ACCELERATED COMMUNICATION

Structural Basis of Species-Dependent Differential Affinity of 6-Alkoxy-5-Aryl-3-Pyridinecarboxamide Cannabinoid-1 Receptor Antagonists^S

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ABSTRACT

6-Alkoxy-5-aryl-3-pyridinecarboxamides, including the brain-penetrant compound **14g** [5-(4-chlorophenyl)-6-(cyclopropylmethoxy)-*N*-[(1*R*,2*R*)-2-hydroxy-cyclohexyl]-3-pyridinecarboxamide] and its peripherally restricted analog **14h** [5-(4-chlorophenyl)-*N*-[(1*R*,2*R*)-2-hydroxycyclohexyl]-6-(2-methoxyethoxy)-3-pyridinecarboxamide], have been recently introduced as selective, high-affinity antagonists of the human cannabinoid-1 receptor (hCB₁R). Binding analyses revealed two orders of magnitude lower affinity of these compounds for mouse and rat versus

human CB₁R, whereas the affinity of rimonabant is comparable for all three CB₁Rs. Modeling of ligand binding to CB₁R and binding assays with native and mutant (Ile105Met) hCB₁Rs indicate that the Ile105 to Met mutation in rodent CB₁Rs accounts for the species-dependent affinity of **14g** and **14h**. Our work identifies Ile105 as a new pharmacophore component for developing better hCB₁R antagonists and invalidates rodent models for assessing the antiobesity efficacy of **14g** and **14h**.

Introduction

An overactive endocannabinoid/CB₁R system has been implicated in obesity and its metabolic complications (Blüher et al., 2006; Pacher et al., 2006). Globally acting CB₁R antagonists reduce body weight and hepatic fat accumulation and improve insulin sensitivity and dyslipidemia in overweight people who have metabolic syndrome (Despres et al., 2005; Pi-Sunyer et al., 2006) but their therapeutic development was halted because of neuropsychiatric side effects (Le Foll et al., 2009); however, CB₁R in peripheral tissues, including

adipose tissue, liver, skeletal muscle, pancreatic β cells, and tissue macrophages, contribute to the metabolic effects of endocannabinoids (Cota et al., 2003; Liu et al., 2005; Osei-Hyiaman et al., 2005; Kim et al., 2011; Jourdan et al., 2013), suggesting that selective blockade of peripheral CB₁R may provide a therapeutic alternative by avoiding side effects linked to blockade of CB₁R in the central nervous system. Indeed, several recent studies have documented that, when tested in rodent models of obesity and metabolic syndrome or type 2 diabetes, novel CB₁R antagonists with low brain penetrance are devoid of behavioral effects predictive of neuropsychiatric side effects in humans; yet they retained most, if not all, of the metabolic efficacy of globally acting CB₁R antagonists (Tam et al., 2010, 2012; Jourdan et al., 2013).

In a recent study, 6-alkoxy-5-aryl-3-pyridinecarboxamides have been introduced as a new series of orally bioavailable CB₁R antagonists with low nanomolar affinity for the human CB₁R (Röver et al., 2013). Two analogs were tested in that

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ABBREVIATIONS: **14g**, 5-(4-chlorophenyl)-6-(cyclopropylmethoxy)-*N*-[(1*R*,2*R*)-2-hydroxy-cyclohexyl]-3-pyridinecarboxamide; **14h**, 5-(4-chlorophenyl)-*N*-[(1*R*,2*R*)-2-hydroxycyclohexyl]-6-(2-methoxyethoxy)-3-pyridinecarboxamide; CHO, Chinese hamster ovary; DIO, diet-induced obesity; GPCR, G-protein-coupled receptor.

study, one with high and one with low brain penetrance (brain-to-plasma ratios of 1.3 versus 0.13, respectively) in a rat model of high-fat diet-induced obesity (DIO); the analog with high brain penetrance (5-(4-chlorophenyl)-6-(cyclopropylmethoxy)-*N*-[(1*R*,2*R*)-2-hydroxycyclohexyl]-3-pyridinecarboxamide designated as **14g**; Fig. 1) caused significant delays in weight gain and adiposity, but the analog with low brain penetrance [5-(4-chlorophenyl)-*N*-[(1*R*,2*R*)-2-hydroxycyclohexyl]-6-(2-methoxyethoxy)-3-pyridinecarboxamide], designated **14h** (Fig. 1), did not. This finding led the authors to the conclusion that the antiobesity efficacy of CB₁R blockade is primarily centrally mediated (Röver et al., 2013).

The rats fed high-fat diets used in the preceding study represent a weak model of the metabolic syndrome because only a subset of the animals develop modest weight gain beyond the normal growth of these animals, and they do not display an increase in the accumulation of ectopic fat in the liver or changes in plasma leptin and insulin concentrations, which would indicate insulin or leptin resistance (Flamment et al., 2009; Röver et al., 2013). We aimed to test the effects of these two compounds in DIO mice, a more robust model of the metabolic syndrome (Collins et al., 2004), for which we needed to establish affinities for the mouse CB₁R. Unexpectedly, we found that the binding affinity (K_i) of both compounds was one to two orders of magnitude lower for both the mouse and rat CB₁R relative to their affinities for the human CB₁R and accordingly failed to elicit CB₁R blockade in functional assays *in vivo*. This prompted us to analyze these ligands along with the reference compound rimonabant, which has equal high CB₁R binding affinity in the three species (Govaerts et al., 2004), for their interaction with mouse, rat, and human CB₁R using molecular modeling. The resulting binding model, strongly supported by site-directed mutagenesis of a key residue in human versus mouse CB₁R, provides the likely structural basis of these striking species-dependent differences, unprecedented among other CB₁R ligands introduced to date (Govaerts et al., 2004; Thomas et al., 2005).

Materials and Methods

CB₁R Antagonists. Compounds **14g** and **14h** were synthesized as described (Röver et al., 2013). The correct structure was verified by nuclear magnetic resonance spectroscopy and, for compound **14h**, by X-ray crystallography (Supplemental Fig. 1). Rimonabant (SR141716) was provided by the National Institute on Drug Abuse drug supply program.

X-Ray Diffraction. Details are provided in the Supplemental Material.

CB₁R Binding Assays. The binding affinity of different antagonists and inverse agonists was determined in radioligand displacement assays using 1 nM [³H]CP55940 as the agonist radioligand and plasma membrane preparations from mouse or rat brains or from Chinese hamster ovary (CHO) cells transfected with the cDNA encoding the human CB₁R. K_i values were derived by computerized curve fitting and using the Cheng-Prusoff equation to account for the affinity of the radioligand by using the GraphPad Prism 6 program (GraphPad Prism Software Inc., San Diego, CA). Nonspecific binding was determined in the presence of 1 μ M rimonabant and accounted for <20% of total binding. Plasma membranes were prepared as described from mouse and rat brains (Compton et al., 1993) and from cultured CHO-K1 cells (PerkinElmer, Waltham, MA).

CB₁R Functional Antagonism. The inhibitory potency of antagonists and inverse agonists was determined by the concentration-dependent inhibition of 1 μ M CP55940-stimulated [³⁵S]GTP γ S binding, assayed as described earlier (Griffin et al., 1998). In some assays, full agonist dose-response curves were generated in the absence or presence of increasing concentrations of antagonists to generate Schild plots for testing the competitive nature of the antagonism (Sim-Selley et al., 2001). Concentration-response relations were analyzed by sigmoidal curve fitting using the GraphPad Prism 6 program. Crude membrane fractions (10 μ g) of mouse brains were incubated with 0.05 nM [³⁵S]GTP γ S, and the indicated concentrations of ligands in TEM buffer (50 mM Tris-HCl, 0.2 mM EGTA, and 9 mM MgCl₂, pH 7.4) containing 100 μ M GDP, 150 mM NaCl, and 0.1% (w/v) bovine serum albumin in a total volume of 1 ml for 60 minutes at 30°C. Nonspecific binding was determined in the presence of 10 μ M GTP γ S, and at baseline, it represented <10% of total binding. Agonist-stimulated GTP γ S binding was expressed as the percent of increase over baseline. Bound and free [³⁵S]GTP γ S levels were separated by vacuum filtration through Whatman GF/B filters using a Brandel M24 Cell Harvester (Gaithersburg, MD). Filters were washed with 3 $\times$ 5-ml of ice-cold buffer, and radioactivity was detected by scintillation spectrometry (LS6500; Beckman Coulter).

Ligand Modeling. The X-ray structure of **14h** (Supplemental Fig. 1) was used as a template to build **14g**. The compound **32a** was built based on the structure shown in (Röver et al., 2013). The conformers of these ligands were obtained by varying the dihedral angle ϕ as defined in Fig. 3A while relaxing the rest of the structure at the level of B3LYP/6-31G* as implemented in the Gaussian 09 software (http://www.gaussian.com/g_prod/g09.htm). The X-ray structure of rimonabant was retrieved from the Cambridge Crystallographic Data Centre as CCDC 924604 (Perrin et al., 2013). Its 2,4-dichlorophenyl ring was then rotated with respect to the C1-N1 bond axis, and a more stable conformer was used for a docking study. Each of the conformers in the study was fully geometry-optimized without any constraint. The calculated Gibbs free energy of each conformer includes the electronic energy as well as the thermal and entropy contribution at 298.15K.

CB₁R Modeling. A model of human CB₁R was built using Prime software (Schrodinger, LLC, New York, NY). The crystal structure of the sphingosine 1-phosphate receptor fused to T4-lysozyme with a sphingolipid mimic bound was chosen as the template (PDB ID 3V2W) (Hanson et al., 2012). Of the 293 residues modeled in CB₁R (ranging from F89 to M411), 83 (28%) are nearest to identical residues in the template structure (Supplemental Material 1).

Human and Mouse *CNR1* Mutagenesis. The human *CNR1* (NM_007726) open reading frame was inserted into the mammalian expression vector pCI (Promega, Madison, WI). The mouse *Cnr1* (NM_0011602586) open reading frame in pcDNA3 (Life Technologies, Carlsbad, CA) was kindly provided by Dr. Mary E. Abood. Mutagenesis was performed using the QuikChange site directed mutagenesis system from Agilent Technologies (Santa Clara, CA). The human *CNR1* was mutated at amino acid position 105 from Ile to Met using the following primer and its complement (mutated codon is indicated): *hCNR1* I105M: 5'-GAGAACTTCATGGACATGGAGTGTTCATGGTC-3'. The mouse *Cnr1* was mutated at amino acid position 106 from Met to Ile using the following primer and its complement: *mCnr1* M106I: 5'-GGAGAATTTTATGGACATAGAGTGCTTCATGATTCTG-3'. Mutations were verified by sequence analysis (MacroGen USA, Rockville, MD), and plasmids were prepared using the QIAfilter Plasmid Maxi Kit (Qiagen, Limburg, The Netherlands).

Cell Culture and Plasmid Transfection. Human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA) were maintained in EMEM with 10% FBS. Cells were transfected with different plasmids (hCB₁R-Ile105, hCB₁R-mutant met105, mCB₁R-met106, and mCB₁R-mutant Ile106) using LipofectAMINE 2000 (Life Technologies) according to the manufacturer's protocol. Transfected cells were harvested after 48 hours, and membranes prepared for receptor binding assays as described (Abood et al., 1997).

Fig. 1. Chemical structure (A), binding affinity (B), and inhibitory potency (C) of rimonabant and compounds **14g** and **14h** to human, rat, and mouse CB₁ receptors. K_i values for binding and functional antagonism were determined as described in *Materials and Methods*. Points and vertical bars represent means $\pm$ S.E. from four to six independent assays. Hill slopes were not significantly different from 1.0. (B) Specific binding (fmol/mg protein) in the absence of competitor was 2236 $\pm$ 30 (hCB₁CHO), 225 $\pm$ 6 (rat brain), or 222 $\pm$ 14 (mouse brain) and was defined as 100%. (C) Basal GTP γ S specific binding was 187 $\pm$ 5 fmol/mg of protein.

Upper Gastrointestinal Motility Assay. Animal protocols were approved by the Institutional Animal Care and Use Committee of the National Institute of Alcohol Abuse and Alcoholism, National Institutes of Health. Drugs (CB₁R antagonists, arachidonoyl-2'-chloroethylamine, and their combination) or vehicle were administered orally by gavage to male, 8- to 10-week-old C57Bl6/J mice 1 hour before oral administration of the marker (10% charcoal suspension in 5% gum arabic). Thirty minutes later, mice were killed, and the distance traveled by the head of the marker between the pylorus, and the cecum was measured and expressed as the percent of total length of the small intestine.

Hyperambulatory Activity. The locomotor activity of drug-naïve mice treated with an oral bolus dose of a CB₁R antagonist or vehicle was quantified by the number of disruptions of infrared beams in two dimensions in an activity chamber.

Results and Discussion

We synthesized compounds **14g** and **14h** (Fig. 1A) as described (Röver et al., 2013). The CB₁R binding affinity of the compounds was then analyzed in CHO cells transfected with the human CB₁R. The low nanomolar affinity of both compounds was similar to published values (Röver et al., 2013) and also to that of the binding affinity of the reference compound rimonabant (Fig. 1B). In the same cells, **14g** and **14h**

also displayed high potency as competitive inhibitors of CB₁R agonist-stimulated GTP γ S activity, as derived from Schild plots (Supplemental Fig. 2). Because we wanted to test the compounds in a mouse model of DIO, we also tested their binding to mouse brain CB₁R. Surprisingly, the binding affinities of both **14g** and **14h** were 20- to 30-fold lower for mouse CB₁R compared with human CB₁R, but the affinity of rimonabant was comparable for mouse and human CB₁R (Fig. 1B). This finding represents the first known example of a substantial species-specific difference in the affinity of a CB₁R antagonist; we also tested all three compounds for binding to the rat brain CB₁R, which differs in only one amino acid from the mouse CB₁R. In contrast, the human CB₁R, is one amino acid shorter and differs in an additional 13 amino acid residues from the mouse CB₁R. As seen in Fig. 1B, binding to the rat CB₁R revealed the same pattern as seen with the mouse CB₁R; that is, rimonabant retained its high affinity, whereas **14g** and **14h** both had binding affinities two orders of magnitude lower and similar low potencies in a functional assay as inhibitors of agonist-stimulated GTP γ S binding (Fig. 1C).

Peak plasma concentrations of **14g** and **14h** in rats after an oral dose of 8 to 9 mg/kg were reported in the range of 1–3 μM , with 96–98% of the ligands plasma protein bound (Table 5 in

Röver et al., 2013). Thus, the concentration of unbound ligand (20–60 nM) was well below the K_i for the rat or mouse CB₁R for both ligands, making it highly unlikely that functional CB₁R blockade was achieved even at the somewhat higher dose of 30 mg/kg used in the earlier metabolic studies (Röver et al., 2013). To test this hypothesis, we treated normal control mice with a single oral dose of 10 or 30 mg/kg **14g** or **14h** or 10 mg/kg rimonabant and examined their effects in the upper gastrointestinal motility assay (Izzo et al., 2001), the industry standard for quantifying peripheral CB₁R blockade. As shown in Fig. 2A, upper gastrointestinal motility as quantified by the distance traveled in 30 minutes by an oral charcoal bolus along the small intestine was profoundly inhibited by pretreatment with 10 mg/kg of the CB₁R agonist arachidonyl-2'-chloroethylamide, and inhibition was completely prevented by rimonabant but remained unaffected by 10 or 30 mg/kg of either **14g** or **14h** (Fig. 2A). Similarly, rimonabant, but not **14g** or **14h**, caused hyperambulatory activity in drug-naïve wild-type but not CB₁R^{-/-} mice (Fig. 2B). The hyperambulatory activity was associated with stereotypic behavior, such as scratching and grooming (unpublished data), and it is a recognized indicator of blockade of central CB₁R (Kunz et al., 2008; Tam et al., 2010, 2012). Thus, the reported inhibition of the modest diet-induced weight gain in rats by chronic treatment with the brain penetrant **14g** compound (Röver et al., 2013) may not be attributed to CB₁R blockade but rather to an off-target effect. Similarly, the lack of an inhibitory effect of **14h** is not relevant to the question of whether peripheral CB₁R contributes to the metabolic benefit of CB₁R blockade, as proposed (Röver et al., 2013).

The unique pharmacologic property of compounds **14g** and **14h** to discriminate between human and rodent CB₁R could be exploited to gain new insight into the structure of the presumed binding pocket. We did molecular modeling of the CB₁R to explore the possible role of nonhomologous amino acid residues of human versus rodent CB₁R that might account for the differential affinities of these ligands. As illustrated in Fig. 3A, compound **14g** can exist in two conformations (**A** and **B**) because of a small energy difference, as indicated by quantum chemical calculations at the density functional level of B3LYP/6-31G*. Note that **14g** was constructed based on the X-ray structure of compound **14h** (Supplemental Fig. 1). Of the two, conformer **B** rather than **A** is likely recognized by the human, rat, or mouse CB₁R. This hypothesis is based on the comparison of the pharmacologic properties of the core variants of the pyridine ring of **14g**, as indicated in Table 4 in Röver et al. (2013). For example, conformer **A** of the compound **32a** is calculated to be 9.4 kcal/mol more stable than conformer **B** of the same compound, as illustrated in Fig. 3B. This energy difference virtually assures that only **A** of **32a** exists in solution, and thus the reported significant decrease in K_i of **32a** (>7000 nM for human CB₁R) (Röver et al., 2013) likely stems from the unfavorable orientation of the chlorophenyl and/or cyclopropyl moiety of **A** in the binding pocket of CB₁R. A similar trend was observed for compounds **37a** and **51** (Röver et al., 2013). This finding in turn suggests that the conformer **B** of **14h** is also preferentially recognized by the CB₁R, as illustrated in Fig. 3C. It has been noted that **14h** is crystallized as conformer **B**. To rationalize the differential affinity of **14g** and **14h** versus rimonabant toward the human, rat, and mouse

Fig. 2. In vivo assays to assess peripheral and central CB₁R blockade. (A) The upper gastrointestinal motility assay was conducted as described in *Materials and Methods*. Note that rimonabant, but not compound **14g** or **14h**, blocks the CB₁R agonist (Farghali, no. 2150)-induced inhibition of upper gastrointestinal motility. Columns and vertical bars represent means $\pm$ S.E. from four separate experiments. *, #: Significant difference ($P < 0.01$) from values in vehicle (Veh) + vehicle-treated or vehicle + arachidonyl-2'-chloroethylamide (ACEA)-treated mice, respectively. (B and C) Ambulatory activity in drug-naïve mice was quantified as described in *Materials and Methods*. Note that rimonabant, but not **14g** or **14h**, causes long lasting hyperambulatory activity in wild-type mice (B) but not in CB₁R^{-/-} mice (C).

Fig. 3. Geometry optimized A and B conformers of **14g** (A), **32a** (B), and **14h** (C), at the level of B3LYP/6-31G* in the gaseous phase. Values in parentheses represent the Gibbs free energy in kcal/mol relative to that of A at 298.15 K. The conversion of B to A was done by varying the dihedral angle φ centered on the C-C bond as indicated by the arrow. Atom coloring: white, hydrogen; green, carbon; dark green, chlorine; blue, nitrogen; red, oxygen.

CB₁R, a model of human CB₁R was built based on the X-ray structure of a lipid G-protein-coupled receptor (GPCR) (Hanson et al., 2012). Earlier models have been proposed based on the structures of bovine rhodopsin (Hurst et al., 2002; McAllister et al., 2003; Shim et al., 2003; Salo et al., 2004; Silvestri et al., 2008) or the β 2-adrenergic receptor (Shim et al., 2012). The more recent structure used here as template affords a sequence alignment that is almost devoid of insertions and deletions. Thus, extracellular structure can be modeled readily for CB₁R, under the assumption that it is similar to that in the lipid GPCR (Supplemental Material 1).

The mouse CB₁R is one amino acid longer than the human CB₁R, and the two differ in 13 additional amino acids, most of which are located at the N-terminal tail or in extracellular loops (Supplemental Fig. 4). Our attention turned quickly to Ile105 because the side chain of this isoleucine points directly into the pocket of the modeled CB₁R (Fig. 4A). The three compounds of interest were manually docked into the human CB₁R model, taking into account the binding affinity data of rimonabant and other inverse agonists on CB₁R mutants, such as W279A (Sitkoff et al., 2011) and S383A (Kapur et al., 2007; Lin et al., 2008). Figure 4 depicts the putative binding modes of rimonabant and **14h** and suggests that Ser383 can H-bond to the nitrogen of the pyridine ring of **14h**. Moreover, Ile105 in the human CB₁R is positioned to provide van der Waals interaction with the cyclohexanol of **14h**. The mouse and rat CB₁Rs have an equivalent methionine at position 106

(see Supplemental Fig. 4), which likely alters the protein-ligand interactions (Fig. 4B) and thus may explain the 20-fold lower K_i of **14h** for the rat compared with human CB₁R. Note that the 20-fold difference in K_i corresponds to 1.8 kcal/mol, which could result from the different interactions with Ile and Met at position 105/106 and/or the altered packing of adjacent residues.

To test the validity of this model, we mutated Ile105 in the human CB₁R to methionine and Met106 in the mouse CB₁R to isoleucine and transiently expressed the two native receptors and their mutants in human embryonic kidney cells. Figure 5 illustrates the results of radioligand displacement assays using membrane preparations from the four cell lines and rimonabant or compound **14h** as the unlabeled ligand. Whereas rimonabant had similar high affinity for the two native receptors and their mutants (Fig. 5, A and B), the Ile105Met mutation of the human CB₁R resulted in a >100-fold decrease in the K_i of **14h** (Fig. 5C); conversely, the low affinity of **14h** to the native mouse CB₁R increased by two orders of magnitude in the Met106Ile mutants (Fig. 5D). These findings clearly confirm the critical role of Ile105Met in the differential affinity of **14h** for the human versus mouse CB₁R, as postulated in our model. Since the structures of **14g** and **14h** are essentially identical except at 6-alkoxy (Supplemental Fig. 3), **14g** is expected to display similar differential affinity to the wild type and the mutant CB₁Rs.

The putative binding mode of rimonabant is depicted in Fig. 4A. This mode is compatible with the nondiscriminatory binding affinity of rimonabant toward all three CB₁R orthologs. Compared with **14g** and **14h**, the aromatic portion of rimonabant was docked to be closer to the hydrophobic pocket formed by F268, W279, and W356, resulting in a sizable separation between Ile105 and the piperidine ring of

Fig. 4. Model of rimonabant and **14h** binding to human CB₁R. (A) The homology model of human CB₁R is depicted with the protein backbone colored by sequence identity to the template X-ray structure (gold where identical, light blue where different). The side chains of Ile105, Phe268, Trp279, and Trp356 are shown as space-filling and those of Glu94, Val110, Ile175, Arg186, Glu258, and His270 (where human CB₁R differs from rat and/or mouse CB₁R) are shown as ball-and-stick. Rimonabant and **14h** are shown manually docked in the pocket of the receptor as ball-and-stick with carbon atoms colored green and gray, respectively. (B) Close-up, rotated view of the docked ligands in the receptor pocket, colored as in (A). This figure was created using the programs MolScript (Kraulis, 1991) and Raster3D (Merritt and Bacon, 1997).

Fig. 5. Binding of rimonabant (A and B) and compound **14h** (C and D) to wild-type and mutated human and mouse CB₁R. Radioligand binding displacement by compound **14h** was tested using plasma membrane preparations from CHO cells transfected with cDNA for the wild-type human or mouse CB₁R, with an Ile105-Met mutant of human CB₁R, or a Met106Ile mutant of mouse CB₁R. [³H]CP55940 (2 nM) was used as the labeled ligand. Points and vertical bars are means $\pm$ S.E. from four independent assays.

	hCB ₁ R I105	hCB ₁ R mutant M105	mCB ₁ R M106	mCB ₁ R mutant I106
Compound 14h K _i [nM]	5 $\pm$ 1	771 $\pm$ 27	614 $\pm$ 22	8 $\pm$ 4
Rimonabant K _i [nM]	1.8 $\pm$ 0.5	1.9 $\pm$ 0.6	3.4 $\pm$ 0.3	3.4 $\pm$ 0.2

rimonabant. As a consequence, neither Ile105 nor Met105 influences the binding affinity of rimonabant. Figure 4B shows the positions of both the cyclohexanol of **14h** and the piperidine of rimonabant in the binding pocket of the human CB₁R.

In summary, an unprecedented species-dependent difference in the binding affinity of a novel class of CB₁R antagonist to human versus rodent CB₁R was uncovered, and its structural basis was analyzed by molecular modeling based on the structure of a related lipid GPCR. The structural analysis suggested that the mutation of Ile to Met at position 105 most likely accounts for the lower affinity of 6-alkoxy-5-aryl-3-pyridincarboxamide CB₁R antagonists for rodent than for human CB₁R, and this hypothesis was then strongly supported by binding assays performed on site-directed mutants altered at this position. CB₁R antagonists have therapeutic potential in obesity-metabolic syndrome, and peripherally restricted analogs have been reported to retain metabolic efficacy with much reduced neuropsychiatric liability. The present findings highlight the importance of analyzing the interaction of CB₁R antagonists not only with the therapeutic target human CB₁R but also with the CB₁R

of rodents used to generate preclinical proof of principle to reach valid conclusions about mechanism of action.

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Authorship Contributions

Participated in research design: Kunos, Steinbach, Lee, Iyer, Cinar, Ikeda.

Conducted experiments: Cinar, Steinbach, Lee, Iyer, Puhl, Deschamps, Liu, Szanda, Godlewski.

Performed data analysis: Steinbach, Lee, Cinar, Iyer.

Wrote or contributed to the writing of the manuscript: Kunos, Steinbach, Lee, Iyer, Cinar.

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