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Binding thermodynamics at the human cannabinoid CB_1 and CB_2 receptors

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ABSTRACT

The thermodynamic parameters ΔG°, ΔH° and ΔS° of the binding equilibrium of agonists and antagonists at cannabinoid CB_1 and CB_2 receptors were determined by means of affinity measurements at different temperatures and van't Hoff plots were constructed. Affinity constants were measured on CHO cells transfected with the human CB_1 and CB_2 receptors by inhibition assays of the binding of the cannabinoid receptor agonist [^{3}H]-CP-55,940. van't Hoff plots were linear for agonists and antagonists in the temperature range 0–30 °C. The thermodynamic parameters for CB_1 receptors fall in the ranges $17 \leq \Delta H^\circ \leq 59$ kJ/mol and $213 \leq \Delta S^\circ \leq 361$ kJ/mol for agonists and $-52 \leq \Delta H^\circ \leq -26$ kJ/mol and $-12 \leq \Delta S^\circ \leq 38$ kJ/mol for antagonists. The thermodynamic parameters for CB_2 receptors fall in the ranges $27 \leq \Delta H^\circ \leq 48$ kJ/mol and $234 \leq \Delta S^\circ \leq 300$ kJ/mol for agonists and $-19 \leq \Delta H^\circ \leq -17$ kJ/mol and $43 \leq \Delta S^\circ \leq 74$ kJ/mol for antagonists. Collectively, these data show that agonist binding is always totally entropy-driven while antagonist binding is enthalpy and entropy-driven, indicating that CB_1 and CB_2 receptors are thermodynamically discriminated. These data could give new details on the nature of the forces driving the CB_1 and CB_2 binding at a molecular level. Enthalpy, entropy, free energy and binding affinity for each ligand to its receptor can all be assessed and therefore the optimal binding profile discovered. Carrying out these binding investigations as early as possible in the discovery process increases the probability that a lead compound will become a successful pharmaceutical compound.

1. Introduction

The thermodynamic analysis of the binding equilibrium of a drug to its receptor allows us to evaluate the two components, standard enthalpy (ΔH°) and standard entropy (ΔS°), of the standard free energy (ΔG°) of the binding equilibrium [1]. It is often assumed that ΔH° and ΔS° terms represent the two classes of factors responsible for the drug–receptor recognition phenomenon: non-bonded interactions, as hydrogen bonding and multipolar or dispersive interactions (which are mainly to be related to the enthalpic term), and solvent reorganization (which is most properly associated with the entropic one) [2]. There are two main strategies for the evaluation of ΔG°, ΔH° and ΔS° terms. The first consists in determining equilibrium constants $\Delta G^\circ = -RT \ln K_A$ in association with direct microcalorimetric enthalpy measurements, even if this method is not practicable in binding studies for the very low receptor concentration in most tissues. The only method of practical use consists in measurements of K_A carried out at different temperatures followed by van't Hoff analysis. Such a method has proved to be successful in many cases. Until now, elegant studies have been carried out on several receptor systems, demonstrating the value of thermodynamics for investigating receptor–ligand interactions [3,4]. In addition, some of these studies have suggested that measurement of thermodynamic parameters can allow the discrimination of agonist and antagonist ligands. In particular, the binding of agonists may be entropy-driven and that of antagonists enthalpy-driven, or *vice versa*. Such a phenomenon has been defined "thermodynamic discrimination" and has been reported for β-adrenergic, glycine, $GABA_A$, histamine H_3, serotonin 5-HT_3, nicotinic, purinergic $P2X_3$ and A_1, A_{2A}, A_{2B} and A_3 adenosine receptors [5–16]. However, there are also studies suggesting that this general thermodynamic distinction between agonists and antagonists may not be applicable to all receptor systems. It is notable that histamine H_1, cholecystokinin CCK_2,

Abbreviations: ACEA, arachidonyl-2′-chloroethylamide; AM 251, *N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM 281, *N*-(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM 630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone(6-iodopravadoline); CHO, Chinese hamster ovary; CP-55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; hCHO-CB_1, CHO cells transfected with human CB_1 cannabinoid receptor; hCHO-CB_2, CHO cells transfected with human CB_2 cannabinoid receptor; 2-Fl-AEA, arachidonyl-2′-fluoroethylamide; JWH-015, (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone; WIN 55212, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanonemesylate.

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δ- and μ-opioid, purinergic $P2X_1$, dopamine D_2 and serotonin 5-HT_{1A} receptors cannot be thermodynamically discriminated [17–23].

Cannabinoids exert most of their effects by binding to $G_{i/o}$ protein-coupled cannabinoid receptors, called CB_1 and CB_2 [24]. The CB_1 receptor is particularly abundant in discrete areas of the brain, but is also expressed in peripheral nerve terminals and various extra-neural sites. In contrast, the CB_2 receptor was initially described to be present in the immune system, although recently it has been shown that expression of this receptor also occurs in cells from other origins [24]. The broad expression profile of cannabinoid receptors in the central nervous system and periphery suggests that the targeting of specific cannabinoid receptors or their downstream signalling pathways will be an essential consideration in drug development. Therapeutic potential exists for the use of cannabinoid compounds as neuroprotective agents; to alleviate pain; and to treat anxiety, emesis, obesity, movement disorders, and glaucoma. Subtype-specific ligands, as well as the use of potentiators and partial agonists, may help to eliminate side effects associated with classical cannabinoids. However, the pharmacology of cannabinoid ligands is strikingly similar between the two receptors, although their sequence homology at the proposed ligand-binding domains is only 48%, substantially less than the 70–80% usually seen between different types of G protein-coupled receptors [24]. The characterization of the enthalpy and entropy contribution to binding at cannabinoid receptors could provide information important for defining ligand binding sites, with clear implications for further development of cannabinoid ligands. However, there has been no thermodynamic analysis of ligand binding at the cannabinoid CB_1 and CB_2 receptors. Therefore, with the aim of contributing to an evaluation of the molecular mechanism underlying ligand–receptor interactions, this paper reports the results derived from the study of binding thermodynamics of five agonists and three antagonists to human CB_1 and CB_2 receptors transfected in CHO cells. The results provide useful information on the binding mechanisms of cannabinoid receptor ligands and demonstrate that agonists and antagonists at CB_1 and CB_2 receptors could be thermodynamically discriminated.

2. Materials and methods

2.1. Materials

[^{3}H]-CP-55,940 (specific activity 139.6 Ci/mmol) was obtained from PerkinElmer Life Sciences, (Milano, Italy). WIN 55212, JWH-015, ACEA, 2-Fl-AEA, CP-55,940, AM630, AM281 and AM251 were obtained from Vinci-Biochem (Firenze, Italy). CHO cells transfected with the human recombinant CB_1 (hCHO-CB_1) and CB_2 receptor (hCHO-CB_2) were obtained from PerkinElmer (Milano, Italy). All other reagents were purchased from Sigma–Aldrich (Milano, Italy).

2.2. Cell culture

hCHO-CB_1 and hCHO-CB_2 were grown adherently and maintained in Ham's Medium with nutrient mixture F12 (Ham's/F12), containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and geneticin (G418, 0.4 mg/ml) at 37 °C in 5% CO_2/95% air. Cells were split 2 or 3 times weekly at a ratio between 1:5 and 1:20.

2.3. Membrane preparation

For membrane preparation the culture medium was removed. The cells were washed with PBS and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris–HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with Polytron and the homogenate was spun for 10 min at 1000 × *g*. The supernatant was then centrifuged for 30 min at 100,000 × *g*. The membrane pellet was resuspended in 50 mM Tris–HCl–0.5% bovine serum albumin (BSA) containing 5 mM $MgCl_2$, 2.5 mM EDTA or 1 mM EDTA for CB_1 and CB_2 receptors, respectively. The protein concentration was determined according to a Bio-Rad method [25] with BSA as a standard reference. Then the suspension was frozen at −80 °C.

2.4. Kinetics of [^{3}H]-CP-55,940 binding

Kinetic studies of [^{3}H]-CP-55,940 binding were performed by incubating membranes in 50 mM Tris–HCl–0.5% BSA containing 5 mM $MgCl_2$, 2.5 mM EDTA or 1 mM EDTA for CB_1 and CB_2 receptors, respectively pH 7.4 in a thermostatic bath at the appropriate temperatures (10 °C, 20 °C and 30 °C or 0 °C, 20 °C and 30 °C for CB_1 and CB_2 cannabinoid receptors, respectively). Then, the reaction was terminated at different time intervals (from 15 to 300 min) by rapid filtration under vacuum, followed by washing four times with ice-cold buffer. Non-specific binding was defined as binding in the presence of 1 μM WIN55212 and was about 20% of total binding. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass-fiber filters using a Micro-Mate 196 cell harvester (PerkinElmer Life Sciences, Milano, Italy). The filter bound radioactivity was counted using a Microplate Scintillation Counter (Top Count, Meriden, CT) at an efficiency of 57% with Micro-Scint 20 (PerkinElmer Life Sciences, Milano, Italy).

2.5. Saturation and competition binding experiments

Saturation and competition binding assays were performed on hCHO-CB_1 and hCHO-CB_2 membranes at different temperatures in a thermostatic bath assuring a temperature of ±0.1 °C. All buffer solutions were adjusted to maintain a constant pH of 7.4 at the desired temperature. Saturation binding experiments of [^{3}H]-CP-55,940 (0.1–60 nM) to the membranes previously obtained were performed in 50 mM Tris–HCl–0.5% BSA containing 5 mM $MgCl_2$, 2.5 mM EDTA or 1 mM EDTA for CB_1 and CB_2 receptors, respectively pH 7.4 for different incubation times according to the results of the time-course experiments. In particular, the incubation time was 180 min at 10 °C, 150 min at 20 °C and 120 min at 30 °C for CB_1 receptor binding. The incubation time was 180 min at 0 °C, 90 min at 20 °C and 60 min at 30 °C for CB_2 receptor binding. Competition experiments of [^{3}H]-CP-55,940 were performed in duplicate in test tubes containing the buffer, the membranes and at least 8–10 different concentrations of typical cannabinoid receptor agonists and antagonists. Non-specific binding was defined as binding in the presence of 1 μM WIN55212 and was about 20% of total binding. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass-fiber filters using a Micro-Mate 196 cell harvester (PerkinElmer Life Sciences, Milano, Italy). The filter bound radioactivity was counted using a Microplate Scintillation Counter (Top Count, Meriden, CT) at an efficiency of 57% with Micro-Scint 20 (PerkinElmer Life Sciences, Milano, Italy). All binding data were analysed using the non-linear regression curve-fitting computer program LIGAND [26]. The affinity values expressed as K_D or K_i were used in the thermodynamic parameter determination.

2.6. Thermodynamic data determination

Determination of ΔG°, ΔH° and ΔS° values has been obtained by measurements of K_A (equilibrium association constant) carried out at different temperatures, followed by van't Hoff analysis. Two cases are to be distinguished: (1) the standard specific heat difference of the equilibrium (ΔC_p°) is essentially zero. In this case

the van't Hoff equation $\ln K_A = -\Delta H^\circ/RT + \Delta S^\circ/R$ gives a linear plot $\ln K_A$ versus $1/T$. The standard free energy can be calculated as $\Delta G^\circ = -RT \ln K_A$ at 298.15 K; the standard enthalpy (ΔH°) and the standard entropy (ΔS°) can be obtained from the slope ($-\Delta H^\circ/R$) and the intercept ($\Delta S^\circ/R$) of the van't Hoff plot $\ln K_A$ versus $1/T$, respectively, with $R = 8.314$ J/K/mol. The linearity of van't Hoff plots is not common in reactions involving biomacromolecules in solution but appears to be typical as far as membrane receptor binding is concerned [27]. (2) ΔC_p° is not equal to zero [9,28]. In this case the plot ΔG° versus T is often parabolic and other mathematical methods for calculating the thermodynamic parameters of the equilibrium are available.

In the present case the van't Hoff plots can be considered to be essentially linear and the first method was applied.

3. Results

3.1. Kinetic binding assays to human CB_1 and CB_2 cannabinoid receptors

Kinetic behaviour of [^{3}H]-CP-55,940 binding was studied at 10 °C, 20 °C and 30 °C or 0 °C, 20 °C and 30 °C for CB_1 and CB_2 cannabinoid receptors, respectively. Fig. 1 shows that [^{3}H]-CP-55,940 binding reached equilibrium after approximately 180 min at 10 °C, 150 min at 20 °C and 120 min at 30 °C for CB_1 receptor binding. Association studies for [^{3}H]-CP-55,940 binding to CB_2 cannabinoid receptors indicated that the equilibrium was reached after approximately 180 min at 0 °C, 90 min at 20 °C and 60 min at 30 °C for CB_2 receptor binding. [^{3}H]-CP-55,940 binding was then stable for at least 2 h.

3.2. Saturation binding assays to human CB_1 and CB_2 cannabinoid receptors

Saturation binding experiments in CHO cells were performed to characterize human CB_1 and CB_2 cannabinoid receptors and evaluate affinity (K_D) and receptor density (B_{MAX}) values (Table 1). These binding parameters were determined at various temperatures by using [^{3}H]-CP-55,940 as radioligand at different concentrations. At 0 °C no specific binding of [^{3}H]-CP-55,940 to CB_1 cannabinoid receptor was observed. Then, the saturation binding experiments were performed from 10 °C up to 30 °C and from 0 °C up to 30 °C for the CB_1 and CB_2 cannabinoid receptors, respectively. In both cannabinoid receptors examined the K_D values change with temperature while B_{MAX} values appear to be largely independent of it. Fig. 2 shows the representative Scatchard plots obtained at the five (Fig. 2A) and four (Fig. 2B) temperatures investigated for the saturation equilibrium of [^{3}H]-CP-55,940 to human CB_1 and CB_2 cannabinoid receptors, respectively. The plots are essentially linear at all the temperatures investigated and computer analysis of the data failed to show a significantly better fit to a two-site than to a one-site binding model, indicating that only one class of high affinity binding sites is present under our experimental conditions.

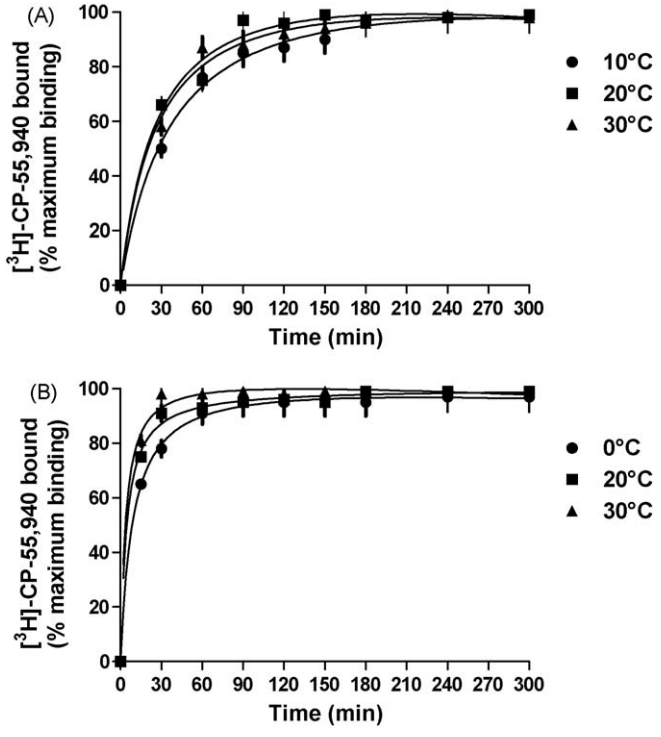

Fig. 1. Kinetics of [^{3}H]-CP-55,940 binding to human CB_1 (panel A) and CB_2 (panel B) cloned receptors expressed in CHO cells at different temperatures.

3.3. Competition binding assays to human CB_1 and CB_2 cannabinoid receptors

Table 2 reports the inhibitory binding constants K_i for agonists and antagonists at human CB_1 and CB_2 cannabinoid receptors stably expressed in CHO cells. These values were determined at various temperatures by displacement of the radiolabeled agonist [^{3}H]-CP-55,940. The order of potency in [^{3}H]-CP-55,940 competition assays for cannabinoid agonists in hCHO-CB_1 was as follows: CP-55,940 > ACEA > 2-Fl-AEA > WIN 55212 > JWH-015. A different order of potency, even if the higher affinity value was always for the agonist CP-55,940, was obtained in hCHO-CB_2: CP-55,940 > WIN 55212 > JWH-015 > 2-Fl-AEA > ACEA. The order of potency of antagonists in hCHO-CB_1 and in hCHO-CB_2 cells was as follows: AM251 > AM281 > AM630 and AM630 > AM251 > AM281, respectively.

3.4. Thermodynamic analysis to human CB_1 and CB_2 cannabinoid receptors

Figs. 3 and 4 illustrate the van't Hoff plots, $\ln K_A$ versus $1/T$, for CB_1 and CB_2 receptor, respectively. All plots appear to be linear in the full temperature range (0–30 °C). In particular, the slopes are systematically negative for agonists and positive for antagonists. The negative slopes of van't Hoff plots for agonists indicate that affinities increase with the increase of temperature while those negative for antagonists demonstrate that affinities are improved by a decrease in temperature (Table 2). Final thermodynamic parameters of the different compounds investigated are reported in Table 3. Fig. 5 summarizes the results in the form of a $-T\Delta S^\circ$ versus ΔH° scatter plot ($T = 298.15$ K). It becomes apparent that all points are arranged on the same diagonal band encompassed between the two dashed lines which represent the loci of points defined by the limiting K_D values of 100 μM and 10 pM. This band is the expression of the enthalpy–entropy (E–E) compensation phenomenon [27]. In CB_1 cannabinoid receptors ΔG° values range from −51.2 to −36.1 kJ/mol for agonists and from −48.8 to −33.2 kJ/mol for antagonists. In CB_2 cannabinoid receptors ΔG° values range from −47.9 to −39.6 kJ/mol for agonists and from −41.2 to −32.2 kJ/mol for antagonists. Equilibrium standard enthalpy (ΔH°) and entropy (ΔS°) values show that the binding of CB_1 and CB_2 receptor agonists is always totally entropy-driven, ΔH° values ranging from 17 to 59 and from 24 to 48 kJ/mol, and ΔS° values ranging from 213 to 361 and from 234 to 300 J/mol/° for CB_1 and CB_2 receptors, respectively. On the contrary, the binding of CB_1 and CB_2 receptor antagonists is enthalpy and entropy-driven, ΔH° values ranging from −52 to −26 and from −19 to −17 kJ/mol, and ΔS° values ranging from −12 to 23 and from 43 to 74 J/mol/° for CB_1 and CB_2 receptor antagonists, respectively. Agonists and

Table 1
(A) Binding parameters, expressed as K_D (nM) and B_{MAX} (pmol/mg protein) values, of [^{3}H]-CP-55,940 to human CB_1 and CB_2 cloned receptors expressed in CHO cells. (B) Thermodynamic parameters for the binding equilibrium of [^{3}H]-CP-55,940 to the same substrates. Equilibrium ΔG°, ΔH° and ΔS° values are given at T = 298.15 K.

[^{3}H]-CP-55,940 binding	0 °C (273.15 K)	10 °C (283.15 K)	15 °C (288.15 K)	20 °C (293.15 K)	25 °C (298.15 K)	30 °C (303.15 K)
(A)						
CHO-hCB_1						
K_D (nM)	N.D.	3.3 ± 0.6	1.7 ± 0.2	1.0 ± 0.1	0.81 ± 0.05	0.66 ± 0.05
B_{MAX} (pmol/mg protein)	N.D.	4.4 ± 0.5	4.2 ± 0.4	4.9 ± 0.5	4.9 ± 0.3	3.9 ± 0.4
CHO-hCB_2						
K_D (nM)	9.1 ± 0.9	6.4 ± 0.9	N.T.	3.7 ± 0.8	N.T.	2.5 ± 0.3
B_{MAX} (pmol/mg protein)	140 ± 10	143 ± 15	N.T.	143 ± 25	N.T.	148 ± 28

Cell lines	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/mol/°)
(B)			
CHO-hCB_1	−51.8 ± 0.2	57 ± 5	365 ± 8
CHO-hCB_2	−48.6 ± 0.2	30 ± 3	265 ± 3

Values are the mean ± S.E.M. from at least four independent experiments performed in duplicate. N.D.: not detectable; N.T.: not tested.

antagonists to CB_1 and CB_2 cannabinoid receptor are therefore thermodynamically discriminated.

4. Discussion

In this study, we have determined the thermodynamic parameters underlying the binding of eight ligands (five agonists and three antagonists) to human CB_1 and CB_2 receptors transfected in CHO cells. The information provided by these data could be useful from a pharmacological point of view to discover new thermodynamic relationships related to drug–receptor interactions and their molecular mechanisms [27].

The most significant results of this paper are:

1. the systematic linearities of van't Hoff plots;
2. the recurrent phenomenon of E-E compensation;
3. the thermodynamic discrimination of agonists from antagonists.

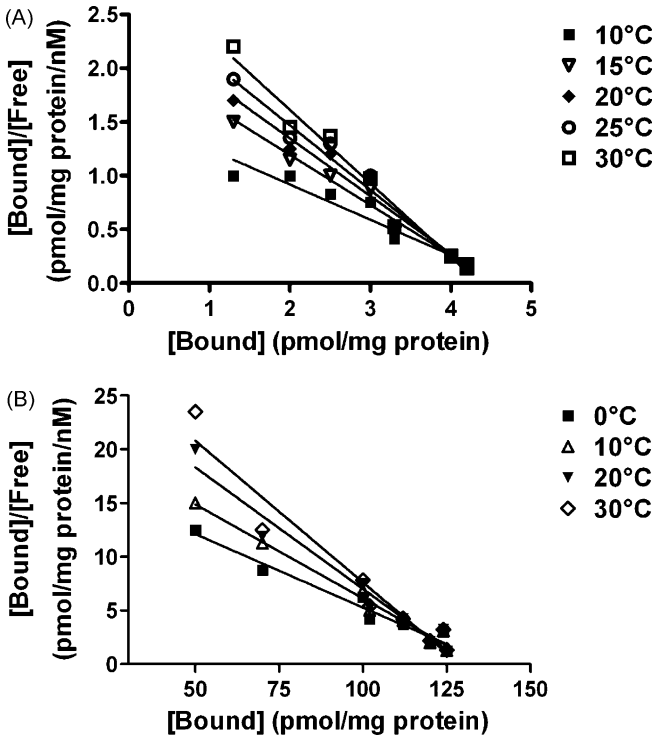

Fig. 2. Representative Scatchard plots for [^{3}H]-CP-55,940 binding to human CB_1 (panel A) and CB_2 (panel B) cloned receptors expressed in CHO cells at different temperatures. The linearity of the plots is indicative of the presence of a single class of high affinity binding sites at all the temperatures investigated.

Regarding the first point, van't Hoff plots turn out to be linear for all compounds considered. This implies that the ΔC_p° for the binding equilibrium approximates zero in all cases, or, in other words, the value of ΔH° is not significantly affected by temperature variations in the range investigated (0–30 °C) [27]. This phenomenon seems to indicate that the conformational changes needed to produce the pharmacological effect are relatively small in this class of macromolecules, most probably because larger modifications would make the association of the receptor with the cell membrane unstable.

The second point concerns the extrathermodynamic interdependence of ΔH° and $-T\Delta S^\circ$ for the CB_1 and CB_2 cannabinoid receptors, clearly apparent from Fig. 5, where all the experimental points appear to be arranged along a same diagonal line, according to the equations:

$$-T\Delta S^\circ\ (\text{kJ/mol}) = -44\ (\pm 2) - 1.02\ (\pm 0.05)\,\Delta H^\circ\ (\text{kJ/mol})$$
$$(n = 8,\ r = -0.991,\ P < 0.0001)$$

and

$$-T\Delta S^\circ\ (\text{kJ/mol}) = -39\ (\pm 2) - 1.12\ (\pm 0.05)\,\Delta H^\circ\ (\text{kJ/mol})$$
$$(n = 8,\ r = -0.989,\ P < 0.0001)$$

for CB_1 and CB_2 cannabinoid receptors, respectively. This equation can be rewritten as

$$\Delta H^\circ = \beta\,\Delta S^\circ,$$

which is the form for a case of enthalpy–entropy compensation [27] with a compensation temperature of 286 K and 262 K, for CB_1 and CB_2 cannabinoid receptors, respectively. This phenomenon seems to be a common feature in all cases of drug–receptor binding [27]. The great number of thermodynamic data available for membrane drug–receptor interactions has made it possible to observe that the enthalpic (ΔH°) and entropic ($-T\Delta S^\circ$) terms of the Gibbs equation are strongly correlated by the linear regression according to the equation [29]:

$$-T\Delta S^\circ\ (\text{kJ/mol}) = -42.4\ (\pm 0.5) - 1.01\ (\pm 0.01)\,\Delta H^\circ\ (\text{kJ/mol})$$
$$(n = 436,\ r = 0.979,\ P < 0.0001).$$

This behaviour, which has been called enthalpy–entropy compensation, is normally imputed to solvent reorganization phenomena accompanying the receptor binding processes [30]. Since ΔG° is related linearly to ΔH° and ΔS° by the Gibbs equation, $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, it is useful to represent the thermodynamic data of drug–receptor interaction in a $-T\Delta S^\circ$ versus ΔH° plot. In this type of representation all the plot allows to obtain information

Table 2
Affinities, expressed as K_i values (nM) of selected cannabinoid compounds to human CB_1 (A) and CB_2 (B) receptors expressed in CHO transfected cells.

Ligand	10 °C (283.15 K)	15 °C (288.15 K)	20 °C (293.15 K)	30 °C (303.15 K)
(A)				
Cannabinoid agonists, K_i (nM)				
WIN 55212	65 ± 5	46 ± 5	20 ± 2	14 ± 2
JWH-015	1190 ± 130	917 ± 95	500 ± 60	397 ± 42
ACEA	16.6 ± 2.1	9.2 ± 1.1	5.5 ± 0.6	3.1 ± 0.3
2-Fl-AEA	12 ± 1	10.5 ± 1.3	9 ± 1	7.4 ± 0.8
CP-55,940	4.4 ± 0.5	1.8 ± 0.2	1.5 ± 0.1	0.8 ± 0.1
Cannabinoid antagonists, K_i (nM)				
AM630	863 ± 92	1000 ± 125	1375 ± 140	1760 ± 190
AM281	4.2 ± 0.5	5.5 ± 0.6	7.5 ± 0.8	11 ± 1
AM251	0.9 ± 0.1	1.5 ± 0.2	1.8 ± 0.2	4.1 ± 0.3
Ligand	0 °C (273.15 K)	10 °C (283.15 K)	20 °C (293.15 K)	30 °C (303.15 K)
(B)				
Cannabinoid agonists, K_i (nM)				
WIN 55212	17 ± 2	11 ± 1	8 ± 1	5 ± 1
JWH-015	346 ± 40	144 ± 15	86 ± 9	40 ± 4
ACEA	422 ± 38	206 ± 18	166 ± 18	87 ± 9
2-Fl-AEA	295 ± 31	196 ± 21	132 ± 14	79 ± 8
CP-55,940	10.0 ± 1.1	6.7 ± 0.7	4.4 ± 0.5	3.6 ± 0.4
Cannabinoid antagonists, K_i (nM)				
AM630	29 ± 3	40 ± 5	53 ± 6	67 ± 7
AM281	1204 ± 115	1546 ± 165	1657 ± 184	3040 ± 318
AM251	330 ± 34	412 ± 37	524 ± 61	705 ± 64

Data are mean ± S.E.M. of four independent experiments performed in duplicate. Inhibition binding experiments were performed as described in Section 2. K_i values represent the concentration of drug able to displace 50% of the radioligand.

on ΔG° and as a consequence on K_A values ($\Delta G^\circ = -RT \ln K_A$). In particular, the same values of ΔG° can be produced by all the linear combination of different ΔH° and $-T\Delta S^\circ$ pairs of values lying on a diagonal of the plot. This type of representation shows the presence or the absence of the thermodynamic discrimination phenomenon between agonists and antagonists for any given receptor. Membrane receptors are well-known to be in enthalpy–entropy compensation, as shown by the correlation equation based practically on all data available, a total of 436 binding experiments performed on 17 membrane receptorial systems with more than 300 different ligands [29]. As it can be seen from the data plotted in Fig. 5, all experimental points are arranged on the same diagonal band encompassed between the two dashed lines which represent the loci of points defined by the limiting K_D values of 100 μM and 10 pM. This band is the expression of the enthalpy–entropy compensation phenomenon probably due to drug–receptor inter-

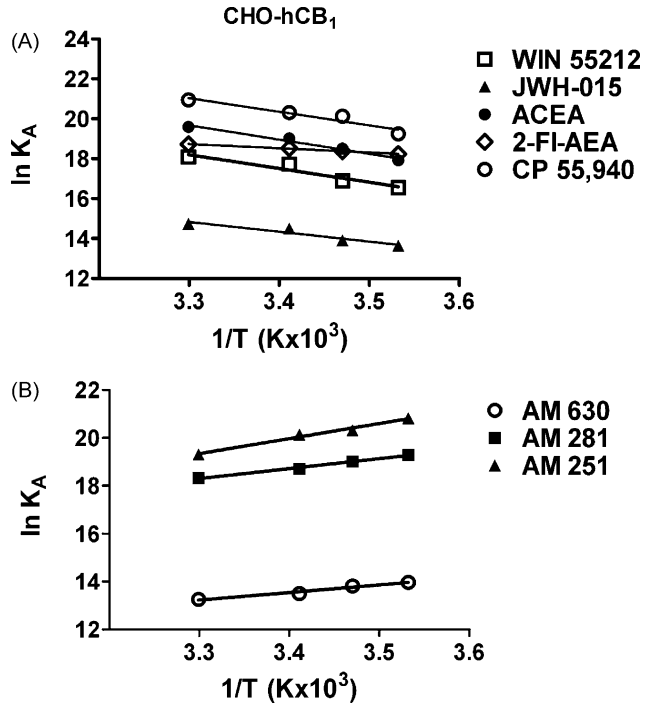

Fig. 3. van't Hoff plots showing the effect of temperature on the equilibrium binding association constants, K_A, for all cannabinoid CB_1 receptor agonists (A) and antagonists (B) studied. All plots are essentially linear ($r \geq 0.91$) in the temperature range of 10–30 °C.

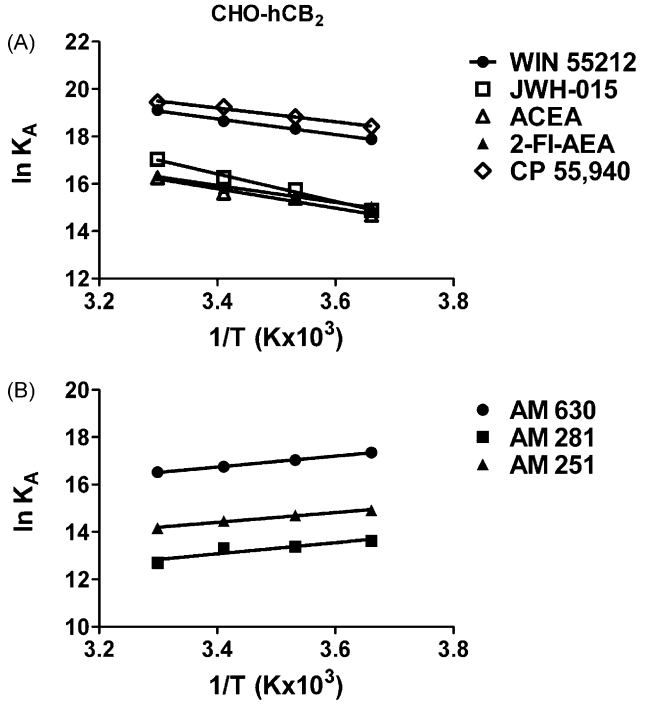

Fig. 4. van't Hoff plots showing the effect of temperature on the equilibrium binding association constants, K_A, for all cannabinoid CB_2 receptor agonists (A) and antagonists (B) studied. All plots are essentially linear ($r \geq 0.91$) in the temperature range of 0–30 °C.

Table 3
Thermodynamic parameters for the binding equilibrium of [^{3}H]-CP-55,940, by cannabinoid agonists and antagonists, to human CB_1 (A) and CB_2 (B) cannabinoid receptors expressed in CHO transfected cells. Equilibrium ΔG°, ΔH° and ΔS° values are given at $T = 298.15$ K.

Ligand	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/mol/°)
(A)			
Cannabinoid agonists			
WIN 55212	−44.2 ± 0.1	57 ± 5	341 ± 12
JWH-015	−36.1 ± 0.1	41 ± 4	258 ± 14
ACEA	−47.8 ± 0.1	59 ± 5	359 ± 26
2-Fl-AEA	−46.1 ± 0.2	17 ± 1	213 ± 17
CP-55,940	−51.2 ± 0.2	56 ± 4	361 ± 24
Cannabinoid antagonists			
AM630	−33.2 ± 0.1	−26 ± 1	23 ± 2
AM281	−45.9 ± 0.2	−35 ± 2	38 ± 4
AM251	−48.8 ± 0.1	−52 ± 4	−12 ± 1
(B)			
Cannabinoid agonists			
WIN 55212	−46.8 ± 0.1	27 ± 2	249 ± 19
JWH-015	−41.4 ± 0.1	48 ± 3	300 ± 27
ACEA	−39.6 ± 0.1	34 ± 2	247 ± 29
2-Fl-AEA	−39.9 ± 0.2	30 ± 3	234 ± 22
CP-55,940	−47.9 ± 0.2	24 ± 2	242 ± 18
Cannabinoid antagonists			
AM630	−41.2 ± 0.2	−19 ± 2	74 ± 6
AM281	−32.2 ± 0.1	−19 ± 2	43 ± 4
AM251	−35.5 ± 0.1	−17 ± 1	61 ± 5

actions and to solvent reorganization that accompanies the receptor binding process [27]. So, the linear relationship between ΔH° and $T\Delta S^\circ$, reported for numerous G-protein-coupled receptors and ligand-gated ion channels (e.g. β-adrenoceptor, adenosine A_1, adenosine A_{2A}, adenosine A_{2B}, adenosine A_3, dopamine D_2, histamine H3, opioid, colecystokinin CCK_2, $P2X_1$, $P2X_3$, serotonin 5-HT_{1A}, serotonin 5-HT_3, glycine, $GABA_A$, and nicotinic receptor) [15,5,11,10,21,18,8,14,13,9], was not unexpected for CB_1 and CB_2 cannabinoid receptors. The linear relationship indicates that enthalpy–entropy compensation exists for the CB_1 and CB_2 cannabinoid receptors, that is, changes in enthalpy are compensated for by changes in entropy (or *vice versa*) such that the free-energy change ΔG° is constant. In the frame of this general enthalpy–entropy compensation, some membrane receptors display the phenomenon of thermodynamic discrimination, for which agonists and antagonists (or rather inverse agonists, as recently suggested) share different regions of the correlation line, being agonist binding enthalpy-driven and antagonist binding entropy-driven, or *vice versa*. Cannabinoid receptors display the same compensation and discrimination effects. The thermodynamic parameters for CB_1 receptors fall in the ranges $17 \leq \Delta H^\circ \leq 59$ kJ/mol and $213 \leq \Delta S^\circ \leq 361$ kJ/mol for agonists and $-52 \leq \Delta H^\circ \leq -26$ kJ/mol and $-12 \leq \Delta S^\circ \leq 38$ kJ/mol for antagonists. The thermodynamic parameters for CB_2 receptors fall in the ranges $27 \leq \Delta H^\circ \leq 48$ kJ/mol and $234 \leq \Delta S^\circ \leq 300$ kJ/mol for agonists and $-19 \leq \Delta H^\circ \leq -17$ kJ/mol and $43 \leq \Delta S^\circ \leq 74$ kJ/mol for antagonists. Collectively, we observed that the binding equilibrium of CB_1 and CB_2 cannabinoid receptor ligands is entropy-driven for agonists, but mostly enthalpy-driven for antagonists. In addition to the CB_1 and CB_2 receptor ligands discussed in this work there are a number of other compounds, some of them not commercially available. Future experiments performed with further ligands, in particular CB receptor antagonists, will allow to validate these results indicating that agonists and antagonists are thermodynamically discriminated for both cannabinoid receptors. They produce a ''thermodynamic signature'' based on enthalpy, entropy, free energy and binding affinity for each ligand to its receptor. From these, the contributions of hydrogen bonding, van der Waals and hydrophobic interactions can all be assessed and therefore the optimal binding

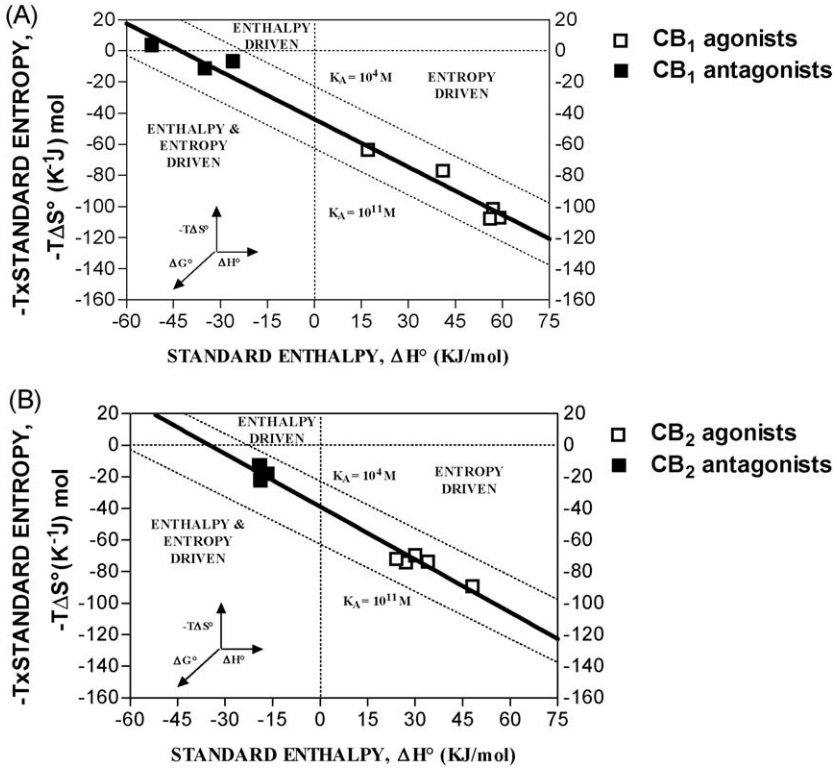

Fig. 5. Scatter plot of $-T\,\Delta S^\circ$ versus ΔH° values for the cannabinoid CB_1 (panel A) and CB_2 (panel B) receptor ligands studied. Full and open symbols indicate antagonists and agonists, respectively. All points lie on a same regression line. The two dashed lines indicate the *loci* of the points representing possible combinations of ΔH° and $-T\,\Delta S^\circ$ values giving rise to the two different equilibrium constants indicated ($K_A = 10^4$ and 10^{11}).

profile discovered. Carrying out these binding investigations as early as possible in the discovery process increases the probability that a lead compound will become a successful pharmaceutical compound.

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