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## Binding thermodynamics at the human Cannabinoid cb- and cb-receptors

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Katia Varani, Pier Andrea Borea



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**BINDING THERMODYNAMICS AT THE HUMAN  
CANNABINOID CB<sub>1</sub>- AND CB<sub>2</sub>-RECEPTORS**

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**Abbreviations**

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6 ACEA, arachidonyl-2'-chloroethylamide; AM 251, *N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-  
7 iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM 281, *N*-(morpholin-4-yl)-1-(2,4-  
8 dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM 630, 6-iodo-2-  
9 methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone(6-  
10 iodopravadoline); CHO, Chinese hamster ovary; CP 55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-  
11 dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; hCHO-CB<sub>1</sub>, CHO cells transfected  
12 with human CB<sub>1</sub> cannabinoid receptor; hCHO-CB<sub>2</sub>, CHO cells transfected with human CB<sub>2</sub>  
13 cannabinoid receptor; 2-FI-AEA, arachidonoyl2'-fluoroethylamide; JWH-015, (2-methyl-1-propyl-  
14 1*H*-indol-3-yl)-1-naphthalenylmethanone; WIN 55212, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-  
15 morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanonemesylate.  
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**Abstract**

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6 The thermodynamic parameters  $\Delta G^\circ$ ,  $\Delta H^\circ$  and  $\Delta S^\circ$  of the binding equilibrium of agonists and  
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8 antagonists at cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors were determined by means of affinity  
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10 measurements at different temperatures and van't Hoff plots were constructed. Affinity constants  
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12 were measured on CHO cells transfected with the human CB<sub>1</sub> and CB<sub>2</sub> receptors by inhibition  
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14 assays of the binding of the cannabinoid receptor agonist [<sup>3</sup>H]-CP-55,940. Van't Hoff plots were  
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16 linear for agonists and antagonists in the temperature range 0-30°C. The thermodynamic parameters  
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18 for CB<sub>1</sub> receptors fall in the ranges  $17 \leq \Delta H^\circ \leq 59 \text{ KJ mol}^{-1}$  and  $213 \leq \Delta S^\circ \leq 361 \text{ KJ mol}^{-1}$  for  
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20 agonists and  $-52 \leq \Delta H^\circ \leq -26 \text{ KJ mol}^{-1}$  and  $-12 \leq \Delta S^\circ \leq 38 \text{ KJ mol}^{-1}$  for antagonists. The  
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22 thermodynamic parameters for CB<sub>2</sub> receptors fall in the ranges  $27 \leq \Delta H^\circ \leq 48 \text{ KJ mol}^{-1}$  and  $234 \leq$   
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24  $\Delta S^\circ \leq 300 \text{ KJ mol}^{-1}$  for agonists and  $-19 \leq \Delta H^\circ \leq -17 \text{ KJ mol}^{-1}$  and  $43 \leq \Delta S^\circ \leq 74 \text{ KJ mol}^{-1}$  for  
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26 antagonists. Collectively, these data show that agonist binding is always totally entropy-driven  
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28 while antagonist binding is enthalpy and entropy-driven, indicating that CB<sub>1</sub> and CB<sub>2</sub> receptors are  
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30 thermodynamically discriminated. These data could give new details on the nature of the forces  
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32 driving the CB<sub>1</sub> and CB<sub>2</sub> binding at a molecular level. Enthalpy, entropy, free energy and binding  
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34 affinity for each ligand to its receptor can all be assessed and therefore the optimal binding profile  
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36 discovered. Carrying out these binding investigations as early as possible in the discovery process  
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38 increases the probability that a lead compound will become a successful pharmaceutical compound.  
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53 **Keywords:** Binding mechanisms; Binding thermodynamics; drug development;  
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55 enthalpy-entropy compensation; Pharmacokinetics.  
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## 1. INTRODUCTION

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6 The thermodynamic analysis of the binding equilibrium of a drug to its receptor allows us to  
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8 evaluate the two components, standard enthalpy ( $\Delta H^\circ$ ) and standard entropy ( $\Delta S^\circ$ ), of the standard  
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10 free energy ( $\Delta G^\circ$ ) of the binding equilibrium [1]. It is often assumed that  $\Delta H^\circ$  and  $\Delta S^\circ$  terms  
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12 represent the two classes of factors responsible for the drug-receptor recognition phenomenon: non-  
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14 bonded interactions, as hydrogen bonding and multipolar or dispersive interactions (which are  
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16 mainly to be related to the enthalpic term), and solvent reorganization (which is most properly  
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18 associated with the entropic one) [2]. There are two main strategies for the evaluation of  $\Delta G^\circ$ ,  $\Delta H^\circ$   
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20 and  $\Delta S^\circ$  terms. The first consists in determining equilibrium constants  $\Delta G^\circ = -RT \ln K_A$  in  
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22 association with direct microcalorimetric enthalpy measurements, even if this method is not  
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24 practicable in binding studies for the very low receptor concentration in most tissues. The only  
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26 method of practical use consists in measurements of  $K_A$  carried out at different temperatures  
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28 followed by van t' Hoff analysis. Such a method has proved to be successful in many cases. Until  
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30 now, elegant studies have been carried out on several receptor systems, demonstrating the value of  
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32 thermodynamics for investigating receptor-ligand interactions [3, 4]. In addition, some of these  
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34 studies have suggested that measurement of thermodynamic parameters can allow the  
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36 discrimination of agonist and antagonist ligands. In particular, the binding of agonists may be  
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38 entropy-driven and that of antagonists enthalpy-driven, or *vice versa*. Such a phenomenon has been  
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40 defined “thermodynamic discrimination” and has been reported for  $\beta$ -adrenergic, glycine, GABA<sub>A</sub>,  
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42 histamine H<sub>3</sub>, serotonin 5-HT<sub>3</sub>, nicotinic, purinergic P2X<sub>3</sub> and A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> adenosine  
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44 receptors [5-16]. However, there are also studies suggesting that this general thermodynamic  
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46 distinction between agonists and antagonists may not be applicable to all receptor systems. It is  
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notable that histamine H<sub>1</sub>, cholecystokinin CCK<sub>2</sub>,  $\delta$ - and  $\mu$ -opioid, purinergic P2X<sub>1</sub>, dopamine D<sub>2</sub> and serotonin 5-HT<sub>1A</sub> receptors cannot be thermodynamically discriminated [17-23].

Cannabinoids exert most of their effects by binding to G<sub>i/o</sub> protein-coupled cannabinoid receptors, called CB<sub>1</sub> and CB<sub>2</sub> [24]. The CB<sub>1</sub> 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<sub>2</sub> 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 to 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<sub>1</sub> and CB<sub>2</sub> 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<sub>1</sub> and CB<sub>2</sub> 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<sub>1</sub> and CB<sub>2</sub> receptors could be thermodynamically discriminated.

## 2. MATERIALS AND METHODS

### 2.1. Materials.

[<sup>3</sup>H]-CP-55,940 (specific activity 139.6 Ci/mmol) was obtained from Perkin Elmer Life Sciences, (Milano, Italy). WIN 55212, JWH-015, ACEA, 2-FI-AEA, CP 55,940, AM630, AM281 and AM251 were obtained from Vinci-Biochem (Firenze, Italy). CHO cells transfected with the human recombinant CB<sub>1</sub> (hCHO-CB<sub>1</sub>) and CB<sub>2</sub> receptor (hCHO-CB<sub>2</sub>) were obtained from PerkinElmer (Milano, Italy). All other reagents were purchased from Sigma-Aldrich (Milano, Italy).

### 2.2. Cell Culture.

hCHO-CB<sub>1</sub> and hCHO-CB<sub>2</sub> 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<sub>2</sub>/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 1,000 x g. The supernatant was then centrifuged for 30 min at 100,000 x g. The membrane pellet was resuspended in 50 mM Tris HCl-0.5% bovine serum albumin (BSA) containing 5 mM MgCl<sub>2</sub>, 2.5 mM EDTA or 1 mM EDTA for CB<sub>1</sub> and CB<sub>2</sub> 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 [<sup>3</sup>H]-CP-55,940 binding

Kinetic studies of [<sup>3</sup>H]-CP-55,940 binding were performed by incubating membranes in 50 mM Tris-HCl-0.5% BSA containing 5 mM MgCl<sub>2</sub>, 2.5 mM EDTA or 1 mM EDTA for CB<sub>1</sub> and CB<sub>2</sub> 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<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors, respectively). Then, the reaction was terminated at different time intervals (from 15 to 300 minutes) 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 (Perkin Elmer 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 (Perkin Elmer Life Sciences, Milano, Italy).

## 2.5. Saturation and competition binding experiments.

Saturation and competition binding assays were performed on hCHO-CB<sub>1</sub> and hCHO-CB<sub>2</sub> 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 [<sup>3</sup>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<sub>2</sub>, 2.5 mM EDTA or 1 mM EDTA for CB<sub>1</sub> and CB<sub>2</sub> 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 minutes at 10°C, 150 minutes at 20°C and 120 minutes at 30°C for CB<sub>1</sub> receptor binding. The incubation time was 180 minutes at 0°C, 90 minutes at 20°C and 60 minutes at 30°C for CB<sub>2</sub> receptor binding. Competition experiments of [<sup>3</sup>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 (Perkin Elmer 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 (Perkin Elmer 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  $\Delta G^\circ$ ,  $\Delta H^\circ$  and  $\Delta S^\circ$  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 ( $\Delta C_p^\circ$ ) 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 ( $\Delta H^\circ$ ) and the standard entropy ( $\Delta S^\circ$ ) 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)  $\Delta C_p^\circ$  is not equal to zero [9, 28]. In this case the plot  $\Delta G^\circ$  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 [<sup>3</sup>H]-CP-55,940 binding was studied at at 10°C, 20°C and 30°C or 0°C, 20°C and 30°C for CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors, respectively. Figure 1 shows that [<sup>3</sup>H]-CP-55,940 binding reached equilibrium after approximately 180 minutes at 10°C, 150 minutes at 20°C and 120 minutes at 30°C for CB<sub>1</sub> receptor binding. Association studies for [<sup>3</sup>H]-CP-55,940 binding to CB<sub>2</sub> cannabinoid receptors indicated that the equilibrium was reached after approximately 180 minutes at 0°C, 90 minutes at 20°C and 60 minutes at 30°C for CB<sub>2</sub> receptor binding. [<sup>3</sup>H]-CP-55,940 binding was then stable for at least 2 hours.

**3.2. Saturation binding assays to human CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors.** Saturation binding experiments in CHO cells were performed to characterize human CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors and evaluate affinity (K<sub>D</sub>) and receptor density (B<sub>MAX</sub>) values (Table 1). These binding parameters were determined at various temperatures by using [<sup>3</sup>H]-CP-55,940 as radioligand at different concentrations. At 0°C no specific binding of [<sup>3</sup>H]-CP-55,940 to CB<sub>1</sub> 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<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors, respectively. In both cannabinoid receptors examined the K<sub>D</sub> values change with temperature while B<sub>MAX</sub> values appear to be largely independent of it. Figure 2 shows the representative Scatchard plots obtained at the five (Figure 2A) and four (Figure 2B) temperatures investigated for the saturation equilibrium of [<sup>3</sup>H]-CP-55,940 to human CB<sub>1</sub> and CB<sub>2</sub> 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.

**3.3. Competition binding assays to human CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors.**

Table 2 reports the inhibitory binding constants K<sub>i</sub> for agonists and antagonists at human CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors stably expressed in CHO cells. These values were determined at various temperatures by displacement of the radiolabeled agonist [<sup>3</sup>H]-CP-55,940. The order of potency in [<sup>3</sup>H]-CP-55,940 competition assays for cannabinoid agonists in hCHO-CB<sub>1</sub> was as follows: CP

55,940 > ACEA > 2-FI-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<sub>2</sub>: CP 55,940 > WIN 55212 > JWH-015 > 2-FI-AEA > ACEA. The order of potency of antagonists in hCHO-CB<sub>1</sub> and in hCHO-CB<sub>2</sub> cells was as follows: AM251 > AM281 > AM630 and AM630 > AM251 > AM281, respectively.

### 3.4. Thermodynamic analysis to human CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors.

Figures 3-4 illustrate the van't Hoff plots,  $\ln K_A$  versus  $1/T$ , for CB<sub>1</sub> and CB<sub>2</sub> 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. Figure 5 summarizes the results in the form of a  $-T\Delta S^\circ$  vs  $\Delta H^\circ$  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  $\mu$ M and 10 pM. This band is the expression of the enthalpy-entropy (E-E) compensation phenomenon [27]. In CB<sub>1</sub> cannabinoid receptors  $\Delta G^\circ$  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<sub>2</sub> cannabinoid receptors  $\Delta G^\circ$  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 ( $\Delta H^\circ$ ) and entropy ( $\Delta S^\circ$ ) values show that the binding of CB<sub>1</sub> and CB<sub>2</sub> receptor agonists is always totally entropy-driven,  $\Delta H^\circ$  values ranging from 17 to 59 and from 24 to 48 kJ/mol, and  $\Delta S^\circ$  values ranging from 213 to 361 and from 234 to 300 J/mol/degree for CB<sub>1</sub> and CB<sub>2</sub> receptors, respectively. On the contrary, the binding of CB<sub>1</sub> and CB<sub>2</sub> receptor antagonists is enthalpy and entropy-driven,  $\Delta H^\circ$  values ranging from -52 to -26 and from -19 to -17 kJ/mol, and  $\Delta S^\circ$  values ranging from -12 to 23 and from 43 to 74 J/mol/degree for CB<sub>1</sub> and CB<sub>2</sub> receptor

antagonists, respectively. Agonists and antagonists to CB<sub>1</sub> and CB<sub>2</sub> 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<sub>1</sub> and CB<sub>2</sub> 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.

Regarding the first point, van't Hoff plots turn out to be linear for all compounds considered. This implies that the  $\Delta C_p^\circ$  for the binding equilibrium approximates zero in all cases, or, in other words, the value of  $\Delta H^\circ$  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  $\Delta H^\circ$  and  $-T\Delta S^\circ$  for the CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors, clearly apparent from Figure 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<sub>1</sub> and CB<sub>2</sub> 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<sub>1</sub> and CB<sub>2</sub> 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 ( $\Delta H^\circ$ ) and entropic ( $-T\Delta S^\circ$ ) terms of the Gibbs equation are strongly correlated by the linear regression according to the equation

$$-T\Delta S^\circ \text{ (kJ/mol)} = -42.4(\pm 0.5) - 1.01(\pm 0.01)\Delta H^\circ \text{ (kJ/mol)}, (n=436, r=0.979, P<0.0001) [29].$$

This behaviour, which has been called enthalpy-entropy compensation, is normally imputed to solvent reorganization phenomena accompanying the receptor binding processes [30]. Since  $\Delta G^\circ$  is related linearly to  $\Delta H^\circ$  and  $\Delta S^\circ$  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  $\Delta H^\circ$  plot. In this type of representation all the plot allows to obtain information on  $\Delta G^\circ$  and as a consequence on  $K_A$  values ( $\Delta G^\circ = -RT\ln K_A$ ). In particular, the same values of  $\Delta G^\circ$  can be produced by all the linear combination of different  $\Delta H^\circ$  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  $\mu$ M and 10 pM.

This band is the expression of the enthalpy-entropy compensation phenomenon probably due to drug-receptor interactions and to solvent reorganization that accompanies the receptor binding process [27]. So, the linear relationship between  $\Delta H^\circ$  and  $T\Delta S^\circ$ , reported for numerous G-protein-coupled receptors and ligand-gated ion channels (e.g.  $\beta$ -adrenoceptor, adenosine  $A_1$ , adenosine  $A_{2A}$ , adenosine  $A_{2B}$ , adenosine  $A_3$ , dopamine  $D_2$ , histamine  $H_3$ , 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  $\Delta G^\circ$  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  $17 \leq \Delta H^\circ \leq 59 \text{ KJ mol}^{-1}$  and  $213 \leq \Delta S^\circ \leq 361 \text{ KJ mol}^{-1}$  for agonists and  $-52 \leq \Delta H^\circ \leq -26 \text{ KJ mol}^{-1}$  and  $-12 \leq \Delta S^\circ \leq 38 \text{ KJ mol}^{-1}$  for antagonists. The thermodynamic parameters for  $CB_2$  receptors fall in the ranges  $27 \leq \Delta H^\circ \leq 48 \text{ KJ mol}^{-1}$  and  $234 \leq \Delta S^\circ \leq 300 \text{ KJ mol}^{-1}$  for agonists and  $-19 \leq \Delta H^\circ \leq -17 \text{ KJ mol}^{-1}$  and  $43 \leq \Delta S^\circ \leq 74 \text{ KJ mol}^{-1}$  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 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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**FIGURE LEGENDS**

1 **Figure 1.** Kinetics of [<sup>3</sup>H]-CP-55,940 binding to human CB<sub>1</sub> (Panel A) and CB<sub>2</sub> (Panel B) cloned  
2 receptors expressed in CHO cells at different temperatures.  
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8 **Figure 2.** Representative Scatchard plots for [<sup>3</sup>H]-CP-55,940 binding to human CB<sub>1</sub> (Panel A) and  
9 CB<sub>2</sub> (Panel B) cloned receptors expressed in CHO cells at different temperatures. The linearity of  
10 the plots is indicative of the presence of a single class of high affinity binding sites at all the  
11 temperatures investigated.  
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18 **Figure 3.** Vant'Hoff plots showing the effect of temperature on the equilibrium binding association  
19 constants, K<sub>A</sub>, for all cannabinoid CB<sub>1</sub> receptor agonists (A) and antagonists (B) studied. All plots  
20 are essentially linear (r ≥ 0.91) in the temperature range of 10-30°C.  
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28 **Figure 4.** Vant'Hoff plots showing the effect of temperature on the equilibrium binding association  
29 constants, K<sub>A</sub>, for all cannabinoid CB<sub>2</sub> receptor agonists (A) and antagonists (B) studied. All plots  
30 are essentially linear (r ≥ 0.91) in the temperature range of 0-30°C.  
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38 **Figure 5.** Scatter plot of -TΔS° versus ΔH° values for the cannabinoid CB<sub>1</sub> (Panel A) and CB<sub>2</sub>  
39 (Panel B) receptor ligands studied. Full and open symbols indicate antagonists and agonists,  
40 respectively. All points lie on a same regression line. The two dashed lines indicate the *loci* of the  
41 points representing possible combinations of ΔH° and -TΔS° values giving rise to the two different  
42 equilibrium constants indicated (K<sub>A</sub> = 10<sup>4</sup> and K<sub>A</sub> = 10<sup>11</sup>).  
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**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  $\Delta G^\circ$ ,  $\Delta H^\circ$  and  $\Delta S^\circ$  values are given at  $T = 298.15$  K.

(A)						
[ $^3$ 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)
CHO-h $CB_1$ $K_D$ (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-h $CB_2$ $K_D$ (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
(B)						
Cell lines	$\Delta G^\circ$ (KJ/mol)	$\Delta H^\circ$ (KJ/mol)	$\Delta S^\circ$ (J/mol/degree)			
CHO-h $CB_1$	-51.8±0.2	57±5	365±8			
CHO-h $CB_2$	-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

**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.**

(A)				
Ligand	10°C (283.15 K)	15°C (288.15 K)	20°C (293.15 K)	30°C (303.15 K)
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-FI-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
(B)				
Ligand	0°C (273.15 K)	10°C (283.15 K)	20°C (293.15 K)	30°C (303.15 K)
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-FI-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 Methods.  $K_i$  values represent the concentration of drug able to displace 50% of the radioligand.

**Table 3** - Thermodynamic parameters for the binding equilibrium of [<sup>3</sup>H]-CP-55,940, by cannabinoid agonists and antagonists, to human CB<sub>1</sub> (A) and CB<sub>2</sub> (B) cannabinoid receptors expressed in CHO transfected cells. Equilibrium  $\Delta G^\circ$ ,  $\Delta H^\circ$  and  $\Delta S^\circ$  values are given at T = 298.15 K.

(A)			
Ligand	$\Delta G^\circ$ (KJ/mol)	$\Delta H^\circ$ (KJ/mol)	$\Delta S^\circ$ (J/mol/degree)
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)			
Ligand	$\Delta G^\circ$ (KJ/mol)	$\Delta H^\circ$ (KJ/mol)	$\Delta S^\circ$ (J/mol/degree)
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

Figure 1.

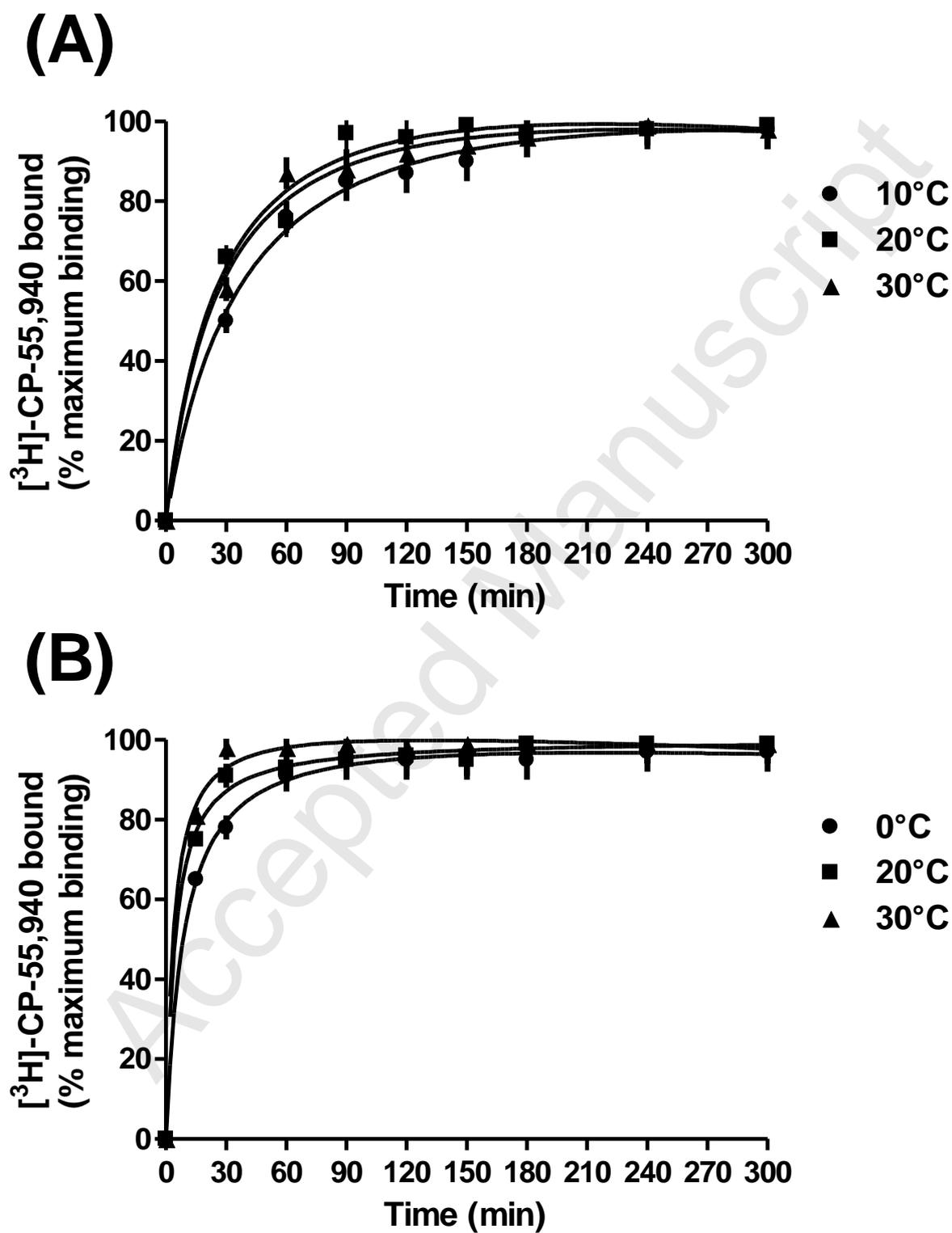


Figure 2.

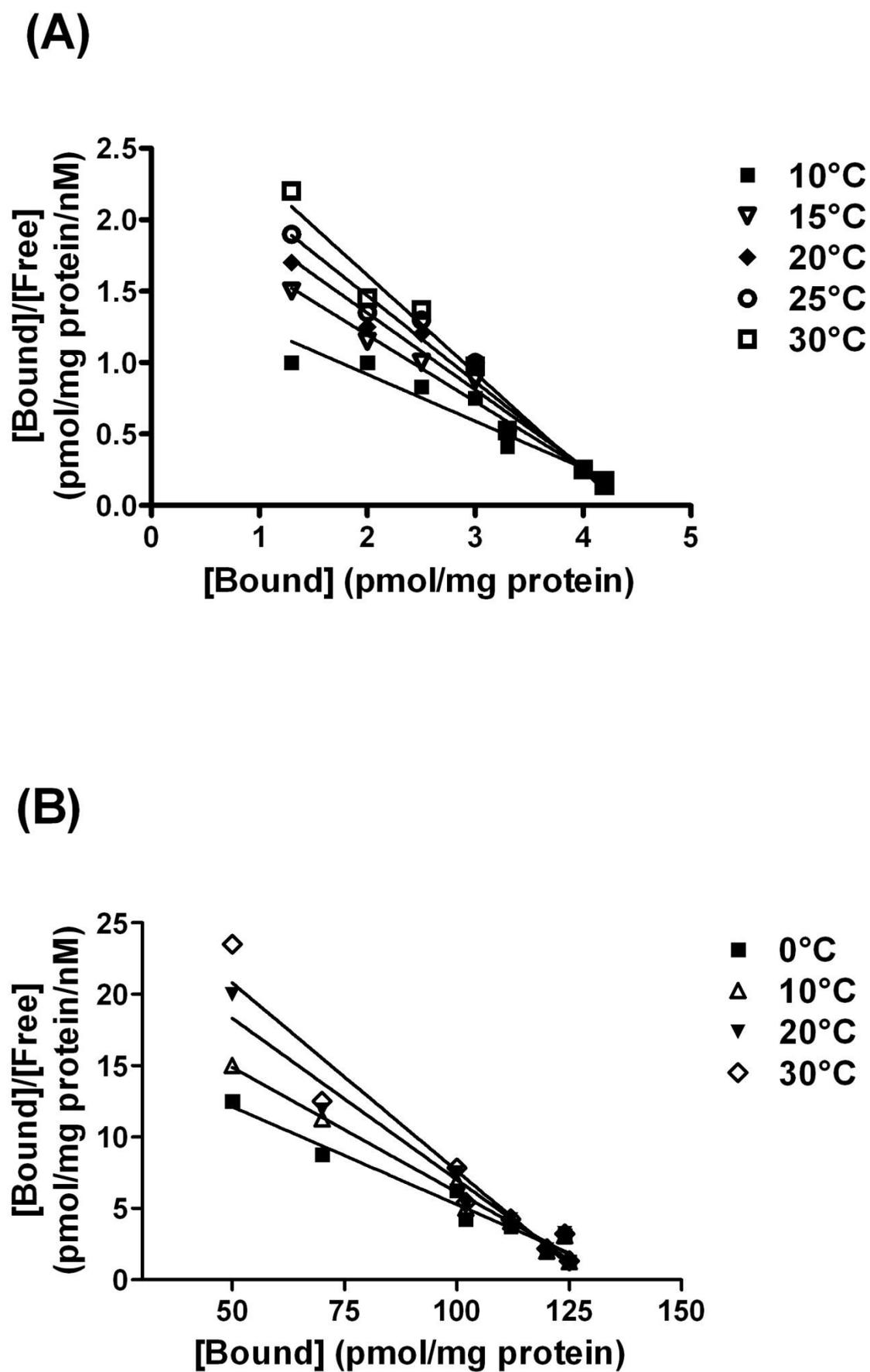


Figure 3.

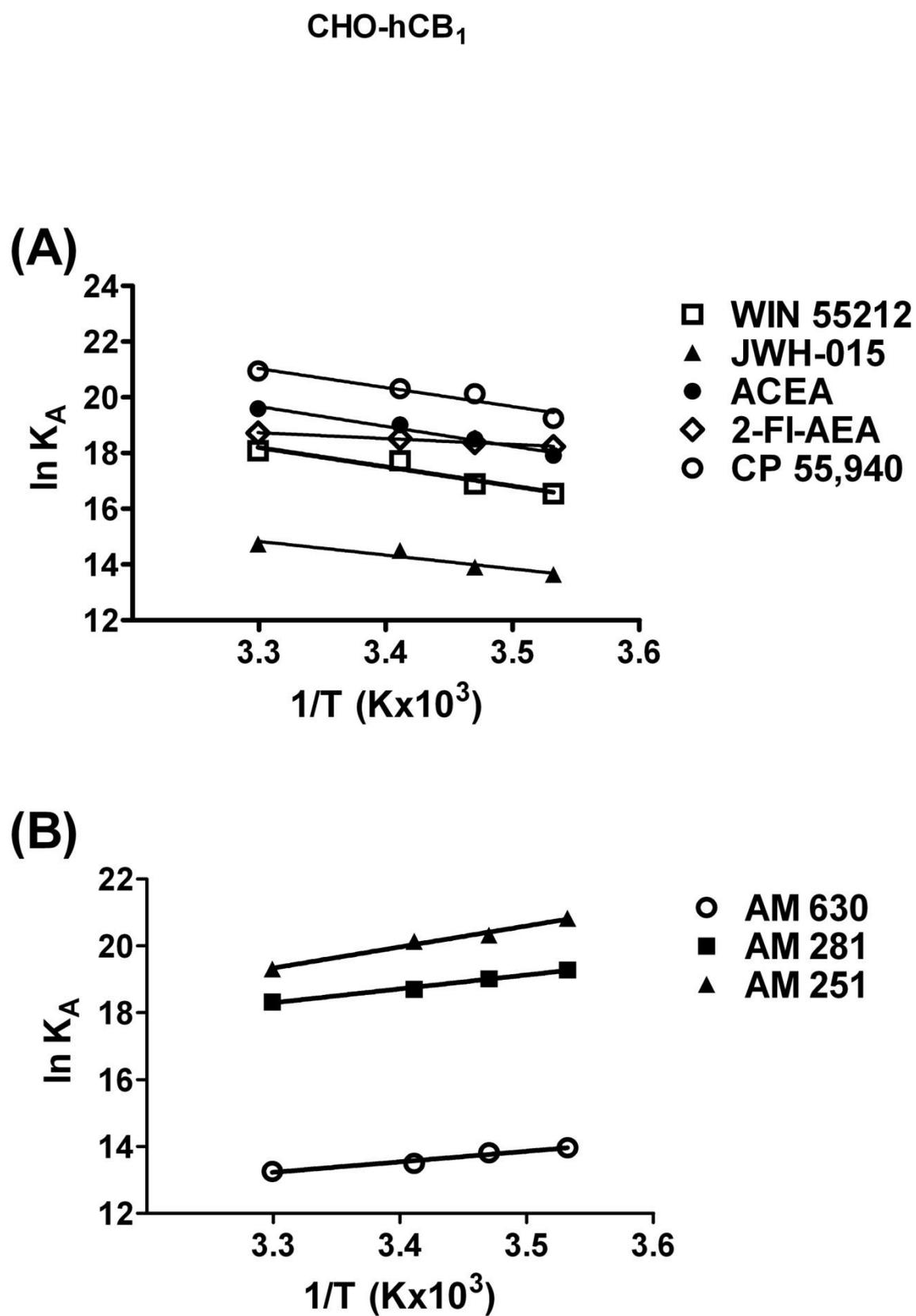


Figure 4.

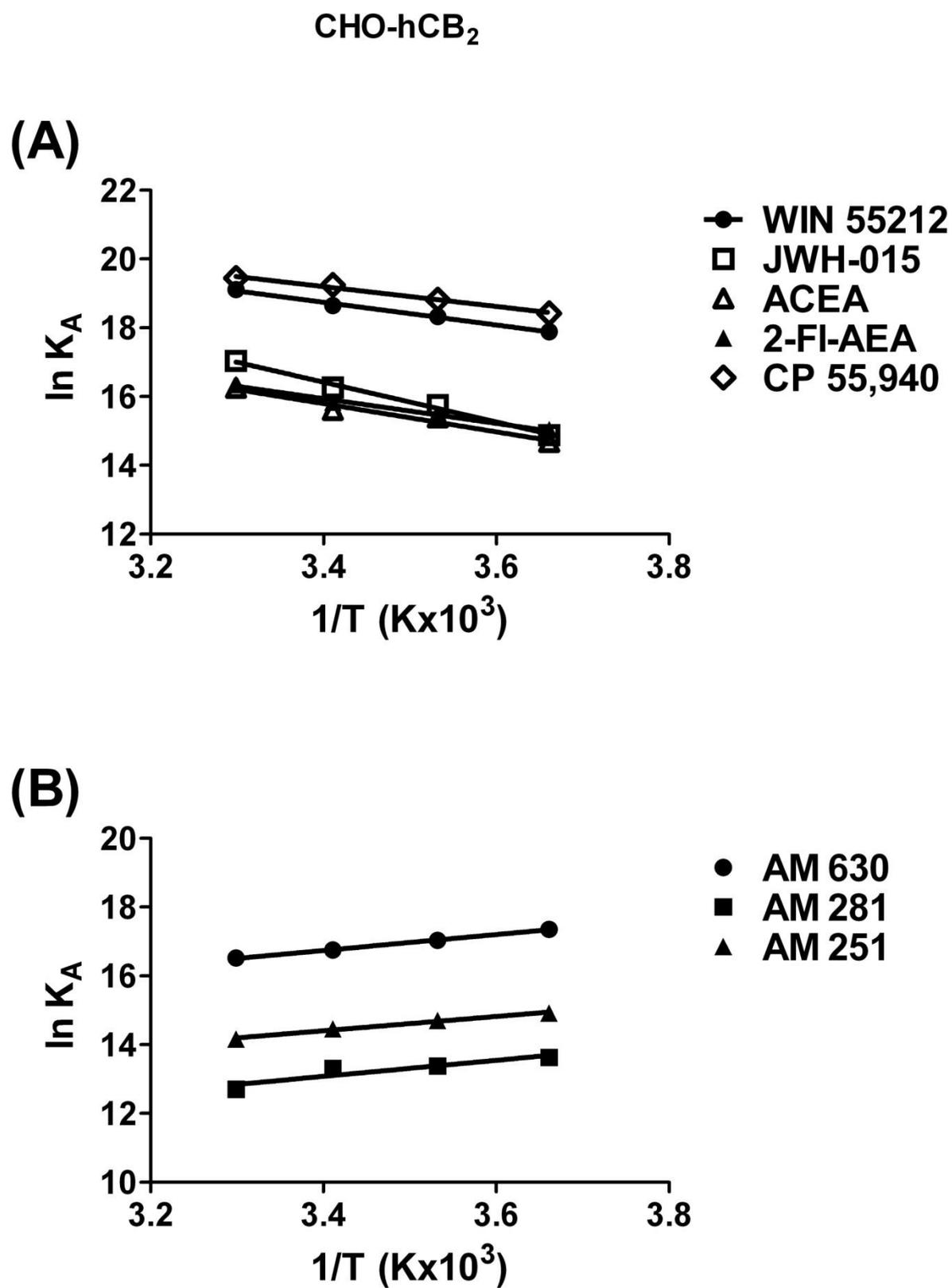
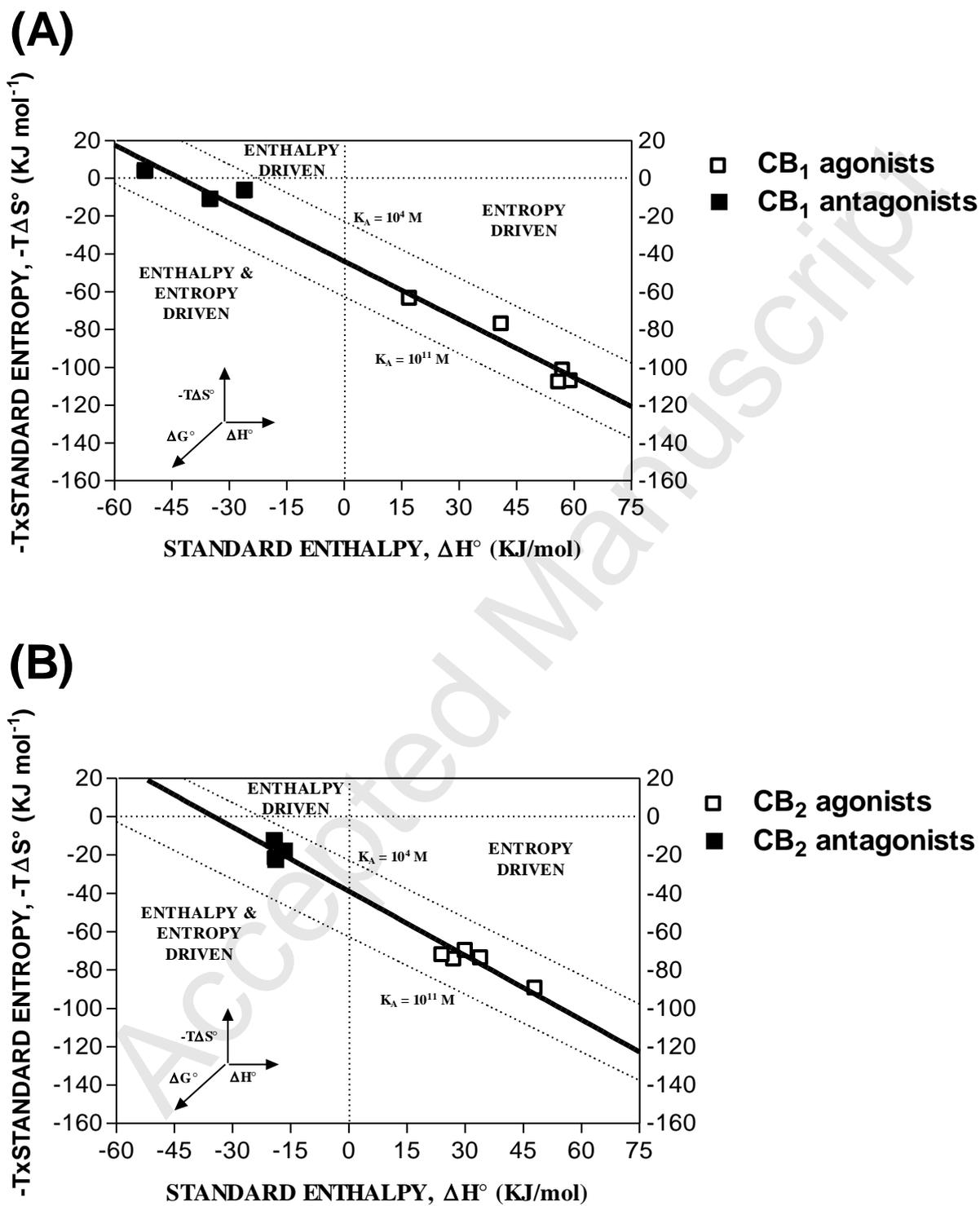


Figure 5.



## Cumulative $\Delta H^\circ$ vs $\Delta S^\circ$ scatter plots for CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors.

