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Stereoselectivity for interactions of agonists and antagonists at mouse, rat and human β_3-adrenoceptors

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Abstract

This study examines the stereoselectivity profile of recombinant mouse, rat and human β_3-adrenoceptors expressed in Chinese Hamster Ovary (CHO-K1) cells using radioligand binding, in comparison with endogenously expressed β_3-adrenoceptors mediating relaxation responses in mouse ileum. The enantiomeric ratios for several β-adrenoceptor agonists and antagonists at the cloned mouse, rat and human β_3-adrenoceptor were less than those reported at the cloned β_1-/β_2-adrenoceptor but higher than those reported in previous studies. The degree of stereoselectivity was relatively low for the agonists isoprenaline and noradrenaline but higher for antagonists and, in particular, tertatolol and propranolol. In mouse ileum, stereoselectivity of propranolol and tertatolol was observed under β_1-/β_2-adrenoceptor blockade. The (−)-enantiomers of propranolol and tertatolol were more effective at antagonism of (−)-isoprenaline-mediated relaxation of mouse ileum than their (+)-enantiomers. The recombinant mouse, rat and human β_3-adrenoceptors display stereoselective interactions for agonists and antagonists similar to the stereoselective profile of β_3-adrenoceptors in mouse ileum. The degree of stereoselectivity varied between species and the human β_3-adrenoceptor displayed higher affinities and enantiomeric ratios than the mouse or rat β_3-adrenoceptors.

Keywords: β_3-Adrenoceptor; Stereoselectivity; Cloned receptor; Ileum, mouse

1. Introduction

The discovery of atypical β-adrenoceptors was based on the low affinity (Harms et al., 1974) and low stereoselectivity indices (Harms et al., 1977) of conventional β-adrenoceptor antagonists at the β-adrenoceptors controlling lipolysis in rat adipose tissue. Further characterization of this receptor supported the conclusion that for conventional β-adrenoceptor agonists and antagonists, the β_3-adrenoceptor displays a low degree of stereoselectivity for their enantiomers (Arch and Kaumann, 1993; Emorine et al., 1994; Zaagsma and Nahorski, 1990). Even in the earliest functional studies (Harms, 1976), differences were identified between human and rat adipocyte β-adrenoceptors with the human response displaying much higher enantiomeric ratios than that in the rat tissue. However, all of the earlier work was conducted in intact tissues or cells where results can be difficult to interpret due to the presence of other β-adrenoceptors. More recent studies have suggested that low enantiomeric ratios may not be a strong defining feature of the β_3-adrenoceptor. Studies with the selective β_3-adrenoceptor antagonist 3-(2-ethylphenoxy)-1-[(1*S*)-1,2,3,4-tetrahydronaphth-1-ylamino]-(2*S*)-2-propanol oxalate (SR59230A) show high enantiomeric ratios between the SS isomer and the RR isomer (SR59483) (De Ponti et al., 1996; Manara et al., 1996; Nisoli et al., 1996; Nisoli and Carruba, 1997) and at the cloned human β_3-adrenoceptor a high degree of stereoselectivity for the enantiomers of propranolol has also been reported (Arch, 2000).

The β_3-adrenoceptor has been cloned from many species including human (Emorine et al., 1989), rat (Granneman et al., 1991) and mouse (Nahmias et al., 1991), which has two isoforms (Evans et al., 1999). Expression of these receptors in cell systems facilitates receptor characterization particularly since there is a lack of a selective high affinity radioligand and a non-specific binding protein has been reported in some

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rodent tissues (Sugasawa et al., 1997, 2001). Here, we examined the stereoselectivity of a number of β-adrenoceptor agonists and antagonists at the human, rat and two mouse variant β_3-adrenoceptors cloned in Chinese Hamster Ovary (CHO-K1) cells as well as β_3-adrenoceptors expressed in mouse ileum. All of the β_3-adrenoceptors displayed higher stereoselectivity indices than previously reported and in the case of the human β_3-adrenoceptor the values approach those reported for β_1- and β_2-adrenoceptors. The human β_3-adrenoceptor also displays higher affinity and stereoselectivity for competitors than the equivalent rodent receptors.

2. Materials and methods

2.1. Generation of rat and human β_3-adrenoceptor clones

Inserts were generated carrying the coding region of the rat and human β_3-adrenoceptor by reverse transcription–polymerase chain reaction on RNA extracted from rat ileum or human SK-N-MC cells, respectively, using *Pfx* polymerase (Life Technologies). The primers used were, forward (rat: 5′-GGAAAGCTTCCCATCCCAGACGC-3′; human: 5′-CGCAAGCTTCGCCATGGCTCCGTGG-3′) and reverse (rat: 5′-GGACCATGGAGATCTAGAAAAGGAGCC-5′; human: CTTCTAGACCTTCAGGCCTA AGAAACTCCC-3′), and included *Hin*dIII or *Xba*I sites, respectively, for subcloning fragments into the mammalian vector pcDNA3.1(+) (Invitrogen). The complete inserts and junctions with pcDNA3.1(+) were checked by DNA sequencing on both strands (Micromon, Monash University, Australia). Inserts of the mouse β_{3a}- and β_{3b}-adrenoceptor were generated as previously described (Hutchinson et al., 2002).

2.2. Cell culture and transient transfection of β_3-adrenoceptor clones

CHO-K1 cells were grown as monolayers in 50:50 Dulbecco's modified Eagle medium (DMEM): Ham's F-12 medium containing 10% (v v^{-1}) foetal bovine serum, glutamine (2 mM), penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). All cells were maintained under 5% CO_2 at 37 °C. For transfection, CHO-K1 cells were seeded overnight at 12×10^6 cells per 150-cm^2 flask. Plasmid DNA (2–5 μg) containing the coding region of rat, human, mouse β_{3a}- or β_{3b}-adrenoceptors and additional pcDNA3.1(+) to a DNA total of 21 μg was added to 1.75 ml of OPTIMEM™ (Life Technologies). This was then added to a solution containing 170 μl of lipofectamine™ (Life Technologies) in 1.75 ml of OPTIMEM™ and incubated for 30 min at room temperature. An additional amount of OPTIMEM™ (14 ml) was then added to the lipid–DNA complexed solution to create the transfection mix. DMEM was removed from the flask, the cells were washed with 10 ml OPTIMEM™ and the transfection mix layered onto the cells and left for 4 h. 17.5 ml of DMEM/Ham's F-12 (50:50) containing 20% (v v^{-1}) foetal bovine serum was then added and incubated overnight. Media was replaced 24 h later with standard (50:50) DMEM/Ham's F-12 and 48 h after the start of transfection membranes were prepared.

2.3. Receptor binding assay

Membranes were prepared from transfected CHO-K1 cells as previously described (Hutchinson et al., 2002). Saturation experiments were performed at room temperature in a total volume of 100 μl binding buffer (50 mM Tris pH 7.4 room temperature, 5 mM $MgCl_2$, 1 mM EDTA, 10 μg ml^{-1} bacitracin, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin A, 0.5 μg ml^{-1} aprotinin) in 96-well microtiter plates. Homogenate (8–14 μg of protein) was incubated with (−)-[^{125}I]-cyanopindolol ([^{125}I]CYP) (5–2000 pM) for 60 min at room temperature in the absence or presence of (−)-alprenolol (1 mM) to define non-specific binding. Competition experiments were performed using a range of concentrations of unlabelled drug and [^{125}I]CYP (500 pM). Reactions were terminated by rapid filtration through GF/C filters presoaked in 0.5% (v v^{-1}) polyethylenimine and washed three times with wash buffer (50 mM Tris pH 7.4, 4 °C) using a Packard Cell Harvester. Filters were dried, 25 μl Microscint-O (Packard) added and radioactivity measured using a Packard Top Count. Experiments were performed in duplicate with n referring to the number of different membrane homogenates used. Data was analyzed using a one-site fit (GraphPad PRISM version 3.0) to obtain pK_D and B_{max} values (saturation experiments) or non-linear curve fitting to obtain pK_i values (competition experiments) expressed as mean ± S.E.M. of n individual experiments. Statistical significance was determined using two-way analysis of variance (ANOVA) tests or Student's t test. Probability values <0.05 were considered significant.

2.4. Organ bath studies

FVB mice (male, 8–14 weeks old) were obtained from Monash University. Mice were anaesthetized with 80% CO_2/20% O_2 and decapitated before tissues were obtained as described previously (Hutchinson et al., 2001). Organ bath experiments were performed as described previously (Hutchinson et al., 2000) with minor modifications: In experiments examining β_3-adrenoceptor-mediated relaxations, β_1- and β_2-adrenoceptors were blocked by the addition of 2-hydroxy-5(2-((hydroxy-3-(4-((1-methyl-4-trifluoromethyl)1*H*-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethane sulfonate (CGP20712A) (100 nM) and erythro-DL-1(7-methylindian-4-yloxy)-3-isopropylaminobutan-2-ol (ICI118551) (100 nM). All responses are measured as a percentage of the maximal relaxation of the tissue to papaverine (10 μM). Non-linear regression was used to fit sigmoid concentration–response curves to the data (GraphPad PRISM version 3.0) and to determine pEC_{50} values. Values are expressed as mean ± S.E.M. of n individual

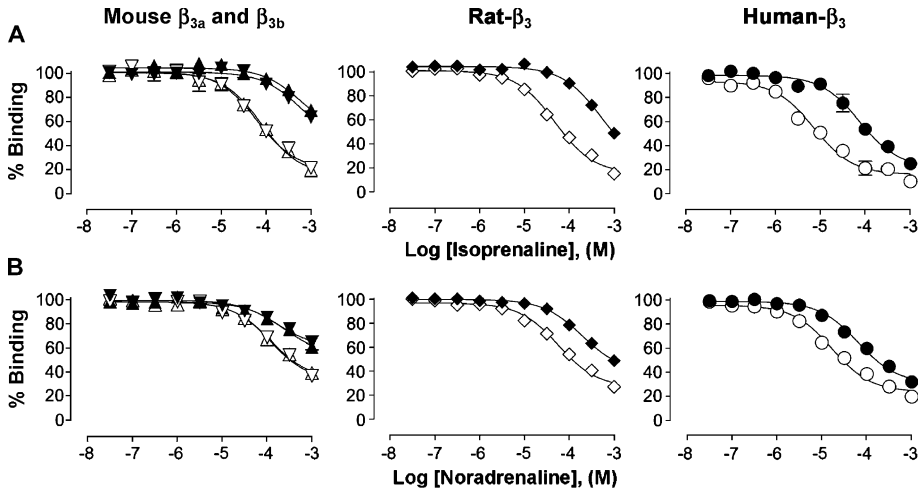

Fig. 1. Competition by the enantiomers of (A) isoprenaline and (B) noradrenaline for [^{125}I]CYP binding to mouse β_{3a}- (△ ▲) and β_{3b}- (▽ ▼), rat (◇◆) and human (○●) β_3-adrenoceptor ($n=4-5$) expressed in CHO-K1 cells. (−)-Enantiomers are represented in open symbols while (+)-enantiomers are represented in closed symbols.

experiments, with each n value referring to the number of individual animals used. In experiments where antagonists were used, pK_B values were calculated according to the method of Furchgott (1972). Student's t test or two-way ANOVA was used to determine statistical significance where $P<0.05$ was considered to be significant.

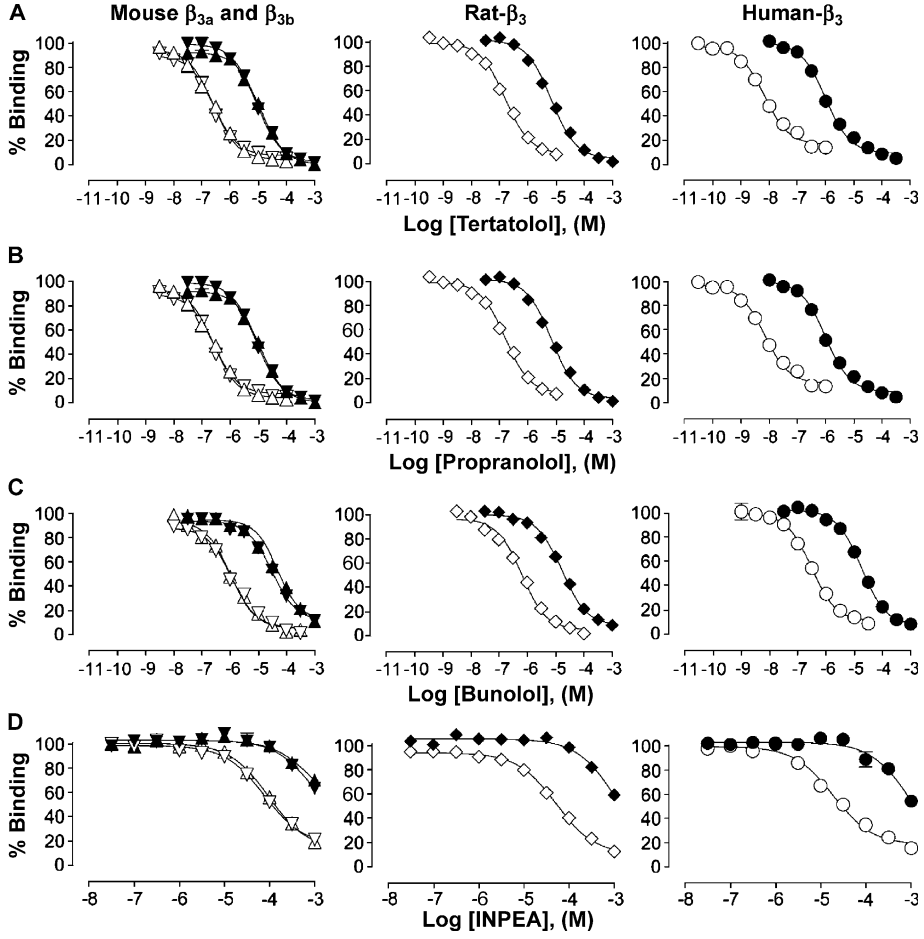

Fig. 2. Competition by the enantiomers of the β-adrenoceptor antagonists (A) tertatolol, (B) propranolol, (C) bunolol and (D) INPEA for [^{125}I]CYP binding to mouse β_{3a}- (△ ▲) and β_{3b}- (▽ ▼), rat (◇◆) and human (○●) β_3-adrenoceptor ($n=3-5$) expressed in CHO-K1 cells. (−)-Enantiomers are represented in open symbols while (+)-enantiomers are represented in closed symbols.

Table 1
pK_i values and enantiomeric ratios for agonists competing for binding of [^{125}I]CYP at mouse, rat and human β_3-adrenoceptors expressed in CHO-K1 cells

Ligand	Mouse β_{3a}		Mouse β_{3b}		Rat β_3		Human β_3	
	pK_i (n)	Enantiomeric ratio	pK_i (n)	Enantiomeric ratio	pK_i (n)	Enantiomeric ratio	pK_i (n)	Enantiomeric ratio
(+)-Isoprenaline	3.06 ± 0.12 (4)[a]	20.6	3.06 ± 0.13 (5)[a]	12.7	3.35 ± 0.04 (4)[a]	11.4	4.18 ± 0.15 (5)[a]	13.9
(−)-Isoprenaline	4.37 ± 0.12 (4)		4.16 ± 0.15 (5)		4.41 ± 0.11 (4)		5.32 ± 0.12 (5)	
(+)-Noradrenaline	3.08 ± 0.05 (4)[a,b]	5.5	2.82 ± 0.22 (4)[a,b]	9.1	3.41 ± 0.02 (4)[a]	5.8	4.10 ± 0.02 (4)[a]	3.7
(−)-Noradrenaline	3.82 ± 0.07 (4)[a]		3.78 ± 0.04 (4)[a]		4.17 ± 0.12 (4)		4.67 ± 0.07 (4)	

[a] Computer extrapolated pK_i. Not enough data available for full binding curve (computer extrapolation based on minimum binding obtained for the (−)-enantiomer of the corresponding ligand).

[b] Computer extrapolated pK_i. Not enough data available for full binding curve (computer extrapolation based on minimum binding obtained for (−)-isoprenaline within the same species).

2.5. Drugs and reagents

The following drugs were gifts: (−) bunolol, (+) bunolol, (−)-INPEA (1-(4-nitrophenyl)-2-isopropylaminoethanol) and (+)-INPEA (Prof. B. Jarrott, Monash University). Drugs and reagents were purchased as follows: (−)-[^{125}I]-cyanopindolol (2200 Ci mmol^{-1}, NEN Life Science Products, Boston, MA, USA); ICI118551 (Imperial Chemical Industries, Wilmslow, Cheshire, England); CGP20712A (Ciba-Geigy, Australia); (−)-alprenolol, bacitracin, (−)-isoprenaline, (+)-isoprenaline, (−)-noradrenaline, (+)-noradrenaline, polyethylemine, (−)-propranolol, (+)-propranolol (Sigma, St. Louis, MO, USA); aprotinin, leupeptin, pepstatin A (ICN, Costa Mesa, CA, USA); (−)-tertatolol, (+)-tertatolol (Servier Laboratories, Neuilly Sur Seine, France). All cell culture medium and reagents were obtained from Trace Biosciences (Castle Hill, NSW, Australia).

3. Results

3.1. Saturation studies of [^{125}I]CYP binding in mouse β_{3a}- and β_{3b}-, rat and human β_3-adrenoceptors expressed in CHO-K1 cells

Saturation binding properties of mouse, rat and human β_3-adrenoceptors were examined using [^{125}I]CYP (5–2000 pM). Binding was saturable to a population of sites with affinity appropriate for binding to β_3-adrenoceptors. Analysis of saturation binding isotherms for the mouse β_{3a}- and β_{3b}-, rat and human β_3-adrenoceptors showed that the receptors were expressed at 420 ± 72 (n = 5), 347 ± 71 (n = 5), 544 ± 57 (n = 4) and 245 ± 25 (n = 8) fmol mg protein^{-1} and displayed pK_D values for [^{125}I]CYP of 9.39 ± 0.03 (n = 5), 9.28 ± 0.07 (n = 5), 9.15 ± 0.05 (n = 4) and 9.17 ± 0.03 (n = 8), respectively.

3.2. Competition binding studies at the cloned mouse β_{3a}- and β_{3b}-, rat and human β_3-adrenoceptors

Competition studies were performed with CHO-K1 cells transfected with β_3-adrenoceptors and [^{125}I]CYP to determine the affinities of the enantiomers of the β-adrenoceptor agonists isoprenaline or noradrenaline, and the enantiomers of the non-selective β-adrenoceptor antagonists tertatolol, propranolol, bunolol and 1-(4-nitrophenyl)-2-isopropylaminoethanol (INPEA) at the cloned mouse β_{3a}- and β_{3b}-, rat and human β_3-adrenoceptors. pEC_{50} values were determined from the competition curves and converted to pK_i values using the Cheng and Prusoff (1973) equation which demonstrated, in all cases, that the (−)-enantiomer displayed a higher affinity than the (+)-enantiomer (Figs. 1 and 2; Table 1 and 2; $P < 0.0001$, two-way ANOVA).

Table 2
pK_i values and enantiomeric ratios for antagonists competing for binding of [^{125}I]CYP at mouse, rat and human β_3-adrenoceptors expressed in CHO-K1 cells

Ligand	Mouse β_{3a}		Mouse β_{3b}		Rat β_3		Human β_3	
	pK_i (n)	Enantiomeric ratio	pK_i (n)	Enantiomeric ratio	pK_i (n)	Enantiomeric ratio	pK_i (n)	Enantiomeric ratio
(+)-Tertatolol	5.29 ± 0.04 (3)	51.3	5.34 ± 0.08 (3)	42.7	5.41 ± 0.01 (5)	39.8	6.25 ± 0.03 (5)	204.2
(−)-Tertatolol	7.00 ± 0.05 (3)		6.97 ± 0.05 (3)		7.01 ± 0.03 (5)		8.56 ± 0.17 (5)	
(+)-Propranolol	4.81 ± 0.10 (3)	60.3	4.65 ± 0.03 (3)	67.6	4.63 ± 0.01 (5)	66.1	5.04 ± 0.02 (5)	141.3
(−)-Propranolol	6.59 ± 0.14 (3)		6.48 ± 0.16 (3)		6.45 ± 0.01 (5)		7.19 ± 0.07 (5)	
(+)-Bunolol	4.71 ± 0.05 (4)	34.7	4.74 ± 0.07 (5)	33.9	4.98 ± 0.04 (5)	28.2	4.98 ± 0.02 (5)	58.9
(−)-Bunolol	6.25 ± 0.03 (4)		6.27 ± 0.05 (5)		6.43 ± 0.06 (5)		6.75 ± 0.04 (5)	
(+)-INPEA	2.91 ± 0.03 (4)[a]	16.2	3.20 ± 0.04 (5)[a]	12.9	3.15 ± 0.06 (5)[a]	20.0	3.32 ± 0.04 (5)[a]	38.0
(−)-INPEA	4.12 ± 0.12 (4)		4.31 ± 0.08 (5)		4.45 ± 0.04 (5)		4.90 ± 0.07 (5)	

[a] Computer extrapolated pK_i. Not enough data available for full binding curve (computer extrapolation based on minimum binding obtained for the (−)-enantiomer of the corresponding ligand).

3.3. Stereoselectivity for agonists at the cloned mouse β_{3a}- and β_{3b}-, rat and human β_3-adrenoceptors

The stereoisomers of isoprenaline and noradrenaline displayed similar affinity values for each of the cloned β_3-adrenoceptors. Although these values were relatively low, isoprenaline appeared to show higher stereoselectivity at all receptors than noradrenaline. Comparisons of enantiomeric ratios between receptor types showed that both agonists displayed similar stereoselectivity between the receptors of different species (Table 1).

3.4. Stereoselectivity for antagonists at the cloned mouse β_{3a}- and β_{3b}-, rat and human β_3-adrenoceptors

The (+)- and (−)-enantiomers of the antagonists tertatolol, propranolol, bunolol and INPEA all displayed stereoselectivity at the cloned β_3-adrenoceptors. The rank order of stereoselectivity at the cloned rat or mouse receptors was propranolol>tertatolol>bunolol>INPEA. The rank order of stereoselectivity for the human receptor and was tertatolol>propranolol>bunolol>INPEA. Comparison of the enantiomeric ratios between the receptors showed that the human receptor displayed approximately two to nine times the stereoselectivity compared to the rat or mouse receptors (Table 2). The relatively high enantiomeric ratios displayed by the human receptor for all of the stereoisomer pairs were a feature that clearly distinguished the human β_3-adrenoceptor from the rat or mouse homologues.

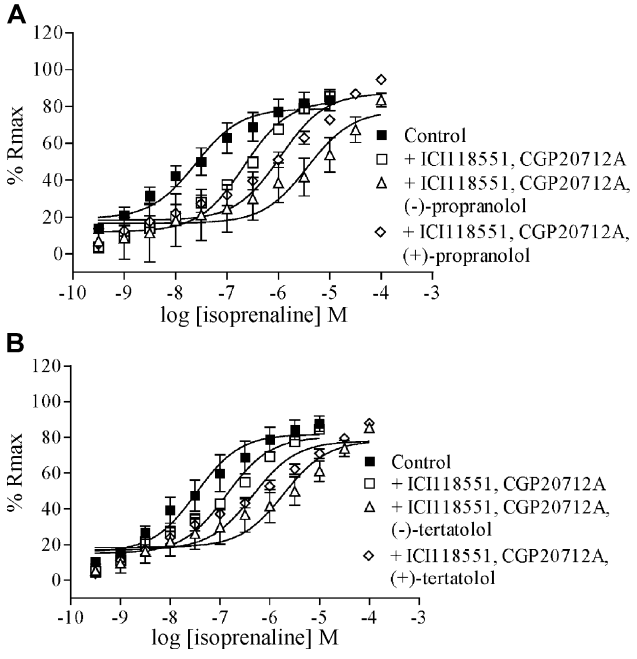

Fig. 3. The effect of enantiomers of propranolol and tertatolol on relaxation responses to isoprenaline in carbachol-contracted mouse ileum. Contributions to the relaxation response from β_1- and β_2-adrenoceptors were blocked with ICI118551 (100 nM) and CGP20712A (100 nM). pK_B values were calculated from the dextral shift produced by (−)- or (+)-propranolol (panel a) or (−)- or (+)-tertatolol (panel b) in the presence of ICI118551 and CGP20712A.

Table 3
pEC$_{50}$ values for (−)-isoprenaline-mediated relaxation of mouse ileum in the absence or presence of the stereoisomers of propranolol or tertatolol under β_1-/β_2-adrenoceptor blockade (n=5–6)

	pEC$_{50}$
Control	7.61 ± 0.17
+ ICI118551 (100 nM), CGP20712A (100 nM)	6.67 ± 0.07
+ ICI118551 (100 nM), CGP20712A (100 nM), (−)-propranolol (10 μM)	5.43 ± 0.30
+ ICI118551 (100 nM), CGP20712A (100 nM), (+)-propranolol (100 μM)	5.96 ± 0.10
Control	7.48 ± 0.16
+ ICI118551 (100 nM), CGP20712A (100 nM)	6.87 ± 0.09
+ ICI118551 (100 nM), CGP20712A (100 nM), (−)-tertatolol (10 μM)	5.72 ± 0.20
+ ICI118551 (100 nM), CGP20712A (100 nM), (+)-tertatolol (100 μM)	6.34 ± 0.10

3.5. Stereoselectivity of propranolol and tertatolol at mouse ileum β_3-adrenoceptors

Relaxation responses to (−)-isoprenaline were antagonised by the stereoisomers of propranolol under conditions chosen to block β_1- and β_2-adrenoceptors (ICI118551 100 nM and CGP20712A 100 nM) (Fig. 3; Table 3) with pK_B values of 6.15 ± 0.3 and 4.58 ± 0.2 for (−)- and (+)-propranolol, respectively (Student's t test P<0.001). Additionally, under conditions chosen to block β_1- and β_2-adrenoceptors, responses to (−)-isoprenaline were antagonised by the stereoisomers of tertatolol (Fig. 3; Table 3) giving pK_B values of 6.10 ± 0.3 and 4.40 ± 0.3 for (−)- and (+)-tertatolol, respectively (Student's t test P<0.001). The degree of blockade with the combination of ICI118551 (100 nM) and CGP20712A (100 nM) was similar to that with CGP20712A (100 nM) alone (Hutchinson et al., 2001).

4. Discussion

One of the reported characteristics of the β_3-adrenoceptor is a poor ability to discriminate between (+)- and (−)-enantiomers of β-adrenoceptor ligands (Arch and Kaumann, 1993; Emorine et al., 1994; Strosberg and Pietri-Rouxel, 1996; Zaagsma and Nahorski, 1990) when compared to β_1-/β_2-adrenoceptors which consistently display high degrees of stereoselectivity (for a review, see Ruffolo, 1991). However, examination of a number of studies of β_3-adrenoceptors indicates that these receptors may possess a higher degree of stereoselectivity than previously suggested (Arch, 2000; De

Ponti et al., 1996; Horinouchi and Koike, 2001; Ida et al., 1996; Manara et al., 1996; Nisoli et al., 1996; Oriowo et al., 1996), especially to β-adrenoceptor subtype selective antagonists (De Ponti et al., 1996; Manara et al., 1996; Nisoli et al., 1996), or agonists (Ida et al., 1996), or to non-selective receptor agonists such as noradrenaline, isoprenaline or trimetoquinol under conditions chosen to block endogenous β_1/β_2-adrenoceptors (Horinouchi and Koike, 2001; Lezama et al., 1996). Differences between the human and rat β_3-adrenoceptor were also observed in early functional studies in adipose tissue using several general β-adrenoceptor antagonists (Harms, 1976), where enantiomeric ratios were considerably higher in human adipocyte β-adrenoceptors than in rat adipocyte β-adrenoceptors. However, studies using intact tissues may include a variable contribution from other β-adrenoceptor subtypes that are known to display higher enantiomeric ratios. There can also be problems with the interpretation of data from comparisons that use data from multiple studies to determine enantiomeric ratios. In this study, we have used radioligand binding techniques and a number of mammalian β_3-adrenoceptor homologues expressed in CHO-K1 cells or endogenously expressed in tissues to establish that the β_3-adrenoceptor and in particular the human β_3-adrenoceptor can display high enantiomeric ratios.

[^{125}I]CYP bound to each of the β_3-adrenoceptor homologues with characteristically low pK_D values as previously reported (Emorine et al., 1989; Granneman et al., 1993; Hutchinson et al., 2002; Nahmias et al., 1991). Competition for [^{125}I]CYP binding by (−)-isoprenaline and (−)-noradrenaline produced pK_i values in close agreement with previous studies (Féve et al., 1991; Granneman et al., 1993; Hutchinson et al., 2002; Muzzin et al., 1992). Competition for [^{125}I]CYP binding by antagonists showed that (−)-propranolol had similar affinities at the human and mouse β_3-adrenoceptor (Blin et al., 1993, 1994; Hutchinson et al., 2002), whereas our reported pK_i value at the rat receptor is 6.45, somewhat higher than the 5.82 reported by Muzzin et al. (1991). A difference may reflect differing expression levels of the receptors in the studies. (−)-Isoprenaline and (−)-noradrenaline had similar affinity for the rat and human β_3-adrenoceptors (Emorine et al., 1989; Muzzin et al., 1994), and this was also demonstrated here for the mouse receptors. Finally, in accordance with previous work (Hutchinson et al., 2002), the two splice isoforms of the mouse β_3-adrenoceptor displayed identical binding affinity for the ligands examined and demonstrated the same degree of stereoselectivity.

Agonists generally displayed lower stereoselectivity than antagonists and isoprenaline displayed more stereoselectivity than noradrenaline. The enantiomeric ratios of 11.4–20.6 for isoprenaline reported here for all the β_3-adrenoceptor homologues appear lower than the 30-fold ratio reported in the human β_3-adrenoceptor (Emorine et al., 1989). However, this largely results from a higher pK_i (6.2) for (−)-isoprenaline, in the previous study compared to 5.32 in the current study. pK_i values for the (+)-isomer were similar across all studies. Other binding studies using (−)-isoprenaline have reported affinity values of 5.2 (Sennitt et al., 1998) and 5.4 (Mejean et al., 1995) in close agreement with the values reported here.

We have shown that the β-adrenoceptor antagonists tertatolol and propranolol display high enantiomeric ratios at the cloned rat, human and mouse β_{3a}- and β_{3b}-adrenoceptor. The ratios were greatest at the human β_3-adrenoceptor with enantiomeric ratios of 204.2 and 141.3 for the (−)- and (+)-enantiomers of tertatolol and propranolol, respectively. These values were two to four times greater than values obtained at the rodent equivalents. The result with the human receptor confirms the report of an enantiomeric ratio of 154 (Arch, 2000) for the enantiomers of propranolol at the cloned human β_3-adrenoceptor. The greater enantiomeric ratios for tertatolol and propranolol at the human β_3-adrenoceptor in comparison to rodent receptors were echoed by the enantiomers of bunolol and INPEA. Although the β_3-adrenoceptor homologues share an amino acid sequence homology of 80–90%, the differences between the human and rodent receptors may reflect structural differences (Strosberg and Pietri-Rouxel, 1996). The difference between the human and rodent β_3-adrenoceptor extends to the pharmacological profile, which has had a major impact on the development of β_3-adrenoceptor agonists for the treatment of obesity and diabetes. Examples include drugs such as disodium (*R*,*R*)-5-[2-[[2-3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL316243), which is highly efficacious at the rodent β_3-adrenoceptor but has little effect at the human receptor (for a review, see Strosberg, 1997). Studies using bovine and monkey β_3-adrenoceptors may help to identify the structural features involved since they share close amino acid sequence homology with the human receptor (Strosberg and Pietri-Rouxel, 1996).

Comparison of the pK_i values for the agonists and antagonists at the β_3-adrenoceptor species homologues showed that the ligands displayed higher affinity at the human β_3-adrenoceptor. It has been suggested that ligand affinity may be linked to high enantiomeric ratios (Pfeiffer, 1956). This suggestion is largely in accord with the present results since compounds generally displayed highest affinity at the human β_3-adrenoceptor and had their highest enantiomeric ratios at the same receptor; agonists competed for [^{125}I]CYP binding with relatively low affinity and displayed low enantiomeric ratios. However, this generalization did not hold in every case since tertatolol had lower enantiomeric ratios than propranolol at the rodent receptors even though it had a higher pK_i. The major increase in enantiomeric ratio for tertatolol at the human β_3-adrenoceptor may reflect a greater selectivity of this antagonist at this receptor.

Enantiomeric ratios were also determined in a functional assay; (−)-isoprenaline induced relaxation of carbachol-precontracted mouse ileum. The possible contribution by β_1- or β_2-adrenoceptors in this tissue was prevented by

blockade of these receptors by CGP20712A and ICI118551, respectively. The (−)- and the (+)-enantiomers of propranolol and tertatolol were examined in this system and found to have enantiomeric ratios of 37.2 and 50.1, respectively. Although the enantiomeric ratio for tertatolol is in good agreement with the value of 43.7 determined in a study in rat ileum (Roberts et al., 1999) the enantiomeric ratio for propranolol was somewhat higher than previous reports in rat adipocytes (Bojanic et al., 1985; Harms, 1976; Harms et al., 1977). This difference may be due to interference from β_1- and β_2-adrenoceptors which are also expressed in adipocytes, which was addressed in this study through the use of the selective antagonists ICI118551 and CGP20712A, or to differences between the β_3-adrenoceptor found in rat adipose tissue and that in the mouse ileum. Although the general conclusion from functional studies is that there are low enantiomeric ratios for compounds acting at β_3-adrenoceptors there is much conflicting data. Most studies demonstrate a modest preference for the (−)-isomer of isoprenaline (Carpéné et al., 1994; Emorine et al., 1989; Nahmias et al., 1991; Van der Vliet et al., 1990; Horinouchi and Koike, 2001), noradrenaline (Horinouchi and Koike, 2001), cyanopindolol (Langin et al., 1991), alprenolol (Harms, 1976; Harms et al., 1977; Roberts et al., 1999) and tertatolol (Bojanic et al., 1985; Harms, 1976; Harms et al., 1977; Roberts et al., 1999), which are all general β-adrenoceptor ligands. Many of the ligands considered here also fall into this category and have a single chiral centre and relatively low affinity for β_3-adrenoceptors. These compounds were used in the initial characterisation of β_3-adrenoceptors and the source of many reports of low enantiomeric ratios. However, β_3-adrenoceptor specific ligands can display high degrees of stereoselectivity in functional studies, indicating that high stereoselectivity is observed when examining receptor subtype selective ligands. Studies carried out in rat adipose and gastrointestinal tissues (Oriowo et al., 1996) investigated the enantiomers of the β_3-adrenoceptor ligand (±)-(*R**,*R**)-(4-[2-([2-(3-chlorophenyl)-2-hydroxyethyl]amino)propyl]phenoxy)acetic acid (BRL37344), showing that the RR isomer was 1000- to 3000-fold more selective for mediation of β_3-adrenoceptor effects than the SS isomer. Studies with the β-adrenoceptor agonist trimetoquinol also reveal high functional, but not binding, selectivity for the (−)-isomer for β_3-adrenoceptor mediated responses in rat tissues, and at the cloned rat and human receptor (Konkar et al., 1996, 1999; Fraundorfer et al., 1994). At β_1-/β_2-adrenoceptors, the degree of stereoselectivity with trimetoquinol was equal in both functional and radioligand assays. The SS isomer of the β_3-adrenoceptor antagonist SR59230A is over 10,000 times more potent than its RR isomer (SR59483A) in antagonising β_3-adrenoceptor-mediated increases in cAMP in rat brown adpocyte membranes and relaxation in rat proximal colon (Manara et al., 1996; Nisoli et al., 1996). The majority of β_3-adrenoceptor selective ligands are relatively bulky (the β_3-adrenoceptor agonists have large alkylamine side chains) and bichiral. Often bichiral compounds can fit into the β_3-adrenoceptor binding pocket which is less sterically hindered than that of the β_1-/β_2-adrenoceptor since there are fewer bulky amino acid side chains that protrude into the pocket (Blin et al., 1993; Granneman et al., 1998).

When comparing functional to binding data at the mouse β_3-adrenoceptor, it can be seen that although the stereoselectivities are within a similar range, there appears to be a reversal in the order of stereoselectivity. Comparison with previous data shows that in functional experiments in rat ileum the enantiomeric ratios are of a similar order to those reported here being 43.7 for tertatolol and 19.5 for alprenolol (Roberts et al., 1999). However, in binding studies, little, if any, stereoselectivity was noted with stereoisomers of several ligands in rat ileum (Roberts et al., 1995). This apparent anomaly may be explained by the presence in rat gastrointestinal tract of an abundant 34-kDa binding protein belonging to the TM9SF superfamily which has similar affinity for [^{125}I]CYP to the β_3-adrenoceptor (Sugasawa et al., 1997, 2001).

This study shows that β_3-adrenoceptors display varying degrees of stereoselectivity for several β-adrenoceptor ligands. They display a low degree of stereoselectivity for the agonists isoprenaline and noradrenaline, but show a higher degree of stereoselectivity for antagonists, and, in particular, tertatolol and propranolol. Thus, low stereoselectivity should not be considered a defining characteristic of the β_3-adrenoceptor since there is a relatively high degree of stereoselectivity for high affinity β-adrenoceptor antagonists. In addition, it is clear from the comparison of human, rat and mouse β_3-adrenoceptor that the effect varies between species. At the human β_3-adrenoceptor, in particular, β-adrenoceptor ligands tend to show higher affinities and higher enantiomeric ratios than at mouse or rat receptors.

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