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European Journal of Pharmacology 448 (2002) 123–131

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Temperature dependence of the affinity enhancement of selective adenosine A_1 receptor agonism: a thermodynamic analysis

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Received 28 January 2002; received in revised form 12 June 2002; accepted 18 June 2002

Abstract

The 2-amino-benzoylthiophene derivatives LUF 5468 [(2-amino-4-ethyl-5-methyl-3-thienyl)[3-(trifluoromethyl)phenyl]methanone] and LUF 5484 [(2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)(3,4-dichlorophenyl)methanone] have been shown to allosterically enhance the adenosine A_1 receptor agonist binding. We report a thermodynamic analysis of the agonist affinity obtained at human adenosine A_1 receptors, in the presence and absence of LUF 5468 and LUF 5484. Moreover, an analysis of the temperature dependence for association and dissociation rates of N^6-cyclohexyladenosine (CHA) binding was performed in the absence and presence of LUF 5484. Thermodynamic data were obtained by affinity measurements performed at different temperatures followed by van't Hoff analysis. The results indicate that the agonist binding is always totally entropy-driven, and that the modulators contribute to decrease the ΔG°, ΔH° and ΔS° values. It is concluded that the enhancers are able to increase the non-bonded interactions of the binding site with agonists as CHA, N^6-cyclopentyladenosine (CPA), 2′-methyl-N^6-cyclopentyladenosine (MeCPA) and 2-chloro-2′methyl-N^6-cyclopentyladenosine (MeCCPA).

Keywords: Adenosine A_1 receptor; Adenosine A_1 receptor agonist; Allosteric; Modulation; Temperature dependence; Binding thermodynamics

1. Introduction

Extracellular adenosine is a nucleoside with profound physiological effects. Its properties are mediated by adenosine receptors, which have been classified into four subtypes: A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 1994). The adenosine A_1 receptor subtype is widely found in the central nervous system, in heart and adipose tissues (Ralevic and Burnstock, 1998). Activation of adenosine A_1 receptors has been found to produce ischemic tolerance and protection in neuronal and cardiac tissues (Heurteaux et al., 1995; Tucker and Linden, 1993) and to reduce free fatty-acid levels in the blood (Merkel et al., 1995). These pharmacological opportunities can turn into side effects given the ubiquitous distribution of the adenosine A_1 receptors in the body (IJzerman et al., 2001). Several approaches have been adopted with the aim to reduce these unwanted phenomena. As an example, the existence of partial agonists for adenosine A_1 receptor has been demonstrated (Borea et al., 1994) and their use abolishes some of the side effects that accompany adenosine A_1 receptor activation by exogenous agents (Van Schaick et al., 1998).

A fundamentally different point of view has been proposed and recently developed (Bruns and Fergus, 1990; Van der Klein et al., 1999) according to the following considerations. The formation of extracellular adenosine is a local phenomenon induced by a tissue at risk under hypoxic or anoxic conditions. These situations can cause heart failure or stroke and, in this context, the adenosine production is tissue protective (Fredholm, 1997). The opportunity to enhance the adenosine action *locally* could have a better therapeutic profile than agonists that activate adenosine A_1 receptors irrespective of localization. In fact, a local enhancement of adenosine action can produce the desired

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PII: S0014-2999(02)01982-9

therapeutic effect only in the tissues where a protective action is required, so that this is where adenosine itself is produced. This selective effect should therefore allow to prevent the adverse effects typical of adenosine A_1 receptor agonists. In the early 1990s, various 2-amino-3-benzoylthiophene derivatives were described to be able to enhance the binding and activity of adenosine A_1 receptor agonists (Van der Klein et al., 1999; Bruns et al., 1990). One potent derivative, PD 81,873—(2-amino-4,5-dimethyl-trienyl)[3-(trifluoromethyl)phenyl]methanone—has been more extensively studied (Kollias-Baker et al., 1994, 1997; Mizumura et al., 1996; Bhattacharya and Linden, 1996; Jarvis et al., 1999) and it has very recently been proposed that it contributes both to enhance the binding of human adenosine A_1 receptor agonists and to inhibit the binding of inverse agonists (Kourounakis et al., 2001). This behavior suggests the presence of an allosteric site, on adenosine A_1 receptor, different from the binding site for agonists and antagonists (Kourounakis et al., 2001). Further analysis appears therefore necessary to improve the knowledge about the molecular mechanisms which are induced by the enhancers and modulate the drug affinities in the binding site of adenosine A_1 receptors. In this context, a series of novel PD 81,723 analogues have been developed as allosteric enhancers of agonist binding to the rat adenosine A_1 receptor and as antagonists on this receptor (Van der Klein et al., 1999; Baraldi et al., 2000). Among them, LUF 5484—(2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)(3,4-dichlorophenyl)methanone—and LUF 5468—(2-amino-4-ethyl-5-methyl-3-thienyl)[3-(trifluoromethyl)phenyl]methanone(3,4-dichlorophenyl)methanone—were characterized, respectively, with superior enhancing activity and diminished antagonist behavior with respect to PD 81,723. EC_{50} value for LUF 5484 to stimulate agonist binding is 6.2 μM; 10 μM LUF 5468 causes 112% enhancement with respect to PD 81,723 set at 100% (Van der Klein et al., 1999). This paper reports a thermodynamic analysis of the effects of the two enhancers LUF 5484 and LUF 5468 (Fig. 1) on human adenosine A_1 receptors expressed by Chinese hamster ovary cells (CHO A_1). The aim of the present study was to characterize the differences which arise, in the absence and in the presence of the modulators, for standard free energy (ΔG°), standard enthalpy (ΔH°) and standard entropy (ΔS°) values referred to the binding of selective adenosine A_1 receptor agonists. The ligands analyzed were (Fig. 1) N^6-cyclohexyladenosine (CHA), N^6-cyclopentyladenosine (CPA), 2′ -methyl-N^6-cyclopentyladenosine (MeCPA) and 2-chloro-2′ methyl-N^6-cyclopentyladenosine (MeCCPA). The two last ligands are characterized as highly A_1 selective with respect to the adenosine A_3 receptor (Franchetti et al., 1998).

The standard free energy of the binding equilibrium $\Delta G^\circ = -RT\ln K_A$ (K_A = drug-receptor affinity constant) consists of two different terms according to the Gibbs equation $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$. In a simplified form, it can be assumed such separate ΔH° and ΔS° terms represent the two classes

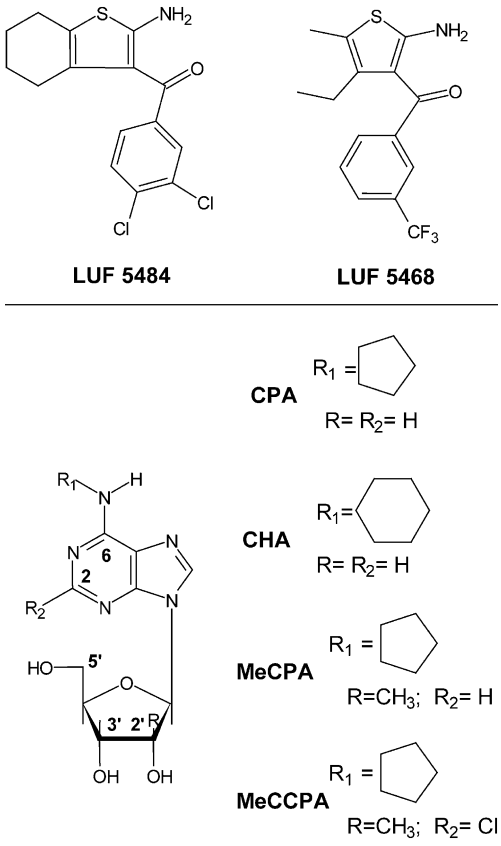

Fig. 1. Chemical formulas of the enhancers and the adenosine A_1 receptor agonists analysed.

of factors responsible for the drug-receptor recognition phenomenon (Borea et al., 2001): 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 associable with the entropic one). The thermodynamic analysis reported in this paper allows us, therefore, to evaluate how the molecular mechanism of agonist interaction with adenosine A_1 receptor changes in the presence of enhancers.

The values of the thermodynamic parameters have been obtained by K_A measurements carried out at different temperatures, followed by van't Hoff analysis (Borea et al., 1996). In this context, a detailed analysis of the temperature dependence for association and dissociation rates of agonist binding to human adenosine A_1 receptors was also performed in the absence and in the presence of the modulator LUF 5484.

2. Materials and methods

2.1. Materials

[^{3}H]CHA ([^{3}H]N^6-cyclohexyladenosine, 32.3 Ci/mmol) was obtained from NEN Research Products (Boston, MA, USA). CPA and CHA were purchased from RBI (Natick,

MA, USA). LUF 5484, LUF 5468, MeCPA and MeCCPA were obtained as previously described (Van der Klein et al., 1999; Franchetti et al., 1998).

Chinese hamster ovary cells transfected with human adenosine A_1 receptors (CHO A_1) were a kind gift of Prof. Peter R. Schofield (Garvan Institute of Medical Research, Darlinghurst, Australia) (Townsend-Nicholson and Shine, 1992; Townsend-Nicholson and Schofield, 1994).

All cell culture reagents were obtained from Gibco Laboratories (Life Technologies Italia, Milan, Italy). All other chemicals were obtained from standard sources.

2.2. 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:

(1) The standard specific heat difference of the equilibrium ($\Delta C_p°$) is essentially zero. In this case, the van't Hoff equation $\ln K_A = -\Delta H°/RT + \Delta S°/R$ gives a linear plot $\ln K_A$ vs. $1/T$. 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 $\ln K_A$ vs. $1/T$, respectively, with $R = 8.314$ J/K mol.

(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 (Eliard and Rousseau, 1984).

2.3. Cell culture

Cells were grown in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 10% Foetal Bovine Serum, streptomycin (50 μg/ml) and penicillin (50 IU/ml) at 37 °C in 5% CO_2. Cells were subcultured twice weekly at a ratio of 1:10 and transferred to large 14-cm diameter plates.

2.4. Membrane preparation

Cells were washed with phosphate-buffered saline and detached from plates by 5-min incubation at 37 °C in the presence of 2 ml of trypsol (0.25% trypsin, 4.4 mM EDTA in phosphate-buffered saline). A 5-ml sample of medium was added to each plate after incubation and the cells were collected and centrifuged for 10 min at $68 \times g$. Pellets derived from six plates were pooled and resuspended in 20 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.4, and homogenized in a Polytron homogenizer (setting 6) for 5 s. Plasma membranes and the cytosolic fraction were separated by centrifugation at $18{,}000 \times g$ in a Sorvall supercentrifuge RC-5B at 10 °C for 30 min. The pellets were resuspended in the Tris buffer at approximately 1×10^8 cells/3 ml and 2 IU/ml of adenosine deaminase were added. After 30 min incubation at 37 °C, the membranes were stored in 200-μl aliquots at −80 °C. Membrane protein concentrations were measured with the bicinchoninic acid method (Smith et al., 1985).

2.5. Receptor binding assays

Membrane aliquots containing 40 μg of proteins were incubated in 400 μl of 50 mM Tris–HCl at 0, 10, 20, 25, 30 °C with incubation times ranging from 1.5 h (30 °C) to 4 h (0 °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 10 different concentrations of CHA ranging from 2 to 500 nM using [^{3}H]CHA ranging from 2 to 100 nM and kept constant at 100 nM. All saturation experiments were performed in the absence and in the presence of 10 μM LUF 5484 or 10 μM LUF 5468. Displacement experiments were performed using at least nine different concentrations of cold drug in the presence of [^{3}H]CHA. A 10-nM sample of [^{3}H]CHA was used at all temperatures in the presence of 10 μM LUF 5484, whereas

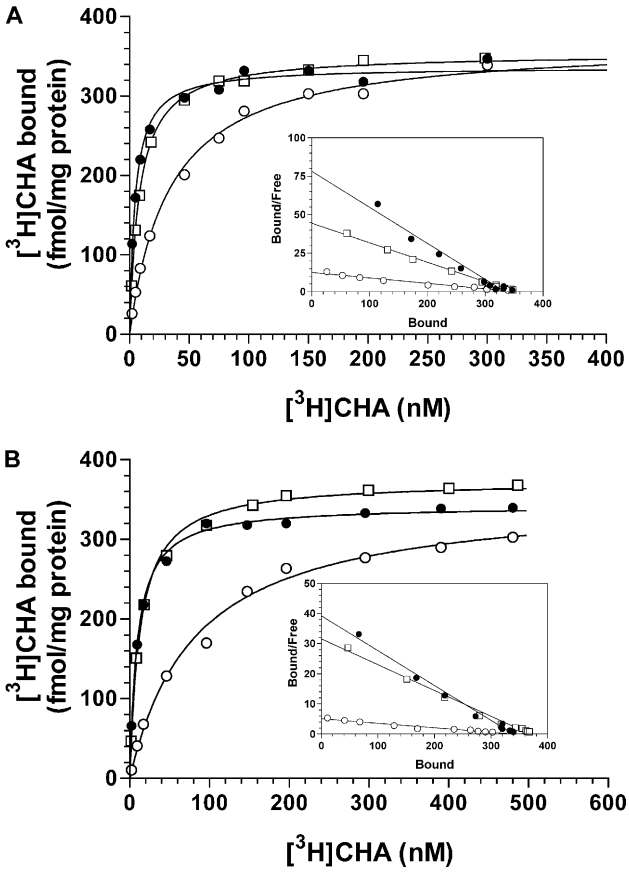

Fig. 2. Saturation curves and their Scatchard plots (inset) obtained at 25 °C (A) and at 0 °C (B) for the binding equilibrium of [^{3}H]CHA to membrane preparation of CHO A_1 cells. Data were obtained in the absence (○) and in the presence of the modulators LUF 5484 (●) and LUF 5468 (□). The linearity of the Scatchard plots indicates the presence of a single class of binding sites in all cases investigated.

in the absence of modulator, 20 nM [^{3}H]CHA was used at 20, 25 and 30 °C and 40 nM [^{3}H]CHA was used at 0 and 10 °C. Nonspecific binding was measured using 10 μM CPA. 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.

2.6. Kinetic assays

Dissociation assays were performed preincubating CHO A_1 cell membranes at 25 °C for 2 h with 20 nM [^{3}H]CHA and at 0° for 4 h with 40 nM [^{3}H]CHA. After the incubation time, the displacement was initiated by 100 μM CPA in the presence or absence of 10 μM LUF 5484. The amount of [^{3}H]CHA still bound to the receptor was measured after time intervals ranging from 5 min to 6 h.

Association experiments were performed incubating, at 0 and 25 °C, CHO A_1 cell membranes with [^{3}H]CHA in the presence and in the absence of LUF 5484. A 10-nM sample of [^{3}H]CHA was used at all temperatures, if the enhancer was present, whereas in its absence, 20 and 40 nM [^{3}H]CHA were used at 25 and 0 °C, respectively. The amount of [^{3}H]CHA bound to the receptor was measured after time intervals ranging from 1 min to 5 h.

2.7. Calculations

2.7.1. Receptor binding assays and thermodynamic data

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 and Prusoff (1973) equation $K_i = IC_{50}/(1+[C^*]/K_D^*)$ were $[C^*]$ is the concentration of the radioligand and K_D^*, its dissociation constant. In this respect, care was taken that total binding never exceeded 10% of the total amount of radioligand added.

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.

2.7.2. Kinetic assays

The dissociation constants k_{-1} were obtained by computer analysis of the exponential decay of the percentage (%) of [^{3}H]CHA bound to the receptor and of the corresponding plot "log% vs. time" (Taylor and Insel, 1990).

The association rate constants k_{+1} were calculated according to the equation $k_{+1}=(k-k_{-1})/[L]$, where k_{-1} are the dissociation rate constants, $[L]$ are the amounts of [^{3}H]CHA used for the association experiments and k are the constant values ($k=0.69/t_{1/2}$, $t_{1/2}$ = half life referred to the asymptotic behaviour of the drug–receptor equilibrium) obtained by computer analysis of the exponential association of [^{3}H]CHA bound to the receptor vs. time (Taylor and Insel, 1990).

Data of all assays were analysed using the nonlinear regression curve fitting computer program Graph Pad Prism (Graph Pad, San Diego, CA, USA).

2.7.3. Statistical analysis

One-way analysis of variance (ANOVA) followed by Dunnett's *t*-test and Student's *t*-test were performed using the computer program Graph Pad Prism (Graph Pad). Differences were considered statistically significant at *P* values less than 0.05 or 0.001.

Table 1
Equilibrium binding parameters at five different temperatures expressed as: (i) K_D and B_{max} (fmol/mg protein) for [^{3}H]CHA derived from saturation experiments to membrane preparations of CHO A_1 cell; (ii) inhibitory constants, K_i, for CPA, MeCPA and MeCCPA, obtained by displacement of [^{3}H]CHA from the same membrane preparation

Drug	*T* (°C)	0	10	20	25	30
[^{3}H]CHA	K_D (nM)	86 ± 4	46 ± 3	40 ± 3	34 ± 2	26 ± 2
	B_{max}	355 ± 21	348 ± 19	366 ± 23	368 ± 19	361 ± 23
[^{3}H]CHA + LUF 5484	K_D (nM)	11.2 ± 0.5[a]	7.1 ± 0.2[a]	6.4 ± 0.3[a]	5.2 ± 0.2[a]	4.5 ± 0.2[a]
	B_{max}	342 ± 14	358 ± 17	352 ± 16	337 ± 13	355 ± 19
[^{3}H]CHA + LUF 5468	K_D (nM)	15.2 ± 0.4[a]	10.7 ± 0.4[a]	9.7 ± 0.3[a]	8.3 ± 0.2[a]	6.7 ± 0.2[a]
	B_{max}	374 ± 12	373 ± 13	363 ± 16	353 ± 13	368 ± 18
CPA	K_i (nM)	26.2 ± 1.6	21.3 ± 2.0	13.6 ± 0.8	11.2 ± 0.6	9.8 ± 0.5
CPA + LUF 5484	K_i (nM)	3.6 ± 0.2[a]	3.6 ± 0.3[a]	2.7 ± 0.2[a]	2.0 ± 0.1[a]	1.9 ± 0.1[a]
MeCCPA	K_i (nM)	1337 ± 90	480 ± 28	303 ± 15	229 ± 14	165 ± 8
MeCCPA + LUF 5484	K_i (nM)	61 ± 3[a]	59 ± 3[a]	36 ± 2[a]	28 ± 2[a]	25 ± 1[a]
MeCPA	K_i (nM)	1800 ± 76	919 ± 39	370 ± 20	262 ± 10	186 ± 9
MeCPA + LUF 5484	K_i (nM)	65 ± 3[a]	53 ± 3[a]	50 ± 2[a]	40 ± 2[a]	37 ± 1[a]

Values are means (± S.E.M.) of three experiments performed in duplicate.
Saturation experiments have been performed in the presence (10 μM) and in the absence of the modulators LUF 5484 and LUF 5468; K_i values have been obtained in the presence and in the absence of the modulator LUF 5484.
[a] $P<0.001$ vs. values obtained for the same drug in the absence of enhancer (LUF 5484 or LUF 5468).

3. Results

Fig. 2 illustrates representative saturation experiments and the corresponding Scatchard plots obtained in the absence and in the presence of the enhancers LUF 5484 and LUF 5468 at 0 and 25 °C for the binding equilibrium of [^{3}H]CHA to human adenosine A_1 receptors. All Scatchard plots were linear in the concentration range investigated ($r \geq 0.98$, $P \leq 0.0001$) and, analogously, computer analysis of the saturation experiments suggested a one-site, rather than a two-site, binding model, either in the absence or in the presence of both enhancers.

Table 1 reports the dissociation binding constants (K_D) and B_{max} values, derived from the saturation experiments of [^{3}H]CHA to CHO A_1 membrane preparation performed in the absence and in the presence of enhancers at five chosen temperatures (0, 10, 20, 25 and 30 °C). It can be observed that similar B_{max} values (around 350 fmol/mg protein) have been obtained at all temperatures investigated either in the absence or in the presence of the two investigated enhancers. On the other hand, K_D values obtained in the presence of LUF 5484 or LUF 5468 appear around six to eight or four to six times lower, respectively, than those obtained in their absence. Moreover, the decrease of temperature induces the affinity reduction of CHA to adenosine A_1 receptor, both in the absence and in the presence of the enhancers.

Association and dissociation kinetics experiments were performed at 0 and 25 °C in the absence and in the presence of the modulator LUF 5484, with the aim to better investigate the temperature and enhancer effects on CHA binding to human adenosine A_1 receptors. The exponential decays of [^{3}H]CHA binding and the corresponding semilogarithmic plots are reported in Fig. 3. All semilogarithmic plots were linear in the time range investigated ($r \geq 0.98$, $P \leq 0.0001$) and, analogously, computer analysis of exponential decay experiments suggested a one-phase rather than a two-phase exponential decay, both in the absence and in the presence of the enhancer. Table 2 reports the dissociation kinetics (k_{-1}) values obtained by the experiments represented in Fig. 3. It can be observed that the temperature decrease from 25 to 0 °C reduces the dissociation constants from 0.145 min^{-1} (dissociation half life = 5 min) to 0.08 min^{-1} (dissociation half life = 9 min) in the absence of LUF 5484; in its presence, the reduction ranges from 0.021 min^{-1} (dissociation half life = 32 min) to 0.0097 min^{-1} (dissociation half life = 71 min). It is interesting to note that LUF 5484 markedly contributes for a reduction of the dissociation rate of CHA from adenosine A_1 receptors. In fact, the CHA dissociation half lives increase seven to eight times in the presence of the enhancer at both the temperatures investigated.

Table 2 also reports the kinetic constant values (k) obtained by the analysis of the exponential association of [^{3}H]CHA bound to human adenosine A_1 receptors vs. time. The time needed for reaching the equilibrium ranged from

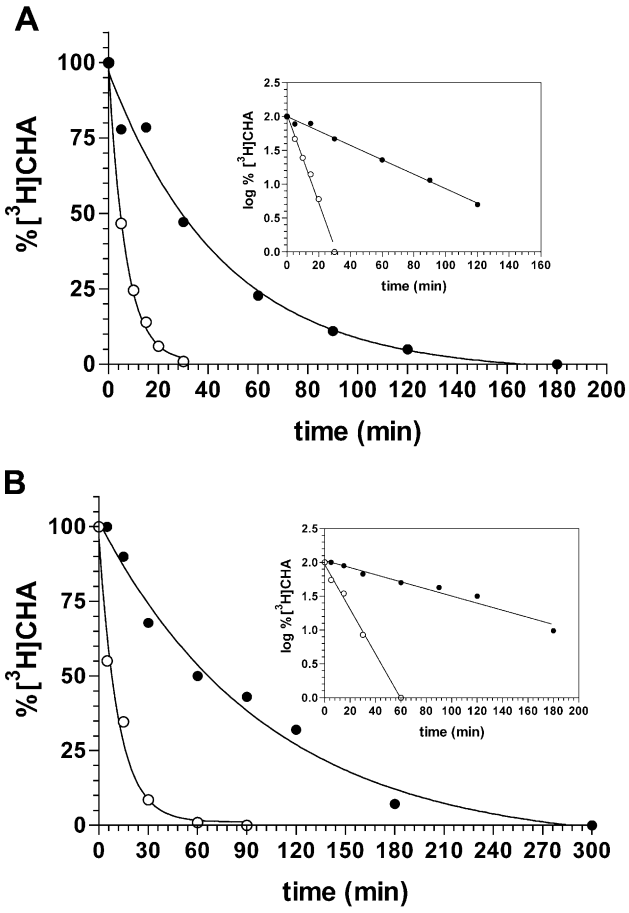

Fig. 3. Exponential decay of the percentage (%) of [^{3}H]CHA bound to human adenosine A_1 receptors and the corresponding log % vs. time plots (inset). Data were obtained at 25 °C (A) and at 0 °C (B) in the absence (○) and in the presence of the modulator LUF 5484 (●).

40 min to 4 h among all cases investigated. The k and k_{-1} values shown in Table 2 allowed us to obtain the kinetic association constants k_{+1} (Table 2). The decrease of temperature from 25 to 0 °C drastically reduces the k_{+1} values. In fact, in the absence of LUF 5468, k_{+1} values range from 0.012 to 0.0007 min^{-1} and in the enhancer presence from 0.014 to 0.0006 min^{-1}. In this case, the temperature strongly influences the CHA association rate, whereas the enhancer contribution to change k_{+1} values appears very weak at both the temperatures investigated.

The affinity values for human adenosine A_1 receptor of the three selective adenosine A_1 receptor agonists CPA, MeCPA and MeCCPA are reported in Table 1 as inhibitory binding constants (K_i) for the displacement of [^{3}H]CHA (Fig. 4). The values were obtained at five different temperatures in the absence and in the presence of the enhancer LUF 5484. All K_i values obtained in the presence of the modulator appear markedly lower than those obtained in its absence. In fact, CPA affinity appears to increase five to seven times in the presence of LUF 5484 at all the investigated temperatures. MeCPA and MeCCPA show a similar behaviour at the higher temperatures investigated and their

Table 2
Kinetic parameters obtained at 0 and 25 °C for the binding of [^{3}H]CHA to human adenosine A_1 receptors

Compound	Temperature (°C)	k (min^{-1})	k_{-1} (min^{-1})	k_{+1} (min^{-1})
CHA	0	0.11 ± 0.01	0.08 ± 0.02	0.0007 ± 0.0005
CHA + LUF 5484	0	0.016 ± 0.001[a]	0.0097 ± 0.002[b]	0.0006 ± 0.0002
CHA	25	0.38 ± 0.06	0.145 ± 0.008	0.012 ± 0.003
CHA + LUF 5484	25	0.16 ± 0.01[b]	0.021 ± 0.004[a]	0.014 ± 0.002

The values were obtained in the presence and in the absence of the modulator LUF 5484. The values of the constant k were obtained by analysis of the exponential association of [^{3}H]CHA bound to human adenosine A_1 receptor vs. time; k_{-1} are the kinetic dissociation constants; k_{+1} are the kinetic association constants.

Values are means (± S.E.M.) of three experiments performed in duplicate.

[a] $P < 0.001$ vs. values obtained for CHA in the absence of LUF 5484.

[b] $P < 0.05$ vs. values obtained CHA in the absence of LUF 5484.

affinity enhancement appears to be amplified by a drop in temperature. The K_i values reported in Table 1 also indicate that, both in the absence and in the presence of LUF 5484, an affinity reduction of CPA and its derivatives is induced by a temperature decrease.

The temperature dependence of the affinity constants K_A ($K_A = 1/K_D$ for CHA; $K_A = 1/K_i$ for CPA, MeCPA and MeCCPA) is exemplified by the van't Hoff plots, $\ln K_A$ vs. $1/T$ of Fig. 5. All van't Hoff plots display a negative slope and are linear in the overall temperature range investigated both in the absence and in the presence of LUF 5484 and LUF 5468.

Table 3 reports the final thermodynamic parameters of the agonists investigated in the absence and in the presence of modulators. Standard free energy ($\Delta G°$) values range from −45.2 to −37.3 kJ mol^{-1} in the absence of enhancers; in their presence $\Delta G°$ values range from −49.2 to −42.0 kJ mol^{-1}. Equilibrium standard enthalpy ($\Delta H°$) and entropy ($\Delta S°$) values show the agonist binding always totally entropy-driven, both in the absence and in the presence of the enhancers. In fact, $\Delta H°$ values range from 13 to 53 kJ mol^{-1} and $\Delta S°$ from 183 to 304 J mol^{-1} K^{-1}. It is interesting to underline that, in the modulator absence, $\Delta H°$ values range from 24 to 56 kJ mol^{-1} and $\Delta S°$ values from 225 to 304 J mol^{-1} K^{-1}, whereas in the modulator presence, $\Delta H°$ values range from 13 to 19 kJ mol^{-1} and $\Delta S°$ values from 183 to 223 J mol^{-1} K^{-1}. In general, the

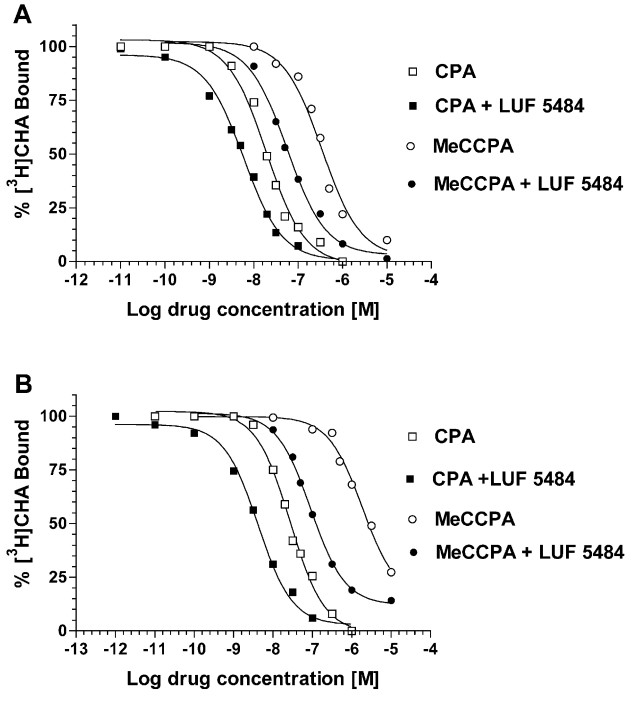

Fig. 4. Competition experiments of CPA and MeCCPA for specific [^{3}H]CHA binding performed at 25 °C (A) and 0 °C (B) in the presence and in the absence of LUF 5484. A 10-nM sample of [^{3}H]CHA was used at all temperatures in the presence of 10 μM LUF 5484, whereas in absence of modulator, 20 nM [^{3}H]CHA was used at 25 °C and 40 nM [^{3}H]CHA was used at 0 °C.

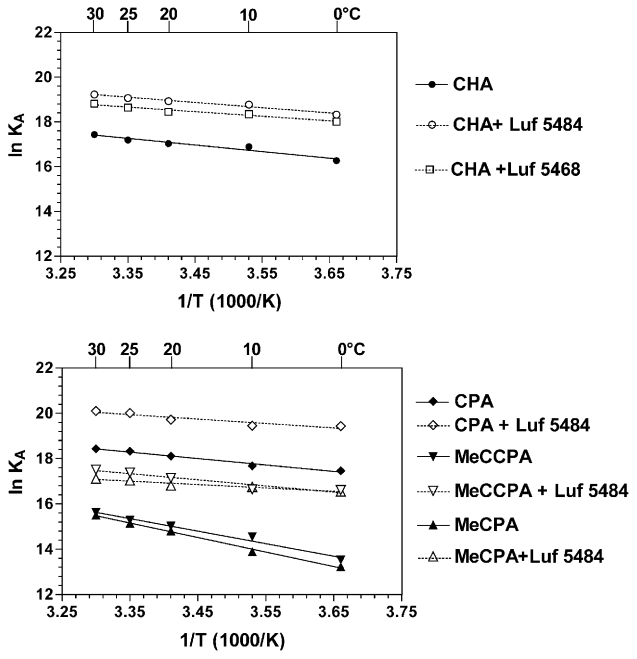

Fig. 5. van't Hoff plots showing the effect of temperature on the equilibrium binding association constants, K_A, for the selective adenosine A_1 receptor agonists analysed on human adenosine A_1 receptors in the presence and in the absence of the modulators LUF 5484 and LUF 5468. All plots are fairly linear in the full temperature range from 0 to 30 °C.

Table 3
Thermodynamic parameters for the binding equilibrium of selective adenosine A_1 receptor agonists to human adenosine A_1 receptors obtained in the presence and in the absence of modulators LUF 5484 and LUF 5468

Compound	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J mol^{-1} K^{-1})
[^{3}H]CHA	− 42.5 ± 0.1	24 ± 3	225 ± 11
[^{3}H]CHA + LUF 5484	− 47.1 ± 0.1[a]	19 ± 2	223 ± 7
[^{3}H]CHA + LUF 5468	− 46.0 ± 0.1[a]	17 ± 2[b]	211 ± 7
CPA	− 45.2 ± 0.1	24 ± 2	230 ± 7
CPA + LUF 5484	− 49.2 ± 0.2[a]	16 ± 4[b]	220 ± 12
MeCCPA	− 37.9 ± 0.1	46 ± 4	281 ± 13
MeCCPA + LUF 5484	− 42.8 ± 0.1[a]	23 ± 4[b]	219 ± 14[b]
MeCPA	− 37.3 ± 0.2	53 ± 2	304 ± 7
MeCPA + LUF 5484	− 42.0 ± 0.1[a]	13 ± 2[a]	183 ± 7[a]

$\Delta G°$, $\Delta H°$ and $\Delta S°$ values (± S.D.) are given at 298.15 K.
[a] $P < 0.001$ vs. values obtained for the same drug in the absence of enhancer (LUF 5484 or LUF 5468).
[b] $P < 0.05$ vs. values obtained for the same drug in the absence of enhancer (LUF 5484 or LUF 5468).

enhancer presence induces a reduction of the $\Delta G°$, $\Delta H°$ and $\Delta S°$ values.

4. Discussion

The first part of our research work was focused on the analysis of the modulator and temperature effects on CHA binding to human adenosine A_1 receptors. In this context, [^{3}H]CHA saturation experiments were performed on a membrane preparation of CHO A_1 cells at five temperatures ranging from 0 to 30 °C, either in the absence or in the presence of the enhancers LUF 5484 and LUF 5468. The results concerning [^{3}H]CHA alone suggest the presence of a single class of high affinity binding sites at all temperature investigated. This same pattern was recently found using adenosine A_1 receptor-radiolabelled agonists and the same cellular system (Kourounakis et al., 2001; Dalpiaz et al., 2001) or rat brain homogenates (Borea et al., 1992; Dalpiaz et al., 1999). Adenosine A_1 receptor belongs to the family of GPCRs and, as consequence, it exists in equilibrium between two different conformations, which correspond to a high and a low affinity binding state (Lohse et al., 1984; Milligan et al., 1995). The single class of high affinity binding sites found by us can be attributed to the presence of the radiolabelled agonist which is able induce the equilibrium position toward the high affinity state (Milligan et al., 1995). We have distinguished the high and low affinity states of adenosine A_1 receptor for CHA performing displacement experiments using an antagonist as radiolabelled ligand (1 nM [^{3}H]DPCPX-[^{3}H]1,3,-dipropyl-8-cyclopentylxanthine—a selective adenosine A_1 receptor antagonist). The results of these experiments indicate the K_i values of CHA to be 25 ± 3 nM for the high affinity state and 590 ± 40 nM for the low affinity state of the receptor (data not shown).

As reported in Table 1, the changes of temperature do not affect the B_{max} values, whereas the CHA affinity systematically increases from 0 to 30 °C. This last behaviour was found for other adenosine A_1 receptor agonists using the same receptor system (Dalpiaz et al., 1998).

Our results indicate, moreover, that B_{max} values do not appear to be influenced by LUF 5484 or LUF 5468. On the other hand, their presence induces consistent enhancements of [^{3}H]CHA affinity toward adenosine A_1 receptors at all the investigated temperatures. In particular, our results suggest that the enhancers allow to increase the agonist binding for the high affinity adenosine A_1 receptor state. A similar effect, even if weaker, has been recently found by us for the radiolabelled agonist [^{3}H]CCPA ([^{3}H]2-chloro-N^6-cyclopentyladenosine) in the presence of the enhancer PD 81,723 (Kourounakis et al., 2001). These last results appear in accordance with the hypothesis that this type of enhancers stabilises a high affinity receptor state for agonists (Kollias-Baker et al., 1997; Kourounakis et al., 2001; Bhattacharya and Linden, 1995; Musser et al., 1999). The modulator LUF 5484 appears to enhance by approximately one order of magnitude (see Table 1) the agonist affinity to human adenosine A_1 receptors expressed by Chinese hamster ovary cells. To the best of our knowledge, this is the first report which describes such a significant effect for this class of enhancers.

As reported in Table 1, the increase of temperature or the enhancer presence induce the affinity improvement of agonists for adenosine A_1 receptors. With the aim to investigate the molecular mechanisms which cause these affinity enhancements, we performed kinetic experiments at 0 and 25 °C to evaluate how the temperature increase or the modulator presence influence the association and dissociation rates of [^{3}H]CHA to adenosine A_1 receptor. Data reported in Table 2 indicate that the enhancement of temperature strongly increases the association rates, whereas it weakly influences the dissociation rates. This happens both in the presence and in the absence of modulator. On the other hand, it can be observed that the presence of LUF 5484 induces a strong decrease of the dissociation rates and very weak effects on the association rates at both the temperatures investigated. Similar decreases of dissociation rate, concerning rat and dog adenosine A_1 receptors, were

previously described in the presence of other enhancers (Bruns et al., 1990; Mizumura et al., 1996).

We therefore conclude that the molecular events on the adenosine A_1 receptor induced by either an increase in temperature or enhancer presence are totally different, even if they cause, on the whole, an agonist affinity increase. This effect is in fact obtained by the enhancement of association rates in the case of a rise in temperature, whereas it is obtained by a reduction of dissociation rates in the case of enhancer presence.

The thermodynamic data obtained by the analysis of van't Hoff plots (Fig. 5) and reported in Table 3 allow us to describe in a more detailed manner these phenomena, according to the following considerations. ΔG°, ΔH° and ΔS° data obtained in the absence and in the presence of modulators indicate that the binding of the agonists is totally entropy-driven, with positive standard enthalpy and entropy values which are typical for adenosine A_1 receptor agonist binding (Borea et al., 1996, 2000, 2001; Dalpiaz et al., 1998, 2001). This is the indication that the driving force of the agonist binding is governed by solvent reorganization phenomena which, according to the Gibbs equation ($\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$; T = temperature, $\Delta G^\circ = -RT\ln K_A$), give favourable entropic ($-T\Delta S^\circ < 0$) and unfavourable enthalpic contributions ($\Delta H^\circ > 0$) to the binding. A model of agonist–adenosine A_1 receptor interaction has been proposed on the basis of thermodynamic behaviour. The presence of the ribose moiety allows the adenosine A_1 receptor agonists to assume a position in the binding site which permits it to displace a network of water molecules, depleting it and releasing a number of solvent molecules which cause the observed entropy change (Borea et al., 1992, 2001). In this context, a related positive enthalpy change is observed, according to the enthalpy–entropy compensation phenomenon, which was attributed to the solvent reorganisation that accompanies the receptor binding processes (Grunwald and Steel, 1995). Data reported in Table 3 indicate that the presence of a methyl group in the 2′ position of the ribose moiety induces an enhancement of positive standard enthalpy and entropy values, suggesting a related increase in the reorganization of water molecules.

The presence of the enhancer induces the increase of affinities (ΔG°, Table 3), but this effect is not associated with an enhancement of standard entropy (ΔS°) values. On the contrary, standard entropy values remain constant, or even decrease in the presence of LUF 5484. It can instead be observed that this presence reduces markedly the positive standard enthalpy (ΔH°) values for all agonists analysed. This change of ΔH° values indicates that in the presence of enhancers, the agonists are better able to accommodate in the binding site, thus improving non-bonded interactions with the receptor. These interactions appear to partially compensate the unfavourable enthalpic contributions to the binding caused by the solvent reorganization. Such a phenomenon may be related to the decrease of the dissociation rate between agonist and adenosine A_1 receptors registered in the presence of LUF 5484 or other enhancers. On the other hand, the receptor conformational changes induced by the enhancers do not seem to facilitate the removal of the network of water molecules, as indicated by the absence of standard entropy increase in the presence of the enhancers. Such a phenomenon should be on the contrary facilitated by a temperature enhancement, which is in fact related to an increase of the association rate between agonist and adenosine A_1 receptor.

Our data suggest the presence of an allosteric site, on adenosine A_1 receptor, different from the binding site for agonists and antagonists. A similar pattern was previously hypothesized after analysis of differential effects of the allosteric enhancer PD 81,723 on agonist and antagonist binding and function at human wild-type and mutant adenosine A_1 receptors (Kourounakis et al., 2001). We can conclude that the thermodynamic results suggest that the enhancers, acting on an allosteric site different from the agonist binding site, are able to induce conformational changes of adenosine A_1 receptors. These changes allow to increase the non-bonded interactions of the binding site with agonists.

Acknowledgements

This research was supported by Italian Ministry of University. The authors thank Prof. Peter Schofield of the Garvan Institute of Medical Research, Sydney, Australia and Dr. Andrea Townsend-Nicholson of the Department of Anatomy & Developmental Biology, University College, London, for providing the CHO A_1 cells.

References

Baraldi, P.G., Zaid, A.N., Lampronti, I., Fruttarolo, F., Pavani, M.G., Tabrizi, M.A., Shryock, J.C., Leung, E., Romagnoli, R., 2000. Synthesis and biological effects of a new series of 2-amino-3-benzoylthiophenes as allosteric enhancers of A_1-adenosine receptor. Bioorg. Med. Chem. Lett. 10, 1935–1937.

Bhattacharya, S., Linden, J., 1995. The allosteric enhancer, PD 81,723, stabilizes human A_1 adenosine receptor coupling to G proteins. Biochim. Biophys. Acta 1265, 15–21.

Bhattacharya, S., Linden, J., 1996. Effects of long-term treatment with the allosteric enhancer, PD 81,723, on Chinese hamster ovary cells expressing recombinant A_1 adenosine receptor. Mol. Pharmacol. 50, 104–111.

Borea, P.A., Varani, K., Guerra, L., Gilli, P., Gilli, G., 1992. Binding thermodynamics of A_1 adenosine receptor ligands. Mol. Neuropharmacol. 2, 273–281.

Borea, P.A., Varani, K., Dalpiaz, A., Capuzzo, A., Fabbri, E., Ijzerman, A.P., 1994. Full and partial agonistic behaviour and thermodynamic binding parameters of adenosine A_1 receptor ligands. Eur. J. Pharmacol., Mol. Pharmacol. Sect. 267, 55–61.

Borea, P.A., Dalpiaz, A., Varani, K., Gessi, S., Gilli, G., 1996. Binding thermodynamics at A_1 and A_{2A} adenosine receptors. Life Sci. 59, 1373–1388.

Borea, P.A., Dalpiaz, A., Varani, K., Gilli, P., Gilli, G., 2000. Can thermodynamic measurements of receptor binding yield information on drug affinity and efficacy? Biochem. Pharmacol. 60, 549–556.

Borea, P.A., Varani, K., Gessi, S., Gilli, P., Dalpiaz, A., 2001. Thermodynamics of Adenosine (A_1 and A_{2A})–receptor interactions. In: Raffa, R.B. (Ed.), Drug–Receptor Thermodynamics: Introduction and Applications. Wiley, New York, pp. 221–244.

Bruns, R.F., Fergus, J.H., 1990. Allosteric enhancement of adenosine A_1 receptor binding and function by 2-amino-3-benzothiophenes. Mol. Pharmacol. 38, 939–949.

Bruns, R.F., Fergus, J.H., Coughenour, L.L., Courtland, G.G., Pugsley, T.A., Dodd Tinney, F.J., 1990. Structure–activity relationships for enhancement of adenosine receptor binding by 2-amino-3-benzoylthiophenes. Mol. Pharmacol. 38, 950–958.

Cheng, Y.C., Prusoff, W.H., 1973. Relationships between the inhibition constant (K_i) and the concentration of inhibitor which cause 50 percent inhibition (IC_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Dalpiaz, A., Townsend-Nicholson, A., Beukers, M., Schofield, P., IJzerman, A.P., 1998. Thermodynamics of full agonist, partial agonist and antagonist binding to wild-type and mutant adenosine A_1 receptors. Biochem. Pharmacol. 56, 1437–1445.

Dalpiaz, A., Scatturin, A., Pavan, B., Varani, K., IJzerman, A.P., Borea, P.A., 1999. Thermodynamic in vitro studies as a method to investigate the pharmacodynamic behaviour of adenosine A_1 receptor ligands. Pharm. Res. 16, 1054–1058.

Dalpiaz, A., Scatturin, A., Menegatti, E., Bortolotti, F., Pavan, B., Biondi, C., Durini, E., Manfredini, S., 2001. Synthesis and study of 5′ -esters prodrugs of N^6-cyclopentyladenosine, a selective A_1 receptor agonist. Pharm. Res. 18, 531–535.

Eliard, P.H., Rousseau, G.G., 1984. Thermodynamics of steroid binding to the human glucocorticoid receptor. Biochem. J. 218, 395–404.

Franchetti, P., Cappellacci, L., Marchetti, S., Trincavalli, L., Martini, C., Mazzoni, M.R., Lucacchini, A., Grifantini, M., 1998. 2′ -*C*-Methyl analogues of selective adenosine receptor agonists: synthesis and binding studies. J. Med. Chem. 41, 1708–1715.

Fredholm, B.B., 1997. Adenosine and neuroprotection. Int. Rev. Neurobiol. 40, 259–280.

Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P., Williams, M., 1994. Nomenclature and classification of purinoceptors. Pharmacol. Rev. 46, 143–156.

Grunwald, E., Steel, C., 1995. Solvent reorganisation and thermodynamic enthalpy–entropy compensation. J. Am. Chem. Soc. 117, 5687–5692.

Heurteaux, C., Lauritzen, I., Widmann, C., Lazdunski, M., 1995. Essential role of adenosine, adenosine A_1 receptors, and ATP-sensitive K^+ channels in cerebral ischemic preconditioning. Proc. Natl. Acad. Sci. U. S. A. 92, 4666–4670.

IJzerman, A.P., Kourounakis, A., Van der Klein, P., 2001. Allosteric modulation of G protein-coupled receptors. Il Farmaco 56, 67–70.

Jarvis, M.F., Gessner, G., Shapiro, G., Merkel, L., Myers, M., Cox, B.F., Martin, G., 1999. Differential effects of the adenosine A_1 receptor allosteric enhancer PD 81,723 on agonist binding to brain and adypocyte membranes. Brain. Res. 840, 75–83.

Kollias-Baker, C., Rubl, J., Dennis, D., Bruns, R.F., Linden, J., Belardinelli, L., 1994. Allosteric enhancer PD 81,723 acts by novel mechanism to potentiate cardiac actions of adenosine. Circ. Res. 75, 961–971.

Kollias-Baker, C.A., Ruble, J., Jacobson, M., Harrison, J.K., Ozeck, M., Shryock, J.C., Belardinelli, L., 1997. Agonist-independent effect of an allosteric enhancer of the A_1 adenosine receptor in CHO cells stably expressing the recombinant human A_1 receptor. J. Pharmacol. Exp. Ther. 281, 761–768.

Kourounakis, A., Visser, C., De Groote, M., IJzerman, A.P., 2001. Differential effects of the allosteric enhancer (2-amino-4,5-dimethyl-trienyl)[3-(trifluomethyl)phenyl]methanone (PD81,723) on agonist and antagonist binding and function at the human wild-type and a mutant (T277A) adenosine A_1 receptor. Biochem. Pharmacol. 67, 137–144.

Lohse, M.J., Lenschow, V., Schwabe, U., 1984. Two affinity states of R_i adenosine receptors in brain membranes. Analysis of guanine nucleotide and temperature effects on radioligand binding. Mol. Pharmacol. 26, 1–9.

Merkel, L.A., Hawkins, E.D., Colussi, D.J., Greenland, B.D., Smits, G.J., Perrone, M.H., Cox, B.F., 1995. Cardiovascular and antilipolytic effects of GR79236. Pharmacology 51, 224–236.

Milligan, G., Bond, R.A., Lee, M., 1995. Inverse agonism: pharmacological curiosity or potential therapeutic strategy? Trends Pharmacol. Sci. 16, 10–13.

Mizumura, T., Auchampach, J.A., Linden, J., Bruns, R.F., Gross, G.J., 1996. PD 81,723, an allosteric enhancer of the A_1 adenosine receptor, lowers threshold for ischemic preconditioning in dogs. Circ. Res. 79, 415–423.

Musser, B., Mudumbi, R.V., Liu, J., Olson, R.D., Vestal, R.E., 1999. Adenosine A_1 receptor-dependent and independent effects of the allosteric enhancer PD 81,723. J. Pharmcol. Exp. Ther. 288, 446–454.

Ralevic, V., Burnstock, G., 1998. Receptors for purines and pyrimidines. Pharmacol. Rev. 50, 413–492.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Taylor, P., Insel, P.A., 1990. Molecular basis of pharmacologic selectivity. In: Pratt, W.B., Taylor, P. (Eds.), Principles of Drug Action. Livingstone, New York, pp. 1–102.

Townsend-Nicholson, A., Schofield, P.R., 1994. A threonine residue in the seventh transmembrane domain of the human A_1 adenosine receptor mediates specific agonistic binding. J. Biol. Chem. 269, 27900–27906.

Townsend-Nicholson, A., Shine, J., 1992. Molecular cloning and characterization of a human brain A_1 adenosine receptor cDNA. Mol. Brain Res. 16, 365–370.

Tucker, A.L., Linden, J., 1993. Cloned receptors and cardiovascular responses to adenosine. Cardiovasc. Res. 27, 54–61.

Van der Klein, P.A.M., Kourounakis, A.P., IJzerman, A.P., 1999. Allosteric modulation of the adenosine A_1 receptor. Synthesis and biological evaluation of novel 2-amino-3-benzoylthiophenes as allosteric enhancers of agonist binding. J. Med. Chem. 42, 3629–3635.

Van Schaick, E.A., Tukker, H.E., Roelen, H.C.P.F., IJzerman, A.P., Danhof, M., 1998. Selectivity of action of 8-alkylamino analogues of N^6-cyclopentyladenosine in vivo: haemodynamic vs. anti-lipolytic responses in rats. Br. J. Pharmacol. 124, 607–618.