Thermodynamic *In Vitro* Studies as a Method to Investigate the Pharmacodynamic Behavior of Adenosine A_1 Receptor Ligands

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Purpose. A thermodynamic analysis of the binding to rat cortex adenosine A_1 receptor of N^6-substituted (full agonists) and N^6-substituted-deoxyribose (partial agonists) adenosine derivatives was performed. The intrinsic activity of the compounds was evaluated by measurements of the inhibition of forskolin stimulated 3′, 5′-cyclic adenosine monophosphate (c-AMP) levels in isolated epididymal rat adipocytes.
Methods. The thermodynamic parameters $\Delta G°$ (standard free energy), $\Delta H°$ (standard enthalpy), and $\Delta S°$ (standard entropy) of the binding equilibrium were determined by means of affinity measurements carried out at different temperatures (0, 10, 20, 25, 30°C). Levels of c-AMP were evaluated performing competitive protein binding assays.
Results. The binding of the ligands increases with temperature enhancement and, as a consequence, is totally entropy driven. Standard entropy values correlate significantly with intrinsic activity ones.
Conclusions. It is proposed the data obtained by these *in vitro* experiments can be used to investigate the *in vivo* pharmacodynamic of A_1 full and partial agonists.

KEY WORDS: adenosine A_1 receptor; full and partial agonists; binding thermodynamics; intrinsic activity; pharmacodynamic.

INTRODUCTION

Adenosine receptors are ubiquitous in the body. They have been classified into four subtypes: A_1, A_{2A}, A_{2B}, and A_3 (1). Activation of A_1 has been shown to produce a wide variety of effects and, among these, cardiac and neuronal excitability depression (2). As a result, A_1 agonists are able to produce ischemic tolerance and protection in neuronal and cardiac tissues (3,4), but on the other hand, these potential benefits may be accompanied by serious side effects. Adenosine receptor agonists have been extensively studied with the aim to obtain drugs for a potential therapeutic use. All known agonists are structurally related to adenosine and are characterized by the presence of a ribose and a purine moiety. N^6-substituted analogues are potent and selective A_1 agonists (Fig. 1), in particular, N^6-cyclopentyladenosine (CPA) is actually considered the prototypic A_1-selective agonist (5). A_1 receptor activation is known to cause the inhibition of adenylyl cyclase activity (6). Recently, the existence of partial agonists for adenosine A_1 receptors has been demonstrated (7) and it is hypothesized their use could permit the inhibition of some of the side effects that accompany adenosine receptor activation by exogenous agents (8). It has been reported that 2′- and 3′-deoxyribose analogues of N^6-substituted adenosine derivatives are selective A_1 ligands and potential partial agonists (9). Their affinity toward adenosine A_1 receptors appears lower with respect to the parent compounds. It has been subsequently proposed that deoxyribose derivatives of CPA may serve as tools for investigation of adenosine receptor partial agonism *in vivo* (10).

In general, the *in vivo* effects of drug-receptor interactions depend mainly on the drug affinity for the receptor (11,12) and on its intrinsic activity (11,12) (indicating if the drug acts as a full, partial, or inverse agonist, or as an antagonist). It therefore appears important to evaluate if the pharmacological and stability informations obtained *in vitro* for a drug could be extrapolated to the *in vivo* situation (13).

This paper reports an *in vitro* study concerning CPA, CHA, and their 2′ and 3′-deoxyribose homologous. The thermodynamics of the binding to rat cortex adenosine A_1 receptor has been analyzed and the standard free energy ($\Delta G°$), standard enthalpy ($\Delta H°$), and standard entropy ($\Delta S°$) values have been obtained. Moreover, the intrinsic activity of the compounds has been evaluated by measurements of adenylyl cyclase inhibition in isolated epididimal rat adipocytes after stimulation by forskolin. High correlation exists between $\Delta S°$ and intrinsic activity data, indicating the thermodynamic analysis permits us to obtain information about the pharmacodynamic of the compounds. All the results obtained for CPA and its deoxy derivatives were compared with their *in vivo* cardiovascular effects.

MATERIALS AND METHODS

Materials

[^{3}H]CHA (34.4 Ci/mmol) was obtained from NEN Research Products (Boston, Mass., U.S.A.). [^{3}H]c-AMP (24 Ci/mmol) was obtained from Amersham, Amity Srl (Milano, Italy). CPA, CHA, and R-PIA were obtained from RBI (Natick, Mass., U.S.A.).). ADA, BSA, c-AMP, aminophylline, Ro 20-1724, forskolin, and collagenase were obtained from Sigma (St. Louis MO, U.S.A.). 2′- and 3′-deoxy-CPA, 2′- and 3′-deoxy-CHA were synthesized as described previously (9). Male Wistar rats were acquired from Nossan Laboratories (Varese, Italy). All other chemicals used were of analytical grade.

Thermodynamic Data Determination

Determination of $\Delta G°$, $\Delta H°$, and $\Delta S°$ values has been obtained by measurements of K_A carried out at different temperatures, followed by van't Hoff analysis. Two cases are to be distinguished:

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ABBREVIATIONS: CPA, N^6-cyclopentyladenosine; CHA, N^6-cyclohexyladenosine; R/S-PIA, N^6-(R)/(S)-phenylisopropyladenosine; PA, N^6-phenyladenosine; PEA, N^6-phenylethyladenosine; BA, N^6-benzyladenosine; MA, N^6-methyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; DMX 7R, 1,3-dimethylxanthine-7-riboside; 2′/3′dCPA/CHA, 2′/3′-deoxyriboseCPA/CHA; c-AMP, 3′,5′-cyclic adenosine monophosphate; GTP, guanosine-5′-tryphosphate; ADA, adenosine deaminase; BSA, bovine serum albumine; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; TCA, trichloroacetic acid.

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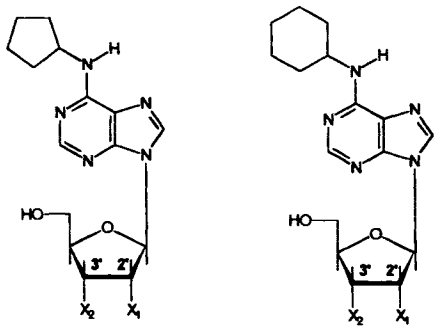

	X_1	X_2
CPA	OH	OH
2'dCPA	H	OH
3'dCPA	OH	H

	X_1	X_2
CHA	OH	OH
2'dCHA	H	OH
3'dCHA	OH	H

Fig. 1. Chemical structures of the adenosine analogues used in the present study.

1) The standard specific heat difference of the equilibrium (ΔC_p°) is essentially zero. In this case the van't Hoff equation $\ln K_A = -\Delta H°/RT + \Delta S°/R$ gives a linear plot $\ln K_A$ versus 1/T. The standard free energy can be calculated as $\Delta G° = -RT \ln K_A$ at 298.15 K; the standard enthalpy ($\Delta H°$) and the standard entropy ($\Delta S°$) can be obtained from the slope ($-\Delta H°/R$) and the intercept ($\Delta S°/R$) 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 (14).

2) ΔC_p° is not equal to zero. In this case the van't Hoff plot is often parabolic and other mathematical methods are available for the analysis (15).

Membrane Preparation

Male Wistar rats (150–200 g) were decapitated and the whole brain (minus brainstem, striatum, and cerebellum) was dissected on ice. The tissue was disrupted in a Polytron (setting 5) in 20 vols of 50 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 48,000 × g for 10 min and the pellet was resuspended in the same buffer, centrifuged and resuspended in Tris-HCl containing 2 IU/ml ADA. After 30 min incubation at 37°C the membranes were centrifuged and pellets were stored at −80°C. Prior to freezing, an aliquot of homogenate was removed for protein assay (16).

Receptor Binding Assays

Membrane aliquots containing 15 μg of proteins were incubated in 1 ml of 50 mM TRIS-HCl at 0, 10, 20, 25, 30° C with incubation times ranging from 3 h (0°C) to 1.5 h (30°C), according to previous time course experiments. All buffer solutions were adjusted to maintain a constant pH of 7.4 at each of these temperatures. Saturation experiments were carried out using eight different concentrations of [^{3}H]CHA ranging from 0.1 to 10 nM. Displacement experiments were perfomed using at least twelve different concentrations of cold drug in the presence of 1 nM [^{3}H]CHA. Non specific binding was measured in the presence of 10 μM R-PIA; this was always ≤10% of the total binding. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters which were washed three times with ice-cold buffer. Filter bound radioactivity was measured by scintillation spectrometry after the addition of 4 ml of Packard Emulsifier Safe.

All the values obtained are means of three independent experiments performed in duplicate.

Preparation of Rat Adipocytes

Isolated rat fat cells were prepared according to the method of Rodbell (17). Wistar male rats were killed by decapitation. The epididymal fat pads were quickly removed, washed in physiological solution, and immediately placed in a beaker with a Krebs-Ringer bicarbonate buffer containing NaCl 125 mM, KCl 5 mM, $CaCl_2$ 1 mM, KH_2PO_4 1 mM, $MgSO_4 \cdot 7H_2O$ 1.2 mM, 1% BSA, gassed with 5% CO_2/95% O_2. The pads were cut in small pieces and transferred, with buffer, into a plastic beaker containing 10 mg collagenase. The mixture was incubated for 45 min, at 37°C, in a shaking bath. The contents were filtered twice through a nylon sieve to remove undigested tissue. Adipocytes were then washed two or three times with buffer and then centrifuged at 100 × g for 15 s. Finally diluted aliquots of fat cells were counted under an optical microscope.

Determination of Cyclic AMP Levels

Fat cells (10^5) were suspended in 400 μl of Krebs-Ringer buffer, pH 7.4, containing 1 IU/ml of ADA and 0.5 mM Ro 20-1724 as phosphodiesterase inhibitor, and incubated for 10 min at 37°C in a shaking bath. Then the compounds and 1 μM (final concentration) forskolin were added and, after 5 min incubation, the reaction was stopped by adding ice-cold 6% (w/v, final concentration) of TCA. TCA suspensions were centrifuged at 200 × g for 10 min at 4°C and supernatant acidity was extracted with water saturated ethyl ether. The final aqueous solution was tested for c-AMP by a competitive protein binding assay carried out essentially according to Nordstedt and Fredholm (18), as previously described (7).

Calculations

Binding Assays

Saturation experiments data (K_D and B_{MAX} values) were obtained by computer analysis of saturation curves and of the corresponding Scatchard plots. The cold drug concentrations displacing 50% of labelled ligand (IC_{50} values) were obtained by computer analysis of displacement curves. Inhibitory binding constants (K_i values) were derived from the IC_{50} values according to the Cheng & Prusoff equation $K_i = IC_{50}/(1 + [C^*]/K_D^*)$ were [C*] is the concentration of the radioligand and K_D^* its dissociation constant (19). In this respect care was taken total binding never exceeded 10% of the total amount of radioligand added. All binding data were analyzed using the non-linear regression curve fitting computer program Graph Pad Prism (Graph Pad, San Diego, CA, U.S.A.).

The values of thermodynamic terms ($\Delta G°$, $\Delta H°$ and $\Delta S°$) were obtained by linear van't Hoff plots regression, as previously described in "Thermodynamic Data Determination" section.

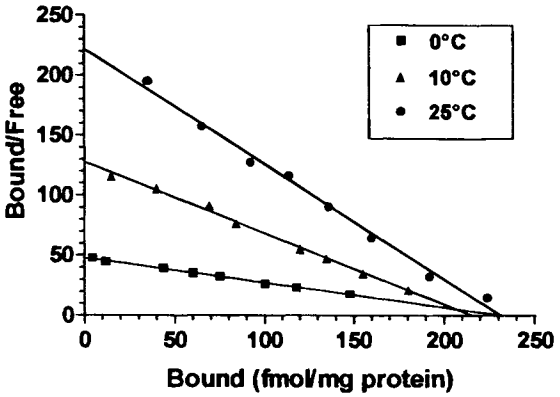

Fig. 2. Representative Scatchard plots of [^{3}H]CHA binding to rat brain membranes. The linearity of all the plots indicates the presence of a single class of high affinity binding sites at all the temperature investigated.

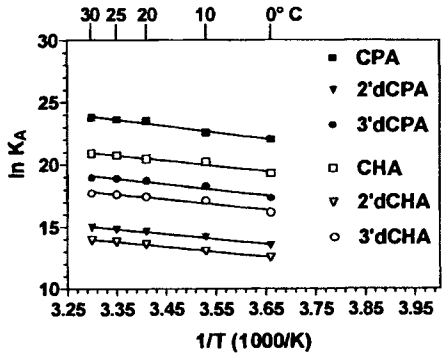

Fig. 3. Van't Hoff plots showing the effect of temperature on the equilibrium binding association constants, K_A, for all adenosine A_1 receptor ligand studied. Linear interpolation over the points connected by the continuous line ($0 < t < 30$°C) gives correlation coefficients, r, in the range of 0.97–0.99.

c-AMP Assays

IC_{50} values in the c-AMP assay were obtained from the concentration-inhibition curves by linear regression analysis after logit-log transformation.

The intrinsic activity (IA) was evaluated for each compound at a concentration equal to one hundred times its inhibitory (Ki) or dissociation (K_D) binding constant (7). The ligands able to inhibit c-AMP accumulation by 100% are considered full agonists (IA = 1); the ligands inhibiting the c-AMP accumulation by a lower value (IA < 1) are considered partial agonists.

RESULTS

Figure 2 shows three representative Scatchard plots at 0, 10, and 25°C for the saturation equilibrium of [^{3}H]CHA. The plots are linear in the concentration range investigated ($r \geq 0.98$) and computer analysis of the saturation experiments suggested a one site, rather than a two site binding model. Similar results were obtained from inhibition curve analysis of the other adenosine derivatives with Hill coefficients not significantly different from unity at all temperatures investigated (data not reported). The presence of one affinity binding site appears a typical result of experiments performed using membrane preparations in the absence of GTP and in the presence of an agonist as radioligand. These experimental conditions permit us to obtain 100% of adenosine A_1 receptors in the high affinity state (20,21).

B_{MAX} values obtained by saturation experiments appear to be independent of temperature (the mean of the values is 206 ± 5 fmol/mg protein).

Table I reports the affinity constants (K_i or K_D), obtained at all temperatures investigated, of the compounds analyzed. Figure 3 illustrates their van't Hoff plots, ln K_A versus 1/T. All plots are linear ($r \geq 0.97$) in the range 0–30°C and their slopes are systematically negative, indicating the affinity of the compounds increases with temperature.

Final thermodynamic parameters of the different compounds investigated are reported in Table II. $\Delta G°$ values range from −58.34 to −34.07 kJ/mol. Equilibrium standard enthalpy ($\Delta H°$) and entropy ($\Delta S°$) values show the binding of all compounds is always totally entropy-driven. In fact, $\Delta H°$ values range from 33 to 43 kJ/mol and $\Delta S°$ values from 226 to 341 J/mol/K.

Table II also reports the IC_{50} values for inhibition by adenosine derivatives of forskolin induced c-AMP accumulation in rat isolated adipocytes. The values have been obtained from the concentration-response curves, reported in Fig. 4, by linear regression analysis after logit-log transformation and range from 1.0 to 597 nM. Figure 4 indicates CPA and CHA are able to fully inhibit the c-AMP accumulation, whereas their 2′- and 3′-deoxy-derivatives partially inhibit it. All inhibitions were fully blocked by the specific adenosine receptor antagonist, DPCPX, which proves an adenosine A_1 mediated effect. Intrinsic activity values of the adenosine derivatives are reported in Table II where it is shown that CPA and CHA act as full

Table I. Equilibrium Binding Parameters at Five Different Temperatures[a]

Drug	T (nM°C)	0	10	20	25	30
[^{3}H]CHA	K_D	4.4 ± 0.2	1.7 ± 0.1	1.31 ± 0.04	0.98 ± 0.04	0.86 ± 0.03
2′dCHA	K_i	3600 ± 100	2100 ± 100	1220 ± 50	1020 ± 30	880 ± 40
3′dCHA	K_i	97 ± 3	38 ± 2	27 ± 1	23.4 ± 0.7	20.2 ± 0.8
CPA	K_i	0.28 ± 0.01	0.16 ± 0.01	0.062 ± 0.005	0.055 ± 0.004	0.048 ± 0.003
2′dCPA	K_i	1370 ± 41	690 ± 30	440 ± 8	375 ± 11	311 ± 12
3′dCPA	K_i	30 ± 1	11.3 ± 0.5	7.7 ± 0.3	6.2 ± 0.2	5.8 ± 0.2

[a] The parameters are expressed as: (i) dissociation constant K_D (nM) for [^{3}H]CHA derived from saturation experiments to rat cortex adenosine A_1 receptors; (ii) inhibitory constants, K_i (nM) for all compounds investigated, obtained by displacement of 1 nM [^{3}H]CHA from the same receptors. Estimated standard errors of at least three determinations are also reported.

Table II. Binding Thermodynamic Parameters, IC_{50} Values Obtained from c-AMP Assays and Intrinsic Activity (IA) Values of Adenosine A_1 Receptor Ligands[a]

Drug	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1}	IC_{50} (nM) c-AMP assay	IA
CHA	−51.22 ± 0.04	36 ± 5	292 ± 17	1.1 ± 0.1	0.98
2'dCHA	−34.07 ± 0.03	33 ± 2	226 ± 6	597 ± 13	0.68
3'dCHA	−43.39 ± 0.03	35 ± 5	263 ± 18	14.4 ± 0.7	0.75
CPA	−58.34 ± 0.07	43 ± 4	341 ± 14	1.0 ± 0.1	1.00
2'dCPA	−36.54 ± 0.03	34 ± 2	236 ± 8	276 ± 6	0.70
3'dCPA	−46.67 ± 0.03	38 ± 5	283 ± 16	9.3 ± 0.5	0.76

[a] The mean values of ΔG°, ΔH° and ΔS° are given at 298.15 K. Estimated standard errors of at least three determinations are also reported.

agonists (IA = 1) whereas their deoxy-derivatives act as partial agonists (0.68 ≤ IA ≤ 0.76). It can be observed that ΔS° values of full agonists are systematically higher than those of partial agonists, suggesting standard entropy values are correlated with IA ones, as previously reported for other adenosine derivatives (7,18). Such a correlation is reported in Fig. 5 where the values of (i) a series of N^6-substituted adenosine derivatives, of (ii) a weak partial agonist (DMX 7R), and of (iii) a series of antagonists (reported as a unique compound with IA = 0 and ΔS° = 18 J K^{-1}mol^{-1}) previously reported (22) are included:

$$IA = 0.0034(\pm 0.0003)\Delta S° - 0.09(\pm 0.06)$$

$$(r = 0.97;\ n = 14;\ P < 0.0001)$$

This equation is identical to that previously reported for the same compounds (21) without the data of deoxyribose adenosine derivatives. The new "ΔS°-IA" correlation includes a wide series of A_1 ligands: (i) the N^6-substituted adenosine derivatives (full and partial agonists), (ii) the deoxyribose adenosine derivatives (partial agonists), and (iii) the xanthine derivatives (partial agonist and antagonists).

DISCUSSION

As reported in Table I and Fig. 3, the affinities of all compounds analyzed increase with the enhancement of temperature. All van't Hoff plots are linear indicating the ΔC_P° (standard specific heat difference of the equilibrium) of the drug-receptor binding equilibrium is nearly zero or, in other words, ΔH° values are not significantly affected by temperature in the range investigated. Such linearity appears to be a typical property of the drug-membrane receptor binding (14), unlike most binding processes between molecules and biomacromolecules occurring in solution (23).

The affinities of CPA and CHA are systematically higher than those of their deoxy derivatives, in particular the 2' ones, in the temperature range investigated. The IC_{50} values obtained by c-AMP assays at 37°C (Table II) indicate a similar weaker effect, on adenylyl cyclase activity, of deoxyribose-adenosine derivatives with respect to their parent compounds CPA and CHA.

Van't Hoff plots analysis indicates the binding of all ligands is totally entropy-driven (Table II), according to a distinctive thermodynamic behaviour of full and partial agonists of adenosine A_1 receptor. It is established that agonist binding is entropy-driven, whereas antagonist binding is essentially enthalpy-driven (7,20,24,25). This behavior has permitted us to propose a model of drug receptor interaction which accounts for the biological role of ribose, the moiety indispensable to confer agonist activity to adenosine A_1 receptor ligands. In particular, it was suggested the ribose ring can assume, in the binding site, a position which permits it to displace a water molecules network, depleting it and releasing a number of solvent molecules which cause the observed entropy change. At the same time, the insertion of the ribose moiety could induce receptor conformational changes which trigger the final effect (22,24). Such a model implies both affinity and efficacy of adenosine

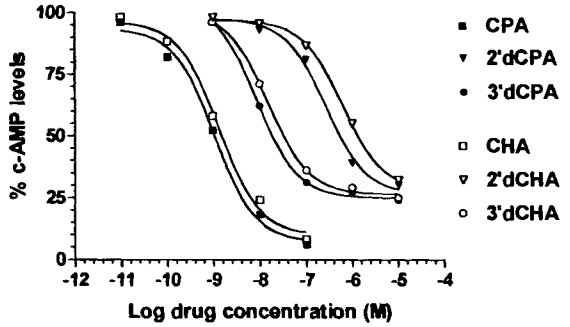

Fig. 4. Inhibition of forskolin-stimulated c-AMP levels in rat isolated adipocytes by CPA, CHA, and their 2'- or 3'-deoxyribose derivatives. This is a single representative experiment carried out in duplicate.

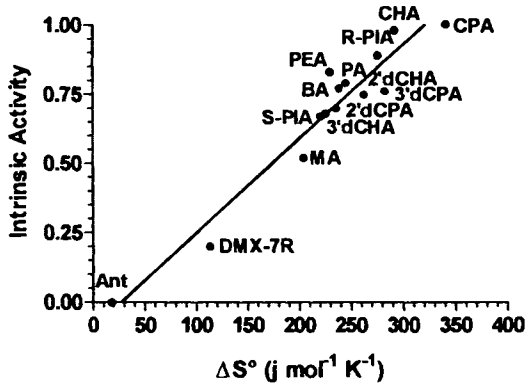

Fig. 5. Intrinsic activities versus standard entropies plot for a series of N^6-substituted adenosine derivatives, the deoxyribose derivatives under examination and DMX-7R. Xanthine antagonists are reported as a single point (ANT) with intrinsic activity of zero and an average entropy of 18 JK^{-1} mol^{-1}. Regression analysis gives a correlation coefficient r = 0.97, with P < 0.0001.

receptor ligands are related. This appears confirmed by the high correlation obtained on the scatter plot shown in Fig. 5, where the intrinsic activity of a wide variety of adenosine A_1 receptor ligands (N^6-substituted-, deoxyribose adenosine analogues, and xanthine derivatives), acting as full or partial agonists and as antagonists, is reported versus their standard entropy. $\Delta S°$ has been included in the plot because it constitutes the driving force of the agonist-receptor interactions. Standard entropy values of adenosine derivatives are therefore the indicators of their pharmacological profile, in terms of affinity and intrinsic activity towards adenosine A_1 receptor. It is reasonable to suppose the *in vivo* effects of the ligands can be reflected by their standard entropy values of the binding to the adenosine A_1 receptor, obtained *in vitro*. In particular, $\Delta S°$ values reported in Table II, indicate a lower activity towards adenosine A_1 receptor of 3′dCPA ($\Delta S° = 283$ J $mol^{-1}K^1$) and of 2′dCPA ($\Delta S° = 236$ J $mol^{-1}K^1$) with respect to CPA ($\Delta S° = 341$ J $mol^{-1}K^1$). As a consequence of the correlation reported in Fig. 5, intrinsic activity values reported in the same table show CPA is agonist (IA = 1), whereas the 2′ (IA = 0.76), and 3′ (IA = 0.70) deoxyribose analogues are partial agonists. Similarly, blood concentration-heart rate relationships, obtained after intravenous infusion of the same compounds in conscious rats (10), indicate the capability of CPA to inhibit heart rate is systematically greater than that of 3′dCPA and much greater than that of 2′dCPA.

According to the above observations, it appears binding thermodynamic of adenosine analogues performed on human adenosine A_1 receptor could give useful indications about the human pharmacodynamic *in vivo* behaviour of these type of compounds.

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