Review Article

Effect of Fenoterol Stereochemistry on the β2 Adrenergic Receptor System: Ligand-Directed Chiral Recognition

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Contribution to the Proceedings of the 22nd International Symposium on Chirality [ISCD 22]

ABSTRACT The β_2 adrenergic receptor (β_2-AR) is a model system for studying the ligand recognition process in G protein-coupled receptors. Fenoterol (FEN) is a β_2-AR selective agonist that has two centers of chirality and exists as four stereoisomers. Radioligand binding studies determined that stereochemistry greatly influences the binding affinity. Subsequent Van't Hoff analysis shows very different thermodynamics of binding depending on the stereoconfiguration of the molecule. The binding of (*S*,*x*′)-isomers is almost entirely enthalpy controlled whereas binding of (*R*,*x*′)-isomers is purely entropy driven. Stereochemistry of FEN molecule also affects the coupling of the receptor to different G proteins. In a rat cardiomyocyte contractility model, (*R*,*R*′)-FEN was shown to selectively activate G_s protein signaling while the (*S*,*R*′)-isomer activated both G_i and G_s protein. The overall data demonstrate that the chirality at the two chiral centers of the FEN molecule influences the magnitude of binding affinity, thermodynamics of local interactions within the binding site, and the global mechanism of β_2-AR activation. Differences in thermodynamic parameters and nonuniform G-protein coupling suggest a mechanism of chiral recognition in which observed enantioselectivities arise from the interaction of the (*R*,*x*′)-FEN stereoisomers with a different receptor conformation than the one with which the (*S*,*x*′)-isomer interacts. *Chirality 23:E1–E6, 2011.*

KEY WORDS: G protein-coupled receptors; β_2 adrenergic agonists; stereoselective binding; affinity; efficacy; Van't Hoff analysis

INTRODUCTION

Fenoterol (FEN) is a well known selective β_2 adrenergic receptor (β_2-AR) agonist that is used as a bronchodilator and tocolytic agent. FEN has two centers of chirality, Figure 1, and exists as four stereoisomers, (*R*,*R*′)-, (*R*,*S*′)-, (*S*,*R*′)-, and (*S*,*S*′) isomers. The marketed product is the racemic mixture of the (*R*,*R*′; *S*,*S*′)-FEN which was selected after initial development studies demonstrated that this racemic mixture was 9–20-fold more active than the (*R*,*S*′; *S*,*R*′)-FEN racemate.[1] Subsequent studies of the relative binding affinities of (*R*,*R*′)- and (*S*,*S*′)-FEN have been reported using rat erythrocytes[2] and membranes obtained from HEK293 cells stably transfected with β_2-AR cDNA, HEK-β_2-AR cells.[3] In the rat erythrocyte studies, the binding affinity, expressed as K_i value, for (*R*,*R*′)-FEN was 2880 nM while (*S*,*S*′)-FEN had no measurable specific binding, and in the studies conducted with membranes obtained from the HEK-β_2-AR cells the calculated K_i values were 350 nM (*R*,*R*′) and 27,800 nM (*S*,*S*′). The enantioselective differences in affinity between (*R*,*R*′)- and (*S*,*S*′)-FEN were also reflected in the efficacy of the two enantiomers in a rat cardiomyocyte contractility model and in the stimulation of cyclic adenosine monophosphate (cAMP) in the HEK-β_2-AR cells.[4,5] (*R*,*R*′)-FEN was a full agonist in both of these systems with EC_{50} values of 73 and 0.3 nM, respectively, while (*S*,*S*′)-FEN produced no significant effect in the rat cardiomyocyte model[4,5] and was a weak but full agonist in the HEK-β2-AR cells with an EC_{50} of 580 nM.[5]

Based on these results, a structure–activity relationship (SAR) study was initiated to elucidate the effect of stereochemistry and the structure of the N-alkyl portion of the FEN molecule on β_2-AR binding affinity and functional efficacy. In these studies, a number of FEN derivatives in various stereoisomeric configurations were synthesized using stereospecific methods.[4] The data from these studies were used to construct quantitative structure–activity relationships (3D-QSAR) models of binding affinity toward β2-AR,[4] which in turn were used to design and synthesize more potent FEN derivatives such as 4-methoxy-1-naphtyl-FEN, one of the most selective β_2-AR agonist ever reported.[5]

Our laboratories have extensively investigated the interactions between the stereoisomers of the N-alkyl-FEN

Contract grant sponsor: National Institute on Aging Intramural Research Program; Contract grant number: HHSN2712010000081
Contract grant sponsor: Foundation for Polish Science (FOCUS and TEAM Programs)
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Received for publication 31 October 2010; Accepted 17 March 2011
DOI: 10.1002/chir.20963
Published online 26 May 2011 in Wiley Online Library (wileyonlinelibrary.com).

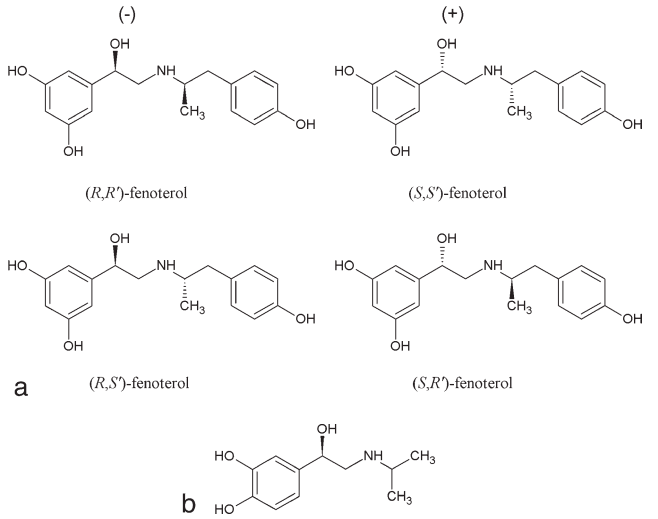

Fig. 1. (**a**) Stereochemistry of fenoterol; (*R*,*x*′)-diastereoisomers are levorotatory (–), (*S*,*x*′)-isomers are dextrorotatory (+).[4] (**b**) Chemical structure of (*R*)-(–)-isoproterenol.

derivatives and the β_2-AR, and this submission reviews an interesting aspect of these studies as they related to the stereoselective and enantioselective molecular interactions between the agonists and the β_2-AR. The data from these studies suggest that the previously described conformation-driven multistep mechanism does not adequately describe the enantioselective interactions of the N-alkyl-FEN agonists with the β_2-AR. This is due in part to the origin of the previous model, which was developed using compounds containing a single chiral center and small alkyl moieties on the N-alkyl portion of the agonist, such as the commonly used agent isoproterenol, Figure 1. The effects on molecular recognition produced by the presence of a second chiral center and aromatic substituents in the N-alkyl portion of the molecule are discussed below.

ENANTIOSELECTIVE BINDING OF AGONISTS TO THE β_2-AR

Binding of β_2-AR Agonists with a Single Chiral Center and Small N-alkyl Substituents

The β_2-AR is a member of the G protein-coupled receptor (GPCR) family. GPCRs are membrane-embedded proteins that contain transmembrane (TM) helices that cross the membrane seven times and which are often labeled as 7TM receptors. The receptors act as molecular switches, which transmit a variety of external signals into the interior of living cell through the initiation of a chain of intracellular events. In the case of the β_2-AR, the binding of an agonist such as isoproterenol produces conformation changes in the receptor which lead to G-protein activation, followed by the activation of adenyl cyclase, increased intracellular cAMP concentrations, and the triggering of further "downstream" signals such as kinase phosphorylation.[6]

The β_2-AR has emerged as a model system for the study of ligand recognition and activation of GPCRs using QSAR, molecular modeling, and site-directed mutation approaches.[7] The data from these studies have identified a key binding site for agonists such as (*R*)-isoproterenol, which is located between the TM helices TM3, TM5, and TM6, Figure 2. Within this site, agonists interact primarily with ASP113 (located on TM3), SER203, SER204 and SER207 (TM5), and PHE390 and ASN293 (TM6).

The binding and receptor activation mechanism initiated by binding at this site has been described as ligand-induced stepwise process in which at least three general steps can be distinguished: (1) binding of catechol-OH groups to TM5 and stabilization of TM5; (2) ion pair interaction of protonated amino moiety with ASP113 residue and stabilization of TM3, and (3) binding of β-hydroxyl group with TM6 and concerted shift of TM6 toward TM5 and TM3.[8] Parallel to the agonist binding process, a cascade of receptor's conformational transitions occurs what eventually leads to opening of the interface for G-protein recognition on the intracellular side of the β_2-AR (i.e., receptor activation). The structure and the ligand–β_2-AR complex developed using indirect methods was confirmed in 2007 with the determination of the crystal structure of the β_2-AR cocrystalized with the inverse agonist, (*S*)-carazolol[9] and by structures reported following the initial report[10] including recently described β_2-AR structure stabilized and crystallized in an active conformation.[11]

A primary structural feature of most β_2-AR agonists is the presence of a hydroxyl moiety on the β-carbon, see Figure 1. The importance of the chirality of the β-carbon stereocenter on agonist activity was initially demonstrated in 1908 by Cushny who determined that (*R*)-(–)-epinephrine constricted the blood vessels of frogs more effectively than its (*S*)-(+)-enantiomer.[12] The initial pharmacologic observations have been supported by numerous studies that have confirmed that the chirality of the β-OH carbon affects binding kinetics, affinities and agonist activities, see Ref. [12]. For example, results from site-directed mutation studies suggest that the differences in binding affinity and stimulation of adenylyl cyclase activity observed with (*R*)- and (*S*)-isoproterenol

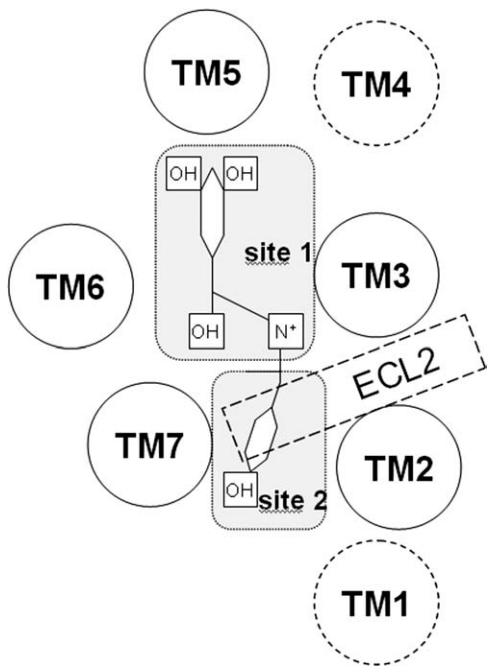

Fig. 2. Schematic representation of interactions of catecholamines with β_2-AR binding sites. Site 1 located between TM3, TM5, and TM6 of transmembrane section is a core binding pocket for compounds like (*R*)-(–)-epinephrine. Site 2 between TM3, TM7, and TM2 helices and capped from the top by ECL2 is auxiliary (allosteric) binding pocket where aminoalkyl moiety of FEN binds; site 2 is putatively responsible for β_2-AR selectivity of FEN. Figure adopted from work by Bokoch et al.[15]

TABLE 1. Binding affinities (K_i values) determined using [^{3}H]CGP-12177 displacement[4] and functional efficacies (EC_{50} values) determined for induced intracellular cAMP accumulation[5] for the stereoisomers of fenoterol: where, α_E is defined as the ratios of the K_i and EC_{50} values of the enantiomeric pairs and α_D is defined as the ratios of the K_i and EC_{50} values of the diasteromeric (*R,S*)/(*R,R*) and (*S,S*)/(*S,R*) pairs

Compound	K_i (nM)	α_E (*S,S′*)/(*R,R′*), (*S,R′*)/(*R,S′*)	α_D (*R,S′*)/(*R,R′*), (*S,S′*)/(*S,R′*)	EC_{50} (nM)	α_E (*S,S′*)/(*R,R′*), (*S,R′*)/(*R,S′*)	α_D (*R,S′*)/(*R,R′*), (*S,S′*)/(*S,R′*)
(*R,R′*)-FEN	345	80	11	0.3	1933	15.7
(*R,S′*)-FEN	3695	2.8		4.7	1.8	
(*S,R′*)-FEN	10,330		2.7	8.5		68
(*S,S′*)-FEN	27,750			580		

The data were obtained using cellular membranes and intact cells from a HEK293 cell line stably transfected with cDNA encoding the human β_2-AR.

were associated with enantioselective binding interactions between the β-OH moiety and the Asn-293 residue in TM6 with the *R*-configuration producing the more favorable complex.[13] Another description of the effect of the chirality of the β-OH carbon on agonist activities and binding kinetics was 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}) and that stereochemistry of the agonist plays a role in the conformational response to binding.[14] In these studies, changes in the spectra of fluorescence-labeled β_2-ARs were used to demonstrate that (*R*)-isoproterenol and (*R*)-norepinehprhine produced and/or stabilized different conformational states than the corresponding (*S*)-enantiomers. However, unlike the site-directed mutation studies, these studies were unable to determine whether the β-OH moiety of (*S*)-norepinephrine and (*R*)-norepinephrine bound to the same amino acid side chain in the β_2-AR.[14]

Binding of FEN Analogs to the β2-AR Agonists with Two Chiral Centers and Large N-alkyl Substituents

The binding of FEN to the β_2-AR has been investigated using a series of 32 FEN analogs in which the primary alteration was in the aromatic substituent of the N-aminoalkyl portion of the molecule. Radioligand displacement assays using high affinity β_2-AR antagonist, [^{3}H]CGP-12177, were performed to determine trends in effects of stereochemistry on the FEN binding affinity, Table 1.[4] The results demonstrate that the calculated K_i values are affected by the chirality at each of the two chiral centers, as the relative order in terms of higher affinity is (*R,R′*) > (*R,S′*) > (*S,R′*) > (*S,S′*), which is reflected by the enantioselectivity ratios (α_E) of 80 and 2.8 for (*S,S′*)/(*R,R′*)-FEN and (*S,R′*)/(*R,S′*)-FEN, respectively. The contribution of each of these centers is suggested by the diastereoselectivity ratios (α_D) of 11 and 2.7, for (*R,S′*)/(*R,R′*)-FEN and (*S,S′*)/(*S,R′*)-FEN, respectively. The results indicate that the presence of the *R* configuration at the β-carbon produces a higher calculated binding affinity in binding studies than the corresponding *S* configuration and that the same trend is observed for the *R* configuration in the chiral center of the aminoalkyl portion of the molecule. The same relative trend is observed in the EC_{50} values associated with FEN-induced accumulation of cAMP, Table 1.

The pK_i values of the FEN analogs were used to develop a comparative molecular field analysis model, CoMFA, to describe and predict the binding of the stereoisomers of FEN and FEN analogs to the β_2-AR.[4] The CoMFA model is consistent with previously described concepts, see Ref. 14, and suggest that there are two separate binding sites, one site defined by the TM helices TM3, TM5, and TM6, where catecholamine part of the molecule bind and the second site enclosed by TM2, TM3, and TM7 and the extracellular loop 2 (ECL2), where aminoalkyl part of FEN binds, Figure 2. Using the previously reported molecular models of the β_2-AR, the CoMFA model indicates that there is an array of key interactions between the aromatic portion of the FEN derivatives and the β_2-AR including hydrogen bond formation between the *p*-oxygen moiety on the phenyl ring in FEN and TYR308 (TM7) and/or ASP192 located on ECL2[4,15] and π–π and/or π–hydrogen bond interactions between the aromatic system of the ligand and aromatic residues located in this area of the binding site.

The CoMFA model indicated that the binding process of the FEN stereoisomers with the β_2-AR includes the interaction of the chiral center on the aminoalkyl portion of the agonist with a sterically restricted site on the receptor. The presence of this site was confirmed by the synthesis of the N-ethyl derivative of FEN, which had a K_i value of >400,000 nM in β_2-AR binding studies conducted using membranes obtained from the HEK-β_2-AR cell line and [^{3}H]CGP-12177 as the marker ligand.[5]

Thermodynamics of FEN–β2-AR Binding

In subsequent study, the effect of temperature on the K_i values of (*R,R′*)-, (*R,S′*)-, (*S,R′*)-, and (*S,S′*)-FEN determined

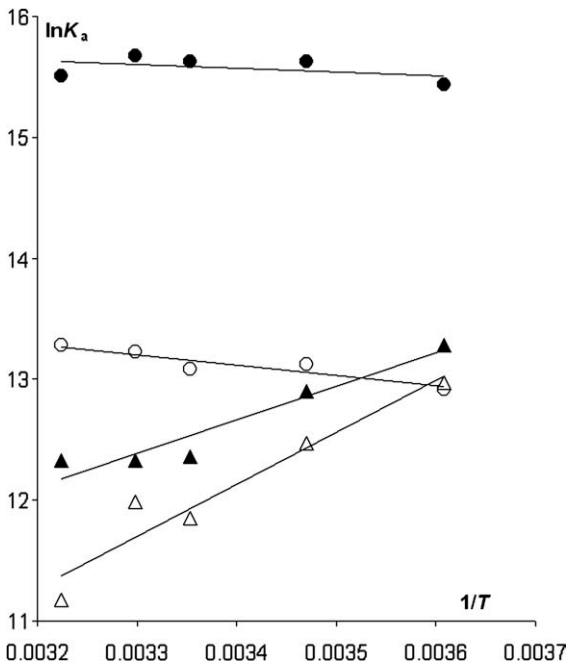

Fig. 3. Van't Hoff plots for (*R,R′*)-fenoterol (●), (*R,S′*)-fenoterol (○), (*S,R′*)-fenoterol (▲), and (*S,S′*)-fenoterol (△).[16]

in [^{3}H]CGP-12177 displacement studies was assessed at five temperature points (4°C, 15°C, 25°C, 30°C, and 37°C).[16] The data were transformed into K_a values, $K_a = 1/K_i$ and used to construct Van't Hoff plots (ln K_a vs. $1/T$, where T is temperature in K), Figure 3. (*R,R′*)-FEN had indeed the highest affinity among all isomers but its ln K_a values did not show statistically significant dependence on $1/T$. The affinity of (*R,S′*)-FEN, the second strongest isomer shows statistically significant dependence: its ln K_a values increase with the increase of temperature. Completely opposite effect was observed for affinity measured for (*S,R′*)-FEN and (*S,S′*)-FEN, as their ln K_a values significantly decreased with increased temperature.[16]

The data were used to calculate the thermodynamic characteristics of the stereoisomers, and the Van't Hoff trends were linearly regressed and ΔH^0 and ΔS^0 functions were calculated according to the equation: $\ln K_a = \frac{\Delta S^0}{R} - \frac{\Delta H^0}{R}\frac{1}{T}$, where R is a gas constant. In case of (*R,R′*)-FEN, where ln K_a did not show temperature dependence, the slope of the curve (and ΔH^0) was assumed to be zero and ΔS^0 was calculated from the curve intercept assumed as ln K_a averaged over all measured temperature points. Table 2 presents enthalpic and entropic components of ΔG^0 function, ($\Delta G^0 = \Delta H^0 - T\Delta S^0$), derived from this analysis for the stereoisomers.

The ΔG^0 values in Table 2 confirm the trend of complex stability for FEN stereoisomers observed in the initial ligand binding studies, that is, (*R,R′*)- > (*R,S′*)- > (*S,R′*)- > (*S,S′*)-FEN. The analysis of thermodynamic functions relative to the chirality at the two chiral centers indicates that the configuration of the chiral center at the β-hydroxy carbon plays a larger role than just the determination of the magnitude of the affinity for the β_2-AR. When the configuration at this center is *R*, that is, (*R,R′*)- and (*R,S′*)-FEN, the formation of the complex is entropy driven ($-T\Delta S^0 \ll 0$; $\Delta H^0 \geq 0$) while the *S* configuration, that is, (*S,R′*)- and (*S,S′*)-FEN, produces a binding process that is almost exclusively controlled by enthalpy ($\Delta H^0 \ll 0$; $-T\Delta S^0 \sim 0$). This suggest that the chirality of the first chiral center determines different modes of intermolecular interactions in the β_2-AR–FEN complex: highly negative entropic component of ΔG^0 for the (*R,x′*) isomers indicates domination of hydrophobic type of interactions with possibility of significant conformational change of ligand and/or receptor on binding. On the other hand, highly negative enthalpic component for the (*S,x′*) isomers suggests domination of hydrogen bond and Van der Waals interactions in these complexes. It is evident that binding of different isomers is not only quantitatively different but also thermodynamics suggests that there is fundamental difference in modes of interactions between FEN stereoisomers and β_2-AR protein.

TABLE 2. Enthalpic (ΔH^0) and entropic ($-T\Delta S^0$) components of the free-energy change (ΔG^0) of the complex formation derived from Van't Hoff analysis for stereoisomers of fenoterol[16]

Compound	ΔH^0 (kJ/mol)	$-T\Delta S^0$ (kJ/mol)	ΔG^0 (kJ/mol)
(*R,R′*)-fenoterol	0	–38.8 (±0.2)	–38.8 (±0.2)
(*R,S′*)-fenoterol	+7.1 (±1.7)	−40.0 (±1.7)	–32.9 (±2.4)
(*S,R′*)-fenoterol	−23.0 (±3.9)	−8.1 (±4.0)	–31.1 (±5.6)
(*S,S′*)-fenoterol	−35.8 (±5.8)	+6.3 (±6.0)	–29.5 (±8.3)

THE EFFECT OF FEN STEREOCHEMISTRY ON AGONIST-INDUCED CARDIOMYOCYTE CONTRACTILITY

Several different in vitro assays were used to characterize functional efficacies of the FEN stereoisomers in β_2-AR-mediated cell activity with a similar trend in functional potencies, that is, (*R,R′*)-FEN is the most potent in these tests, while (*S,S′*)-FEN shows lower or undetectable biological activity.[5,17] One of these studies, the ability of FEN to induce cardiomyocyte contractility in a rat cardiomyocyte model also demonstrated that the configuration at the chiral centers produces different G-protein coupling.

The cardiomyocyte contractility assay[6] measures the potency of a ligand to stimulate contraction of the cell length of rat cardiomyocytes via activation of ARs. Previous studies have shown that activated β_2-AR couples to both G_s and G_i proteins, which results in opposing signaling effects.[18] Coupling to G_s protein promotes cardiomyocyte contractility while G_i coupling decrease this effect. The G_i component of coupling can be selectively blocked by pertussis toxin (PTX) and the use of this agent in cardiomyocyte contractility studies significantly increased the potency of a number of standard agonists. When the effect of PTX on the activity of (*R,R′*)-FEN and (*S,R′*)-FEN was investigated, two different results were obtained. The presence of PTX in the cardiomyocyte experiment produced no significant change in the potency of (*R,R′*)-FEN, that is, no decrease in the EC_{50} value, while there was a significant change in the activity of (*S,R′*)-FEN.[6] These results indicate that activation of β_2-AR by the (*R,R′*)-stereoisomer leads to exclusive activation of G_s protein-mediated signals (no PTX effect) while (*S,R′*)-isomer activates the receptor to the form which couple both G_s and G_i proteins. Similar selective G_s activation by (*R,R′*)-stereoisomers was observed for two further derivatives of FEN, 4-methoxy-FEN[6] and 4-amino-FEN but not for 1-naphtyl- and 4-methoxy-1-naphtyl-derivatives (data not presented). Thus, the data additionally suggest that the mode of binding of the latter two derivatives in (*R,R′*)-configuration is significantly different than the mode of binding observed for (*R,R′*)-FEN.

The mechanism of β_2-AR activation can be described as multistep process; in each step, the agonist molecule interact with transforming binding site of macromolecule and induce further transitions.[8] Local changes within the binding site are further attenuated as global transition of the receptor to a series of active states when the interface for G protein coupling is formed on the intracellular side of the receptor. G_s selectivity of conformation induced by (*R,R′*)-FEN means that this active interface prefer binding to G_s protein in contrast to other stereoisomers where induced intracellular interface may adopt both G_s and G_i. Thus, it appears that FEN isomers induce different β_2-AR active states during binding process and that the resulting receptor conformation is dependent on stereoconfiguration at the β-OH site of the FEN molecule. In addition, it is also possible that the sequence of β_2-AR transition events triggered by the initial binding interactions of FEN is affected by the stereoconfiguration of FEN at both chiral centers.

The Effect of FEN Stereochemistry on Proliferation of 1321N1 Astrocytoma Cells

Recent data has also suggested that the definition of and the degree of enantioselectivity are dependent on the system and the phenomenon used to define the enantioselectivity. In

this study,[17] it was determined that (*R,R′*)-FEN and FEN stereoisomers inhibited the growth of human-derived 1321N1 astrocytoma cells in a dose-dependent manner. The effect was associated with stimulation of the β_2-AR expressed in the cell line, which resulted in an accumulation of cAMP which, in turn, induced a decrease in [^{3}H]-thymidine incorporation. Both (*R,R′*)- and (*S,S′*)-FEN were efficient inhibitors of cellular growth in the 1321N1 cell line with IC_{50} values of 0.4 and 184 nM and stimulated cAMP accumulation with EC_{50} values of 16 nM and 1856 nM, respectively. The enantiomeric difference calculated as EC_{50} (*S,S′*)/EC_{50} (*R,R′*) is 117 using the data from the 1321N1 cell line as compared to 1933 calculated using the data obtained using the HEK293-β_2-AR cell line, Table 1. The quantitative differences in the EC_{50} values and enantioselectivity are consistent with there being fewer β_2-AR in the 1321N1 cells as compared to the HEK-β_2-AR cells that overexpress the receptor. However, the observation that (*R,R′*)-ethyl-FEN is an effective inhibitor of cellular growth and a β_2-AR agonist in 1231N1 cells (IC_{50} 1.4 nM; EC_{50} 2.8 nM)[17] is contrary to the determination that this compound is inactive in the rat cardiomyocyte contractility model. This comparison supports the view that there are different conformations, or perhaps splice variants, in the β_2-AR expressed in the two test systems.

All above results underline the importance of stereoconfiguration in FEN and related molecules in current medicinal chemistry projects. A change in stereochemistry may become as advantageous as a change in chemical constitutions of future drug molecules in targeting highly specific and defined signals in a cell. In fact, as the results of our research, the (*R,R′*)-FEN currently undergoes a clinical trial as a part of novel treatment of congestive heart failure and next molecules are considered for a trial in this therapy as well.

CHIRAL RECOGNITION AS PART OF A LIGAND-SELECTIVE BINDING PROCESS

The current chiral recognition mechanism associated with the binding and activation of the β_2-AR is predicated on the assumption that the (*S*)- and (*R*)-enantiomers of the compound bind to the same conformation of the receptor.[13] Such mechanisms of recognition of enantiomers are typically described using classical point interaction models assuming that either three[19] or four[20] key interactions are necessary to distinguish one enantiomer from the other. The binding subsequently produces different conformational changes in the protein resulting in different binding affinities (K_i values) and potencies (EC_{50} values), with enantioselectivity defined as the ratio of the two K_i or EC_{50} values. This model has been developed using agonists with a single chiral center and small alkyl substituents on the N-alkyl portion of the molecule. The data from the studies with the stereoisomers of FEN indicate that there is a more complex molecular recognition mechanism associated with the interaction of the β_2-AR with an agonist with two chiral centers and aromatic substituents on the N-alkyl portion of the molecule. Indeed, the data suggest that initial step in the molecular recognition mechanism is the binding of the (*R,R′*)- and (*R,S′*)-isomers to a conformational state of β_2-AR that is different from the receptor conformation to which the (*S,R′*)- and (*S,S′*)-isomers bind. Thus, the observed (*S,S′*)/(*R,R′*)-FEN and (*S,R′*)/(*R,S′*)-FEN enantioselectivities reflect binding to different conformational forms of the receptor and are not described by the standard chiral recognition process which is defined as differential binding to a spatially defined site.

This assumption is supported by the observation that (*R,R′*)-FEN selectively activate the receptor to the state(s) preferentially coupling to G_s protein, a highly unique property. In contrast, other FEN stereoisomers do not show this selectivity and this difference strongly suggests that FEN stereoisomers bind to and/or stabilize different active conformations of the receptor, that is, distinct states that are preferred by different G proteins. This possibility was supported by Seifert and Dove[21] who concluded that the data corroborated the concept of ligand-specific GPCR conformations and functional selectivity. The authors also suggested that (*R,R′*)- and (*S,R′*)-FEN may bind in completely different modes, although they added that this might be unlikely as the chirality of the second chiral center, that is, the aminoalkyl moiety, was the same and independent of the chirality of the β-OH carbon. However, the data from the study of the thermodynamics of FEN–β_2-AR binding demonstrate that the inversion of the chirality at the β-OH carbon results in dramatic changes in the binding process while the configuration of the second chiral center does not play a significant role in the determination of the binding thermodynamics. Thus, the results are consistent with the assumption by Seifert and Dove[21] and indicate that (*R,R′*)- and (*R,S′*)-FEN bind in significantly different modes than (*S,S′*)- and (*S,R′*)-FEN.[21]

Binding to distinct conformations of the binding site may also explain the differences in thermodynamics determined in the Van't Hoff analysis. Entropy-controlled binding of (*R,x′*)-isomers may be a sign of massive conformational changes of the receptor during activation of more active isomers. This is in contrast to the attractive interactions with (*S,x′*)-isomers for which binding was determined to be enthalpy driven.

In addition to K_i values determined for different temperatures in Van't Hoff analysis, Hill's *n* coefficients were determined based on dose–response curve of displacement experiments.[16] These coefficients vary significantly for stereoisomers: the *n* values in 15°C were 0.92 (±0.15); 1.08 (±0.19); 1.95(±0.14); 2.06(±0.14) for (*R,R′*)-, (*R,S′*)-, (*S,R′*)-, and (*S,S′*)-FEN, respectively.[16] Similar values were observed in other temperatures and can be generalized that (*R,x′*)-isomers have *n* around unity, and the *n* values for (*S,x′*)-isomers are significantly higher and oscillate between 1.6 and 2.1. Hill's *n* equal to 1 indicates independent binding on the one type of binding site, while *n* significantly greater than 1 is an indication of co-operative mode of binding or more than one class of binding sites. This suggests that (*R,x′*)-FEN predominately bind to a single conformation of the β_2-AR while (*S,x′*)-FEN binds to more than one conformations. It is possible that (*R,x′*)-FEN and (*S,x′*)-FEN bind to the same conformation of the β_2-AR, with this interaction representing only a minor component of the total (*S,x′*)-FEN binding to the β_2-AR. This possibility has not been eliminated by the results of the current studies.

The results from our recent studies of the activity of FEN stereoisomers in functional systems suggests that the determination of enantioselective and/or stereoselective differences has to be defined relative to the system used to determine this property. The data also suggests that SAR studies of biological interactions based on standard molecular recognition models using three-point and/or four-point

interactions may miss the subtle and importance of the conformational structure of the protein.

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