation correlates with the pro-apoptotic action of the β2-adrenergic agonist, (R,R')-4-methoxy-1-naphthylfenoterol in HepG2 hepatocarcinoma cells

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

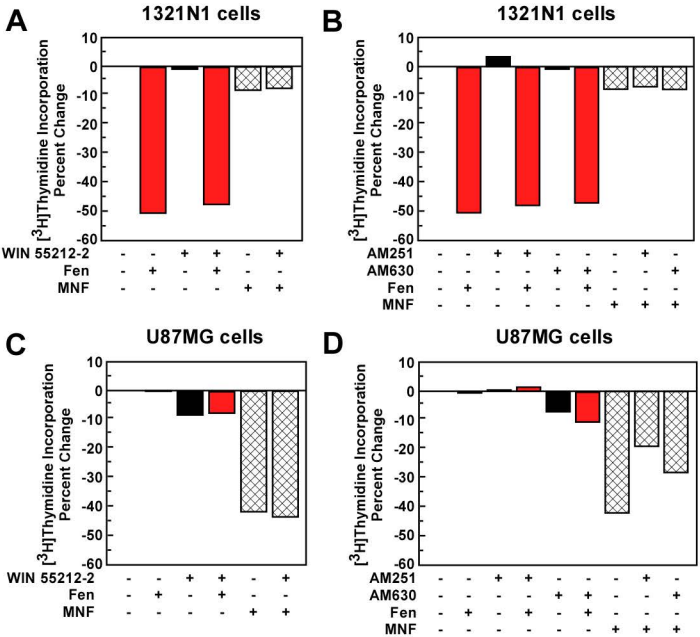
Journal: The Journal of Pharmacology and Experimental Therapeutics



Supplemental Fig. 1. Expression of β_2-AR in HepG2 and 1321N1 cells. A, Total RNA was extracted from HepG2 and 1321N1 cells, and then analyzed semi-quantitatively by PCR. A non-template control (NTC) has been included (lane 1). M, marker standards. B, Cell lysates were immunoblotted with three commercial anti-β_2-AR antibodies, using β-actin as a loading control. The positions of molecular mass markers (in kilodaltons) are shown to the left of the immunoblots.



Supplemental Fig. 2. Selective inhibition of Fen-mediated cell proliferation control by ICI-118,551 (ICI). HepG2, 1321N1 and U87MG cells were incubated with ICI (1 μM) for 1 h followed by the addition of vehicle, Fen (0.5 μM) or MNF (0.25 μM) for 24 h, and levels of [^{3}H]-thymidine incorporation were measured. Quantitation of percent change in [^{3}H]-thymidine incorporation *vs.* control are expressed as means +/- SD and represent the results from 3 independent experiments, each performed in triplicate dishes.



Supplemental Fig. 3. CBRs play no role in cell proliferation control by Fen. 1321N1 (A, B) and U87MG (C, D) cells were incubated either with the CBR agonist, WIN 55,212-2 (0.5-1 μM), or antagonists, AM251 (0.5-1 μM) or AM630 (0.25-0.5 μM) for 1 h followed by the addition of vehicle, Fen (0.5 μM) or MNF (0.25 μM) for 24 h, and levels of [^{3}H]-thymidine incorporation were measured. Quantitation of percent change in [^{3}H]-thymidine incorporation *vs.* control are expressed as means +/- SD and represent the results from 3 independent experiments, each performed in triplicate dishes.

Supplemental Table 1. List of PCR primers and assay conditions.

Human Gene	Primers	Ref #	Initial denaturation	Amplification (35 cycles)			Final extension
				Denaturation	Annealing	Extension	
CNR1	F:5′-CGTGGGCAGCCTGTTCCTCA R:5′-CATGCGGGCTTGGTCTGG	1	95°C/ 5 min	94°C/ 30 sec	57°C/ 30 sec	72°C/ 1 min	72°C/ 5 min
CNR2	F:5′-CGCCGGAAGCCCTCATACC R:5′-CCTCATTCGGGCCATTCCTG	1	95°C/ 5 min	94°C/ 30 sec	57°C/ 30 sec	72°C/ 1 min	72°C/ 5 min
GAPDH	F:5′-ACCACAGTCCATGCCATC R:5′-TCCACCACCCTGTTGCTG	2	94°C/ 4 min	94°C/ 1 min	53°C/ 1 min	72°C/ 1 min	72°C/ 10 min
ADRB2	F:5′-CATGTCTCTCATCGTCCTGGCCA R:5′-CACGATGGAAGAGGCAATGGCA	3	94°C/ 6 min	94°C/ 1 min	58°C/ 30 sec	72°C/ 1 min	72°C/ 5 min

1 Aguado et al., 2007

2 Curran et al., 2005

3 Lutgendorf SK, Cole S, Costanzo E, Bradley S, Coffin J, Jabbari S, Rainwater K, Ritchie JM, Yang M, and Sood AK. (2003) Stress-related mediators stimulate vascular endothelial growth factor secretion by two ovarian cancer cell lines. *Clin Cancer Res* **9:**4514-4521.