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[^{3}H]MRE 3008F20: A Novel Antagonist Radioligand for the Pharmacological and Biochemical Characterization of Human A_3 Adenosine Receptors

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ABSTRACT

The lack of a radiolabeled selective A_3 adenosine receptor antagonist is a major drawback for an adequate characterization of this receptor subtype. This paper describes the pharmacological and biochemical characterization of the tritiated form of a new potent A_3 adenosine receptor antagonist, the pyrazolo triazolo pyrimidine derivative [^{3}H]5*N*-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-*e*] -1,2,4-triazolo[1,5-*c*]pyrimidine ([^{3}H]MRE 3008F20). [^{3}H]MRE 3008F20 bound specifically to the human adenosine A_3 receptor expressed in CHO cells (hA_3CHO), and saturation analysis revealed a single high affinity binding site, K_D = 0.80 ± 0.06 nM, with a B_{max} = 300 ± 33 fmol/mg protein. This new ligand displayed high selectivity (1294-, 165-, and 2471-fold) in binding assay to human A_3 versus A_1, A_{2A}, and A_{2B} receptors, respectively, and binds to the rat A_3 receptors with a $K_i > 10$ μM. The pharmacological profile of [^{3}H]MRE 3008F20 binding to hA_3CHO cells was evaluated using known adenosine receptor agonists and antagonists with a rank order of potency consistent with that typically found for interactions with the A_3 adenosine receptors. In the adenylyl cyclase assay the same compounds exhibited a rank order of potency identical with that observed in binding experiments. Thermodynamic data indicated that [^{3}H]MRE 3008F20 binding to hA_3CHO is entropy- and enthalpy-driven in agreement with the typical behavior of other adenosine antagonists to A_1 and A_{2A} receptors. These results show that [^{3}H]MRE 3008F20 is the first antagonist radioligand with high affinity and selectivity for the human A_3 adenosine receptor and may be used to investigate the physiopathological role of A_3 adenosine receptors.

Adenosine, an endogenous modulator of a wide range of biological functions, interacts with at least four cell surface receptor subtypes classified as A_1, A_{2A}, A_{2B}, and A_3. These receptor subtypes belong to the superfamily of G protein-coupled receptors and have been cloned in several animal species (Fredholm et al., 1994). Typically, G protein-coupled receptors show sequence homologies ranging from 85% to 95% among different species (Ralevic and Burnstock, 1998). On the contrary, the A_3 subtype exhibits a lower degree of homology that is only 74% between rats and humans or sheep and 85% between sheep and humans (Zhou et al., 1992; Linden, 1994). Moreover no considerable changes in binding affinity of several agonists and antagonists have been found between rat and human A_1 and A_{2A} receptors (Dionisotti et al., 1997), whereas the rat A_3 receptor differs from the human in the antagonist binding (Salvatore et al., 1993). Furthermore, A_3 adenosine receptors have large interspecies differences in peripheral distribution. The rat transcript has been detected in testis, lung, kidneys, heart, and brain (Hill et al., 1997; Jacobson et al., 1998b). The human A_3 receptor transcript is widespread, with the most abundant expression being found in the lung and liver. This suggests that numer-

ABBREVIATIONS: AB-MECA, 4-aminobenzyl-5′-*N*-methylcarboxamidoadenosine; MRE 3008F20, 5-*N*-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; NECA, 5′-*N*-ethylcarboxamidoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; SCH 58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; R-PIA, *R*(−)-N^6-(2-phenylisopropyl)adenosine; S-PIA, *S*(−)-N^6-(2-phenylisopropyl)adenosine; CGS 21680, 2-[*p*-(2-carboxyethyl)phenetylamino]-5′-*N*-ethylcarboxamidoadenosine; IB-MECA, N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; CHO, Chinese hamster ovary; CGS 15943, 5-amino-9-chloro-2-(furyl)-1,2,4-triazolo[1,5-*c*]quinazoline; XAC, 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine; MPC-NECA, N^6-(4-methoxyphenylcarbamoyl)-adenosine-5′-*N*-ethyluronamide; MPC-MECA, N^6-(4-methoxyphenylcarbamoyl)adenosine-5′-*N*-methyluronamide; MRE 3010F20, 5-*N*-(3-chlorophenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; hA_3CHO, human adenosine A_3 receptor expressed in CHO cells.

ous physiological effects of adenosine may be mediated by the A_3 adenosine receptor (Jacobson et al., 1995). Several studies indicate that adenosine A_3 receptors may play a basic role in different pathologies such as inflammation and neurodegeneration (Kohno et al., 1996), ischemic brain damage (Von Lubitz et al., 1994), asthma (Jacobson et al., 1998b), and cardiac ischemia (Liang and Jacobson, 1998). To obtain more information on the physiological role of A_3 receptors, new selective agonists and antagonists should be synthesized. The human cloned A_3 adenosine receptor was first characterized with N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine (Salvatore et al., 1993). Subsequently, [^{125}I]AB-MECA has been widely used as a high affinity radioligand for A_3 receptors (Olah et al., 1994; Jacobson, 1998a) even if it shows a moderate A_3/A_1 selectivity (Klotz et al., 1998). The lack of potent and selective radiolabeled A_3 receptor antagonists has been the major obstacle in the characterization of structure, function, and regulation of this adenosine receptor subtype. Antagonists are generally considered more acceptable in the receptor classification than are the results obtained using agonists, the latter being complicated by the different receptor affinity states and cell-dependent effector coupling. In the last few years important progress has been made on the development of selective A_3 receptor antagonists, which have an interesting pharmacological profile (Jacobson et al., 1997; Jiang et al., 1997; Kim et al., 1998; Li et al., 1999). Recently, our group has identified a series of substituted pyrazolo triazolo pyrimidines as potent and selective antagonists to human A_3 adenosine receptors (Baraldi et al., 1999).

This paper describes for the first time the pharmacological characterization of the human A_3 adenosine receptors transfected in Chinese hamster ovary (CHO) cells by using the tritium-labeled form of the most representative compound of this series, [^{3}H]5*N*-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine ([^{3}H]MRE 3008F20) (see Fig. 1). To verify the species specificity of MRE 3008F20, its affinity to rat adenosine receptors was also evaluated. Binding parameters obtained in hA_3CHO using [^{3}H]MRE 3008F20 or [^{125}I]AB-MECA were compared. MRE 3008F20 displayed high selectivity for human A_3 (K_i = 0.85 ± 0.02 nM) when compared with its affinity to human A_1 (K_i = 1100 ± 100 nM), A_{2A} (K_i = 140 ± 15 nM), and A_{2B} (K_i = 2100 ± 300 nM) adenosine receptors. Moreover, the abilities of typical agonists to inhibit cAMP accumulation and the potency of a series of antagonists in blocking the IB-MECA-induced inhibition of adenylyl cyclase have been evaluated. Finally, with the aim of obtaining insights on the forces driving the coupling of the human A_3 adenosine receptor with a selective ligand, a thermodynamic analysis of [^{3}H]MRE 3008F20 binding was performed and the enthalpic ($\Delta H°$) and entropic ($\Delta S°$) contributions to the standard free energy ($\Delta G°$) of the binding equilibrium were determined.

Experimental Procedures

Materials. [^{125}I]AB-MECA (specific activity 2000 Ci/mmol) was obtained from Amersham Laboratories, Milan, Italy. [^{3}H]DPCPX (specific activity, 120 Ci/mmol) was obtained from NEN Research Products (Boston, MA). [^{3}H]SCH 58261 (specific activity, 68 Ci/mmol) was obtained from the Schering-Plough Research Institute (Milan, Italy). NECA, R-PIA, S-PIA, CGS 21680, IB-MECA, AB-MECA, CGS 15943, DPCPX, and XAC were obtained from Research Biochemical International (Natick, MA). SCH 58261, MPC-NECA, MPC-MECA, MRE 3010F20, and MRE 3008F20 were synthesized by Prof. P.G. Baraldi (Department of Pharmaceutical Sciences, University of Ferrara, Italy). CHO cells transfected with the rat recombinant A_3 adenosine receptor were obtained from NEN Life Sciences Products (Boston, MA). HEK-293 cells transfected with the human recombinant A_{2B} adenosine receptor were obtained from Receptor Biology, Inc. (Beltsville, MD). CHO cells transfected with the human recombinant A_1, A_{2A}, and A_3 adenosine receptor were obtained from sources described earlier (Klotz et al., 1998). All other reagents were of analytical grade and obtained from commercial sources.

Synthesis of [^{3}H]MRE 3008F20. The synthesis of [^{3}H]MRE 3008F20 (specific activity, 67 Ci/mmol) (Fig. 1) was performed at Amersham International (Buckinghamshire, UK) from tritium gas through a method developed by Nycomed Amersham plc. The product was purified by reversed-phase high performance liquid chromatography using a water methanol/methanol triethylamine gradient.

Human Cloned A_1, A_{2A}, A_{2B}, and A_3 Adenosine Receptor Binding Assay. The expression of the human A_1, A_{2A}, and A_3 receptors in CHO cells has been previously described (Klotz et al., 1998). The cells were grown adherently and maintained in Dulbecco's modified Eagle's medium with nutrient mixture F12 without nucleosides, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and Geneticin (G418, 0.2 mg/ml) at 37°C in 5% CO_2/95% air. Cells were split two or three times weekly at a ratio of between 1:5 and 1:20.

For membrane preparation the culture medium was removed. The cells were washed with phosphate-buffered saline 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*g*. The supernatant was then centrifuged for 30 min at 100,000*g*. The membrane pellet was resuspended in 50 mM Tris-HCl buffer at pH 7.4 (for A_3 adenosine receptors: 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA) and incubated with 3 I.U./ml of adenosine deaminase for 30 min at 37°C. Then the suspension was frozen at −80°C.

Binding to CHO cells transfected with the human recombinant A_1 and A_{2A} adenosine receptor was performed using [^{3}H]DPCPX and [^{3}H]SCH 58261, respectively, as previously described (Dionisotti et al., 1997; Klotz et al., 1998).

Competition experiments of [^{3}H]DPCPX to HEK-293 cells transfected with the human recombinant A_{2B} adenosine receptor were performed essentially according to the method described by Linden et al. (1998), who used as radioligand [^{3}H] 1,3-diethyl-8-phenylxanthine. In particular, assays were carried out for 60 min at 25°C in 0.1

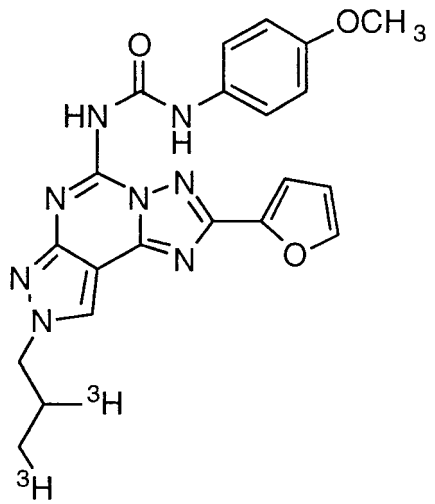

Fig. 1. Chemical structure of [^{3}H]MRE 3008F20.

ml of 50 mM Tris-HCl buffer, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM benzamidine (pH 7.4), 2 I.U./ml adenosine deaminase containing 40 nM [^{3}H]DPCPX, diluted membranes (20 μg of protein per assay) and at least six to eight different concentrations of MRE 3008F20. Non specific binding was determined in the presence of 100 μM NECA and was always $\leq$30% of the total binding.

Binding of [^{125}I]AB-MECA to CHO cells transfected with the human recombinant A_3 adenosine receptors was performed according to Varani et al. (1998a).

[^{3}H]MRE 3008F20 Binding Assay. Kinetic studies of 2 nM [^{3}H]MRE 3008F20 were performed by incubating membranes obtained by CHO cells transfected with the human A_3 receptors in a thermostatic bath at 4°C. For the measurement of the association rate, the reaction was terminated at different times (from 5 to 200 min) by rapid filtration under vacuum, followed by washing with 5 ml of ice-cold buffer four times. For the measurement of the dissociation rate, the samples were incubated at 4°C for 120 min, and then 1 μM MRE 3008F20 was added to the mixture. The reaction was terminated at different times from 5 to 150 min.

Saturation binding experiments of [^{3}H]MRE 3008F20 (0.05 to 10 nM) to CHO cells transfected with the human recombinant adenosine A_3 receptors were performed by incubating membranes (50 μg of protein per assay) for 120 min at 4°C. Competition experiments of 1 nM [^{3}H]MRE 3008F20 were performed in duplicate in a final volume of 100 μl in test tubes containing 50 mM Tris-HCl buffer, 10 mM $MgCl_2$, 1 mM EDTA (pH 7.4), and 100 μl of membranes and at least 12 to 14 different concentrations of typical adenosine receptor agonists and antagonists. Analogous experiments were performed in the presence of 100 μM GTP. Nonspecific binding was defined as binding in the presence of 1 μM MRE 3008F20 and at the K_D value at which the radioligand was approximately 25% 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 (Packard). The filter-bound radioactivity was counted using a microplate scintillation counter (Top Count, Meriden, CT) at an efficiency of 57% with Micro-Scint 20. The protein concentration was determined according to a Bio-Rad method (Bradford, 1976) with bovine serum albumin as a standard reference.

Cyclic AMP Accumulation Assay. Membrane preparation obtained by CHO cells transfected with the human A_3 receptors was suspended in 0.5 ml of incubation mixture (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4) containing 5 μM GTP, 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro-20-1724) as phosphodiesterase inhibitor, and 2.0 I.U./ml adenosine deaminase and preincubated for 10 min in a shaking bath at 37°C. Then the respective agonist and ATP (1 mM) were added to the mixture, and the incubation was continued for 10 min. Adenylyl cyclase was stimulated with 10 μM forskolin, which typically produced a 6- to 8-fold increase of activity over basal levels. Maximal inhibition of adenylyl cyclase by agonists amounted to about 60% of total stimulation. The potencies of antagonists were determined by antagonism of the inhibition of cAMP production induced by 100 nM IB-MECA. The reaction was terminated by transferring the tubes to a boiling water bath for 2 min. Then the tubes were cooled to room temperature, centrifuged at 2,000*g* for 10 min at 4°C, and the supernatants were used for the determination of cAMP by a competition protein binding assay carried out essentially according to the method of Varani et al. (1998b).

Thermodynamic Analysis. For a generic binding equilibrium, $L + r = LR$ (where L = ligand and r = receptor), the affinity association constant $K_A = 1/K_D$ is directly related to the standard free energy $\Delta G°$ ($\Delta G° = -RT \ln K_A$), which can be separated in its enthalpic and entropic contributions according to the Gibbs equation: $\Delta G° = \Delta H° - T\Delta S°$. The standard free energy was calculated as $\Delta G° = -RT \ln K_A$ at 298.15 K, the standard enthalpy, $\Delta H°$, from the van't Hoff plot $\ln K_A$ versus $(1/T)$ (the slope of which is $-\Delta H°/R$) and the standard entropy as $\Delta S° = (\Delta H° - \Delta G°)/T$ with T = 298.15 K and r = 8.314 $JK^{-1}mol^{-1}$.

K_A values were obtained from saturation experiments of [^{3}H]MRE 3008F20 binding to the human A_3 adenosine receptors performed at 0, 10, 15, 20, 25, and 30°C.

Rat A_1, A_{2A}, and A_3 Adenosine Receptor Binding Assay. Binding assays to rat brain and striatum A_1 and A_{2A} receptors were performed according to Borea et al. (1994) and Borea et al. (1995), respectively.

Binding of the A_3 agonist, [^{125}I]AB-MECA, to CHO cells transfected with the rat recombinant A_3 adenosine receptor was performed according to the method described by Olah et al. (1994).

Data Analysis. All binding studies (kinetics, saturation, competition) were analyzed with the program LIGAND (Munson and Rodbard, 1980). EC_{50} and IC_{50} values in the cAMP assay were calculated with the nonlinear least-squares curve fitting program Prism (GraphPAD, San Diego, CA).

Results

The kinetic behavior of [^{3}H]MRE 3008F20 binding was studied at 4°C in CHO cells expressing the cloned human A_3 adenosine receptors. Figure 2A shows that [^{3}H]MRE 3008F20 bind-

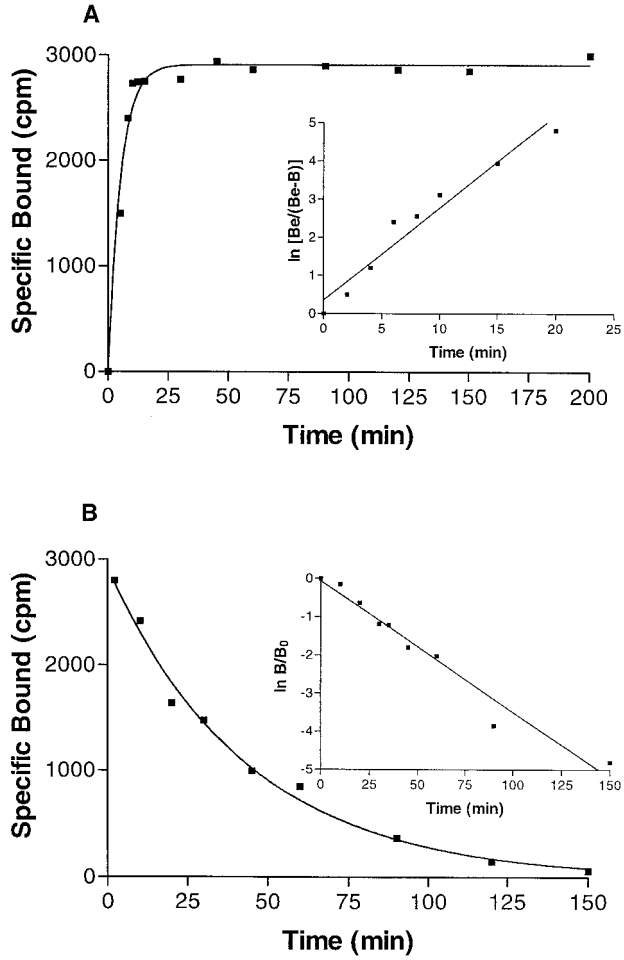

Fig. 2. A, kinetics of 2 nM [^{3}H]MRE 3008F20 binding to human A_3 adenosine receptors with association curves representative of a single experiment. Inset, first-order plots of [^{3}H]MRE 3008F20 binding. Be represents the amount of [^{3}H]MRE 3008F20 bound at equilibrium, and B represents the amount of [^{3}H]MRE 3008F20 bound at each time. Association rate constant was: K_{+1} = 0.076 ± 0.002 min^{-1} nM^{-1}. B, kinetics of [^{3}H]MRE 3008F20 binding to human A_3 adenosine receptors with dissociation curves representative of a single experiment. Inset, first-order plots of [^{3}H]MRE 3008F20 binding. Dissociation rate constant was: K_{-1} = 0.04 ± 0.002 min^{-1}.

ing reached equilibrium after approximately 40 min and was stable for at least 5 h. [^{3}H]MRE 3008F20 binding was rapidly reversed by the addition of 1 μM MRE 3008F20 as shown in Fig. 2B. Association and dissociation curves were fitted to a one-component model significantly better than to a two-component model ($P < .05$). The rate constants were: $k_{obs} = 0.195 \pm 0.020$ min^{-1} and $k_{-1} = 0.042 \pm 0.002$ min^{-1}. From the k_{+1} ($k_{+1} = 0.076 \pm 0.002$ min^{-1} nM^{-1}) and k_{-1} values, the apparent equilibrium dissociation constant (K_D) was estimated to be 0.55 nM. Figure 3A shows a saturation curve of [^{3}H]MRE 3008F20 to the adenosine A_3 receptor with a K_D value of 0.80 $\pm$ 0.06 nM and a B_{max} value of 300 $\pm$ 33 fmol/mg protein ($n = 3$). The Scatchard plot was essentially linear, and computer analysis of the data (Munson and Rodbard, 1980) failed to show a significantly better fit to a two-site as compared with a one-site binding model, indicating that only one class of high affinity binding sites is present under our experimental conditions. Figure 3B shows a saturation curve of [^{125}I]AB-MECA to hA_3CHO cells

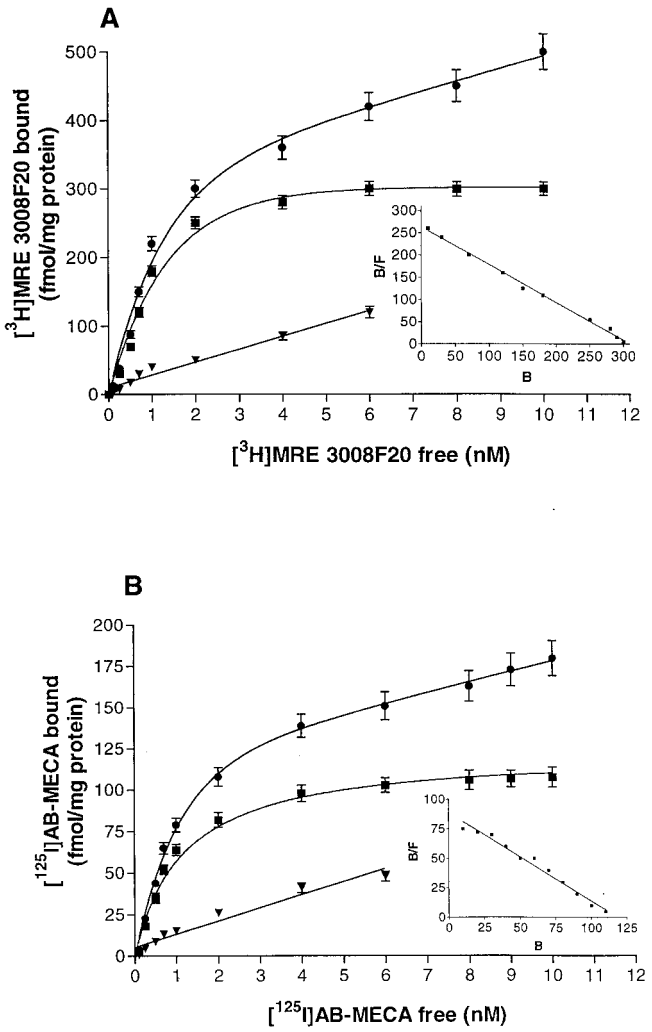

Fig. 3. A, saturation of [^{3}H]MRE 3008F20 binding to human A_3 adenosine receptors. The K_D value was 0.80 $\pm$ 0.06 nM and the B_{max} value was 300 $\pm$ 33 fmol/mg protein. B, saturation of [^{125}I]AB-MECA binding to human A_3 adenosine receptors. The K_D value was 0.85 $\pm$ 0.05 nM and the B_{max} value was 110 $\pm$ 12 fmol/mg protein. Experiments were performed as described in *Experimental Procedures*. Values are the means and vertical lines are the S.E. of the mean of four separate experiments performed in triplicate. In the inset the Scatchard plot of the same data is shown.

with a K_D value of 0.85 $\pm$ 0.05 nM and a B_{max} value of 110 $\pm$ 12 fmol/mg protein. Table 1 shows the affinity, expressed as K_i values, and selectivity of MRE 3008F20 to human and rat adenosine receptors. MRE 3008F20 bound with high affinity ($K_i = 0.85 \pm 0.02$ nM) to human A_3 adenosine receptors, with low affinity ($K_i = 140 \pm 15$ nM) to human A_{2A} adenosine receptors, and with affinity values in the micromolar range to human A_1 ($K_i = 1.1$ μM) and A_{2B} ($K_i = 2.1$ μM) receptors. Moreover, MRE 3008F20 displayed a weak affinity to rat A_{2A} receptors ($K_i = 2.0$ μM) and no affinity to A_1 and A_3 receptors. All these data clearly indicate that MRE 3008F20 is endowed with affinity and selectivity for the human A_3 adenosine receptors. Table 2 shows the comparison of affinities, expressed as K_i, K_H, and K_L values, of selected adenosine receptor agonists and antagonists to human A_3-cloned receptors expressed in CHO cells using [^{125}I]AB-MECA or [^{3}H]MRE 3008F20, and the percentage of receptors in the high affinity state (R_H) is also shown. The resultant order of potency in [^{125}I]AB-MECA displacement assays for adenosine receptor agonists was as follows: IB-MECA > MPC-NECA > AB-MECA > NECA > R-PIA > MPC-MECA > S-PIA > CGS 21680 (Fig. 4A). The order of potency of the receptor antagonists was as follows: MRE 3008F20 > MRE 3010F20 > XAC > CGS 15943 > DPCPX. SCH 58261 showed a K_i value > 10 μM (Fig. 4B). The order of potency in [^{3}H]MRE 3008F20 displacement assays for adenosine receptor agonists was as follows: IB-MECA > MPC-NECA > AB-MECA > NECA > R-PIA > MPC-MECA > S-PIA > CGS 21680 (Fig. 5A). Displacement of [^{3}H]MRE 3008F20 binding was stereoselective, with R-PIA ($K_H = 66$ nM; $K_L = 2800$ nM) being approximately 6- to 7-fold more active than its stereoisomer, S-PIA ($K_H = 482$ nM; $K_L = 16000$ nM). The order of potency of the receptor antagonists was: MRE 3008F20 > MRE 3010F20 > XAC > CGS 15943 > DPCPX. SCH 58261 showed a K_i value > 10 μM (Fig. 5B). To assess adenosine A_3 receptor-G protein interactions, inhibition experiments were performed also in the presence of 100 μM GTP. Its inclusion did not significantly affect antagonist binding. In contrast, agonists were 30- to 50-fold less active in the presence of 100 μM GTP, although the same order of potency was observed as that seen in the absence of GTP. The resultant Hill coefficients of agonists were significantly different from unity, whereas only one affinity state could be detected with a slope factor near unity in the presence of GTP. The Hill coefficients of antagonists were not significantly different from unity both in the absence or in the presence of GTP. Computer analysis of binding curves revealed that, in the presence of GTP, a one-component binding model adequately described the agonist binding curves (data not shown). The Spearman's rank correlation coefficient between affinity values of [^{125}I]AB-MECA and [^{3}H]MRE 3008F20 binding to human A_3 adenosine receptor by selected receptor agonists and antagonists was 0.97 ($P < .01$). Table 2 also shows the inhibition of cAMP accumulation by agonists (EC_{50}) and the capability of the antagonists (IC_{50}) to block the effect of 100 nM IB-MECA on adenylyl cyclase in CHO cells. Figure 6A shows the log-dose-response curve for typical adenosine receptor agonists. All adenosine analogs were able to inhibit cAMP accumulation displaying an order of potency identical with that observed in binding affinities to the adenosine A_3 receptor. IB-MECA appeared to be the most potent compound ($EC_{50} = 14$ nM) followed by MPC-NECA and AB-MECA (EC_{50} in the range of 120–160 nM); R-PIA was more potent than its stereoisomer S-PIA ($EC_{50} = 550$ nM and 2.1 μM, respectively). Figure 6B shows the capability of the

antagonists to block the effect of 100 nM IB-MECA cAMP production in CHO cells by adenosine receptor antagonists. The most potent adenosine receptor antagonists were MRE 3008F20 and MRE 3010F20 (IC_{50} = 4.5 and 5.3 nM, respectively). The linear correlation coefficient between affinity values of [^{125}I]AB-MECA and [^{3}H]MRE 3008F20 binding to the human A_3 adenosine receptor by selected receptor agonists and antagonists was 0.99 ($P < .01$) (Fig. 7A). The comparison of K_i and EC_{50}/IC_{50} values indicates that a high correlation exists between data obtained from binding and functional assays (r = 0.99; $P < .01$) (Fig. 7B). K_D and B_{max} derived from the saturation experiments of [^{3}H]MRE 3008F20 binding to A_3 adenosine receptors performed at the six temperatures selected were found within the following range: K_D = 0.8 to 3.3 nM and B_{max} = 285 to 313 fmol/mg protein. Although the dissociation constant (K_D) changed with temperature, B_{max} values obtained from [^{3}H]MRE 3008F20 saturation experiments appeared to be largely independent of it. Figure 8 shows the van't Hoff plot ln K_A versus $1/T$ of the [^{3}H]MRE 3008F20 binding to the A_3 adenosine receptor and the final equilibrium thermodynamic parameters (expressed as mean values ± S.E. of three independent determinations) were: $\Delta G° = -48.77 \pm 0.12$ kJ mol^{-1}; $\Delta H° = -33.11 \pm 3.13$ kJ mol^{-1}; $\Delta S° = 52.25 \pm 5.53$ J mol^{-1} K^{-1}. The linearity of the plot was statistically significant ($\Delta Cp°$, equilibrium heat capacity change, approximately 0) and its slope ($-\Delta H°/R$) was positive, a property that has been found to be typical for antagonist binding to adenosine receptors (Lohse et al., 1984; Borea et al., 1996).

Discussion

In this study we characterized the human A_3 adenosine receptor on CHO cells utilizing [^{3}H]MRE 3008F20. The results of the paper show that this highly potent and selective antagonist represents a key advance toward the characterization of A_3 adenosine receptors. In saturation experiments [^{3}H]MRE 3008F20 labeled a single class of recognition sites with affinity (K_D) of 0.80 ± 0.06 nM and receptor density (B_{max}) of 300 ± 33 fmol/mg protein (Fig. 3A). Moreover, saturation experiments in hA_3CHO indicate that [^{125}I]AB-MECA interacts with only one recognition site with affinity in the nanomolar range, K_D = 0.85 ± 0.05 nM, and with a

TABLE 1

Affinity and selectivity of MRE 3008F20 to adenosine receptor subtypes

Human A_{2B} receptors were expressed in HEK-293 cells; all other human receptors and rat A_3 receptors were expressed in CHO cells. Rat A_1 and A_{2A} receptors were characterized in cortical and striatal membranes, respectively. K_i values (nM) are shown and represent the mean ± S.E. of three independent determinations performed in duplicate. The radioligand used is stated along with the receptor subtype. The selectivity values versus A_3 receptors were calculated with respect to the K_i value obtained using [^{3}H]MRE 3008F20 as radioligand.

Species	[^{3}H]DPCPX A_1	[^{3}H]SCH 58261 A_{2A}	[^{3}H]DPCPX A_{2B}	[^{125}I]AB-MECA A_3	[^{3}H]MRE 3008F20 A_3	A_1/A_3	A_{2A}/A_3	A_{2B}/A_3
Human	1100 ± 100	140 ± 15	2100 ± 300	0.29 ± 0.03	0.85 ± 0.02	1294	165	2471
Rat	>10000	1993 ± 224	NT[a]	>10000	ND			

[a] NT, not tested; ND, not determinable.

TABLE 2

Affinities, expressed as K_H, K_L, and K_i values, of selected adenosine receptor agonists and antagonists to human A_3-cloned receptors expressed in CHO cells

Displacement of [^{3}H]MRE 3008F20 was determined in the absence and presence of 100 μM GTP. K_H and K_L are the K_i values of the high and low affinity states for agonists, respectively. $\%R_H$ indicates the percentage of A_3 receptors in the high affinity state ± S.E. A comparison is made with inhibition of cAMP assay by agonists (EC_{50}) and the capability of the antagonists (IC_{50}) to blockade the effect of the IB-MECA (100 nM)-induced inhibition of cAMP production.

	[^{125}I]AB-MECA binding (K_i)	[^{3}H]MRE 3008F20 binding (K_H, K_L)	R_H	[^{3}H]MRE 3008F20 binding (K_i + GTP)	cAMP accumulation assay, EC_{50}
	nM	*nM*	%	*nM*	*nM*
Agonists					
IB-MECA	1.2 ± 0.1	1.5 ± 0.2 74 ± 8	27 ± 3	80 ± 6	14 ± 2
MPC-NECA	10 ± 2	8.6 ± 0.8 350 ± 30	29 ± 4	380 ± 40	120 ± 10
AB-MECA	18 ± 2	12 ± 1 460 ± 36	23 ± 3	435 ± 50	160 ± 20
NECA	35 ± 3	30 ± 3 1500 ± 140	28 ± 4	1620 ± 155	200 ± 25
R-PIA	75 ± 5	66 ± 6 2800 ± 240	28 ± 4	2500 ± 350	550 ± 50
MPC-MECA	140 ± 8	128 ± 12 4920 ± 650	34 ± 5	4300 ± 488	1100 ± 100
S-PIA	520 ± 30	482 ± 28 16000 ± 1500	25 ± 3	15000 ± 1350	2100 ± 200
CGS 21680	980 ± 70	920 ± 85 35000 ± 3000	23 ± 4	32500 ± 3140	3600 ± 400
Antagonists					
MRE 3008F20	0.29 ± 0.03	0.85 ± 0.02		0.86 ± 0.03	4.5 ± 0.4
MRE 3010F20	0.48 ± 0.02	0.95 ± 0.05		0.98 ± 0.04	5.3 ± 0.6
XAC	38 ± 6	25 ± 2		28 ± 3	75 ± 7
CGS 15943	90 ± 9	70 ± 4		65 ± 6	400 ± 30
DPCPX	1200 ± 100	1100 ± 120		1210 ± 118	4000 ± 500
SCH 58261	> 10000	> 10000		>10000	> 10000

binding capacity of 110 ± 12 fmol/mg protein (Fig. 3B). One possible explanation for this difference is that the agonist [^{125}I]AB-MECA recognizes only receptors in the high affinity state for agonists, whereas [^{3}H]MRE 3008F20 as an antagonist can bind to both agonist low and high affinity states of the receptor with equal affinity. In inhibition experiments MRE 3008F20 displayed a subnanomolar affinity for A_3 adenosine receptors (K_i = 0.85 ± 0.02 nM) and very high selectivity versus A_1 (K_i = 1100 ± 100 nM), A_{2A} (K_i = 140 ± 15 nM), and A_{2B} (K_i = 2100 ± 300 nM) adenosine receptors, proving to be the most potent and selective antagonist for the characterization of the human A_3 receptor subtype (Table 1). Moreover, competition experiments of MRE 3008F20 indicated that the behavior of this antagonist examined in humans is completely different from that observed in rat A_3-cloned receptors (Table 1). The high interspecies rat-human differences in affinity constants is common to other xanthinic and nonxanthinic antagonists (Salvatore et al., 1993; Ji et al., 1994). In competition binding studies, various adenosine receptor agonists and antagonists bound human A_3 adenosine receptors in CHO cells with a rank order of potency and affinity range similar to that observed for [^{125}I]AB-MECA. Computer analysis of competition curves with [^{3}H]MRE 3008F20 as a radioligand show that agonists interact with two recognition binding sites, whereas adenosine antagonists interact with only one (Fig. 5; Table 2). At human A_3 adenosine receptors, agonist competition isotherms were biphasic and fitted better to a two-site model (Fig. 5A), allowing for the characterization of the high and low affinity components, presumably reflecting binding to G protein-coupled and uncoupled states of the receptor, respectively. The addition of GTP made the curve become steeper and the slope factor approach unity. The K_i of agonists in the presence of GTP was in general reasonable close to the affinity of the low affinity state in the absence of GTP. On the contrary, competition binding curves with antagonists ligands were monophasic for human A_3 adenosine receptors (Fig. 5B). Another aim of this study was to investigate the thermodynamic behavior of [^{3}H]MRE 3008F20 binding and determine the enthalpic ($\Delta H°$) and entropic ($\Delta S°$) contributions to the standard free energy ($\Delta G°$) of the binding equilibrium. The linearity of the van't Hoff plot for [^{3}H]MRE 3008F20 binding in the CHO cells indicates that $\Delta Cp°$ values for the drug interaction are nearly zero, which means that $\Delta H°$ and $\Delta S°$ values are not significantly affected by temperature varia-

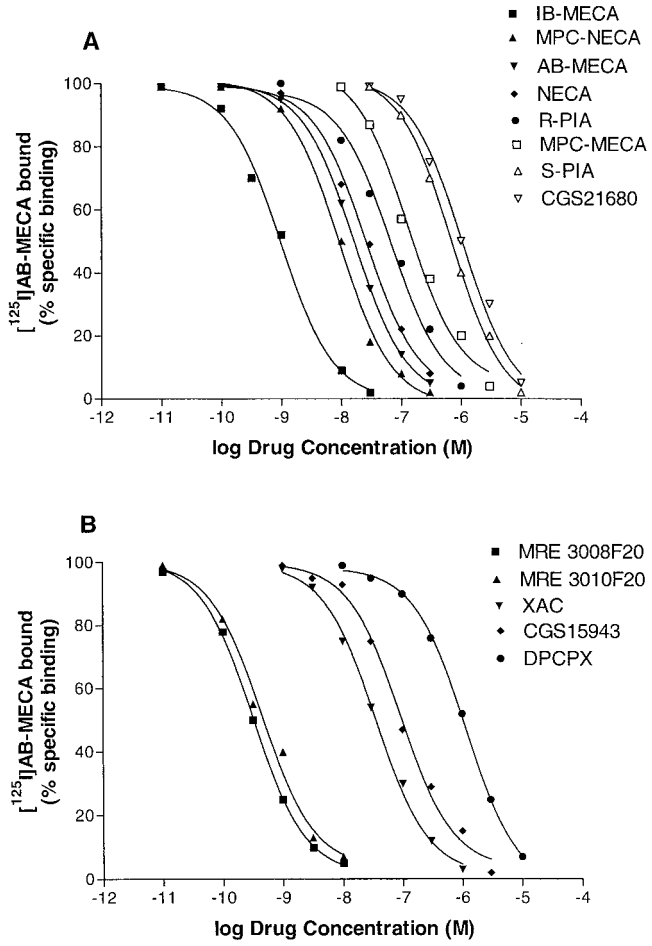

Fig. 4. A, competition curves of specific [^{125}I]AB-MECA binding to human A_3 adenosine receptors by adenosine agonists. B, competition curves of specific [^{125}I]AB-MECA binding to human A_3 adenosine receptors by adenosine antagonists. Curves are representative of a single experiment from a series of four independent experiments. Competition experiments were performed as described in *Experimental Procedures*.

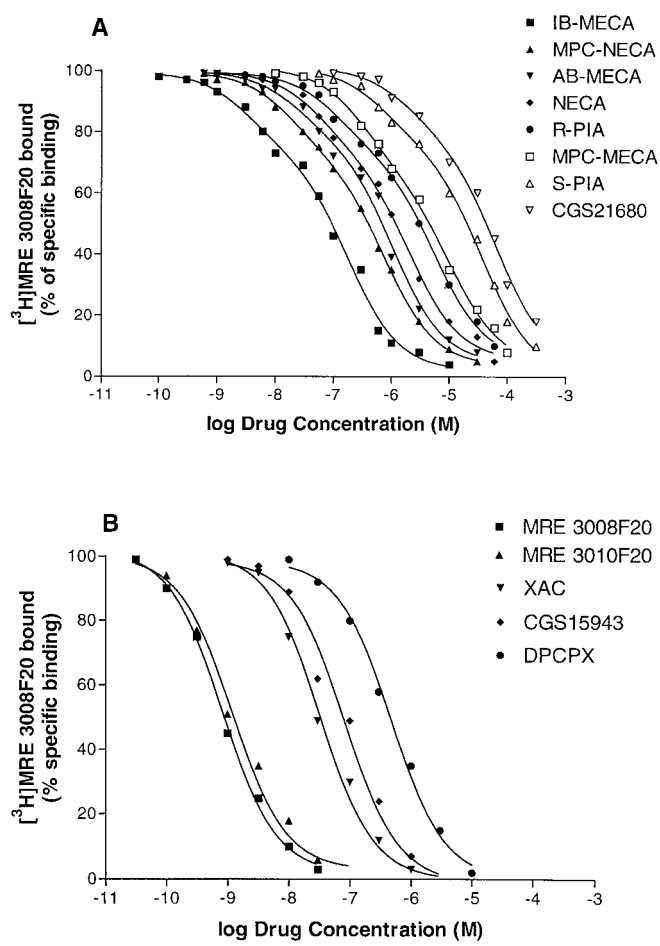

Fig. 5. A, competition curves of specific [^{3}H]MRE 3008F20 binding to human A_3 adenosine receptors by adenosine agonists. B, competition curves of specific [^{3}H]MRE 3008F20 binding to human A_3 adenosine receptors by adenosine antagonists. Curves are representative of a single experiment from a series of four independent experiments. Competition experiments were performed as described in *Experimental Procedures*.

tions at least over the temperature range investigated (Borea et al., 1995).

The linearity of van't Hoff plots in a limited range of temperatures (4–30°) appears to be a common feature of practically all membrane receptor ligands so far studied from a thermodynamic point of view (Gilli et al., 1994). Thermodynamic data obtained from the van't Hoff plot indicate that [^{3}H]MRE 3008F20 binding to human A_3 adenosine receptors is enthalpy- and entropy-driven ($\Delta H° = -33.11 \pm 3.13$ kJ mol^{-1}, $\Delta S° = 52.25 \pm 5.53$ J mol^{-1} K^{-1}). This binding behavior has previously been found to be typical of adenosine A_1 and A_{2A} receptor antagonists (Borea et al., 1996). To evaluate the regulation of adenylyl cyclase activity and to test whether the binding parameters correlated with the functional response, we determined the IC_{50} values obtained for inhibition of cAMP production by receptor agonists and antagonists, respectively. In the cAMP assay, the compounds studied exhibited a rank order of potency similar to that observed in binding experiments. Agonists were generally less potent as inhibitors of cAMP in CHO cells expressing human A_3 receptors than predicted from binding data. This could be related to the fact that, apart from the fact that the conditions of these assays differ, recombinant A_3 receptors could not be well coupled to inhibition of cAMP accumulation in CHO cells. In addition, it has been observed for A_1 adenosine receptors that function correlates with the low affinity rather than the high affinity state (Lohse et al., 1986; Wilken

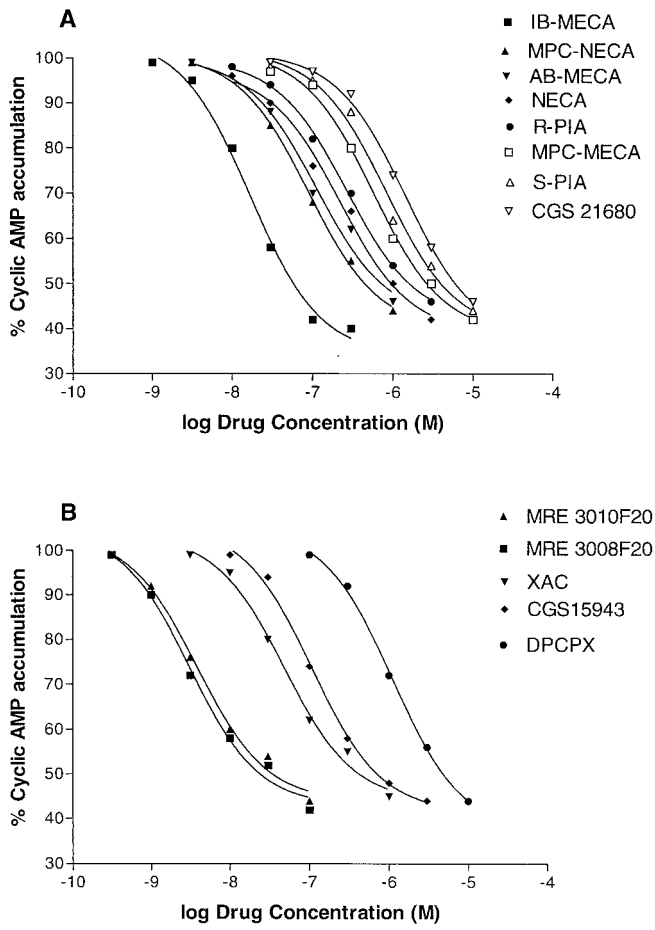

Fig. 6. A, inhibition curves of cAMP accumulation to human A_3 adenosine receptors by adenosine agonists. B, inhibition curves representing the capability of the antagonists to block the effect of 100 nM IB-MECA on adenylyl cyclase to human A_3 adenosine. Curves are representative of a single experiment from a series of four independent experiments.

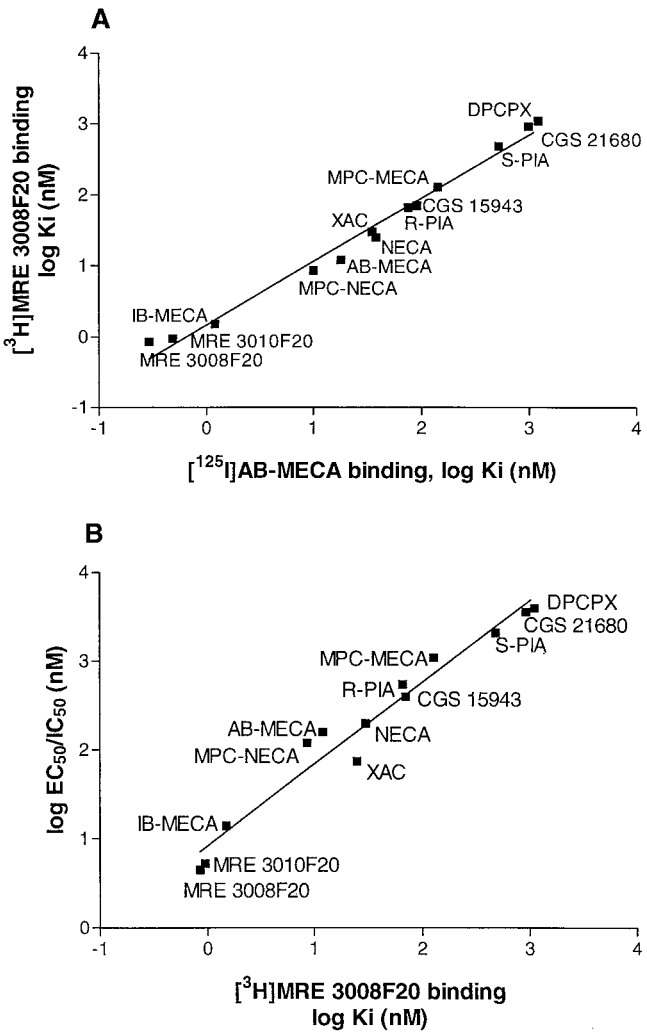

Fig. 7. Comparison between affinity values of [^{3}H]MRE 3008F20 and [^{125}I]AB-MECA binding to human A_3 adenosine receptors ($r = 0.99$; $P < .01$). B, comparison between affinity values of [^{3}H]MRE 3008F20 binding and EC_{50} or IC_{50} obtained in cAMP assays to human A_3 adenosine receptors ($r = 0.99$; $P < .01$).

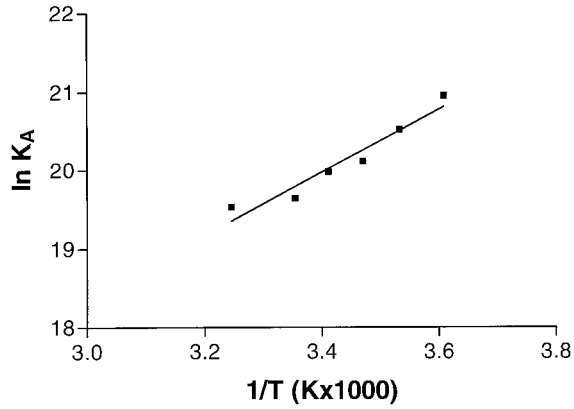

Fig. 8. Van't Hoff plot, ln K_A versus $1/T$, showing the effect of temperature on the equilibrium binding association constant, $K_A = 1/K_D$ of [^{3}H]MRE 3008F20.

et al., 1990). However, the new adenosine receptor antagonists MRE 3008F20 and MRE 3010F20 are potent in binding (K_i = 0.85 and 0.95 nM, respectively) and in functional assays (IC_{50} = 4.5 and 5.3 nM, respectively). The high statistically significant Spearman's rank correlation coefficient between receptor affinity values of [^{3}H]MRE 3008F20 binding and receptor affinity of [^{125}I]AB-MECA to human A_3 adenosine receptors expressed in CHO cells (Table 2) confirmed that both ligands can label the human A_3 adenosine receptor subtype. Likewise the Spearman's rank correlation coefficient between IC_{50} values in the cAMP assay and receptor affinity values showed a highly significant positive correlation.

In conclusion, all these data indicate, for the first time, that MRE 3008F20 is one of the most selective high affinity human A_3 adenosine receptor antagonists ever reported. Furthermore, we demonstrated that its tritiated radiolabeled form, [^{3}H]MRE 3008F20, is a ligand with subnanomolar affinity for the human A_3 adenosine receptor, exhibiting an appropriate A_3 pharmacological profile in human A_3 CHO cells. Due to its high selectivity for A_3 receptors, this new radioligand could be used to detect A_3 receptors in a variety of mammalian tissues and can be considered a new pharmacological tool to better elucidate the physiopathological role of A_3 adenosine receptors.

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