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[^{3}H]-MRE 2029-F20, a selective antagonist radioligand for the human A_{2B} adenosine receptors

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Abstract—MRE 2029-F20 [N-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide] is a selective antagonist ligand of A_{2B} adenosine receptors. For use as a radioligand, 1,3-diallyl-xanthine, the precursor of [^{3}H]-MRE 2029-F20, was synthesized, and tritiated on the allyl groups. [^{3}H]-MRE 2029-F20 bound to human A_{2B} receptors expressed in CHO cells showed a K_D value of 1.65 ± 0.10 nM and B_{max} value of 36 ± 4 fmol/mg protein. [^{3}H]-MRE2029-F20 represents a useful tool for the pharmacological characterization of human A_{2B} adenosine receptor subtype.

The biological activity of adenosine occurs through the activation of specific receptors located on cell membranes and belonging to the extensive family of G-protein coupled receptors.[1,2] Adenosine interacts with four pharmacologically distinct adenosine receptor subtypes designated A_1, A_{2A}, A_{2B}, and A_3.[1,3] The adenosine receptors are associated with different messenger systems: A_1 and A_3 mediate adenylate cyclase inhibition and the decrease of intracellular cyclic AMP levels.[4] A_{2A} and A_{2B} are positively coupled to adenylate cyclase via G_s and mediate the stimulation of the adenylate cyclase activity and the increase of *c*AMP levels. However, coupling to phospholipase C via G_q, resulting in mobilization of intracellular calcium, and direct coupling to calcium channels has also been described.[1,2] A_{2B} adenosine receptors have been implicated in the regulation of mast cell secretion,[5] gene expression,[6] cell growth,[7] intestinal function,[8] neurosecretion,[9] vascular tone,[10] and asthma.[2,11,12] While the A_1, A_{2A}, and A_3 adenosine receptors have been pharmacologically characterized through the use of highly potent and selective agonists and/or antagonists, the study of the A_{2B} receptor has been precluded due to the lack of selective ligands,[13–15] and the absence of an appropriate radioligand binding assay.

Recently, [^{3}H]-ZM241385 has been proposed as a useful radioligand for studying the A_{2B} adenosine receptor subtype.[16]

Building on this research, Jacobson and co-workers have reported some xanthine derivatives endowed with good affinity but limited significant selectivity for the human A_{2B} adenosine receptor subtype.[17,18] An evolution of this study led to the synthesis of 8-phenyl xanthine carboxylic acid congeners. In particular, the derivative named MRS-1754,[19] ([N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purine-8-yl)phenoxy]-acetamide] proved to be the most potent and selective A_{2B} adenosine receptor antagonist yet reported. This result, led to the synthesis and characterization of the tritium labeled form of MRS-1754 as the first radioligand for the A_{2B} receptor.[20]

Very recently our group has reported the synthesis and evaluation of a series of 8-heterocyclic substituted

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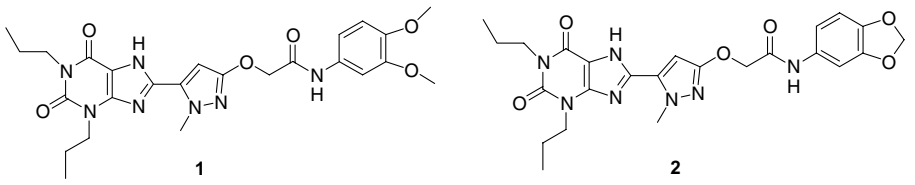


Figure 1. Chemical structures of human A_{2B} adenosine receptor antagonists.

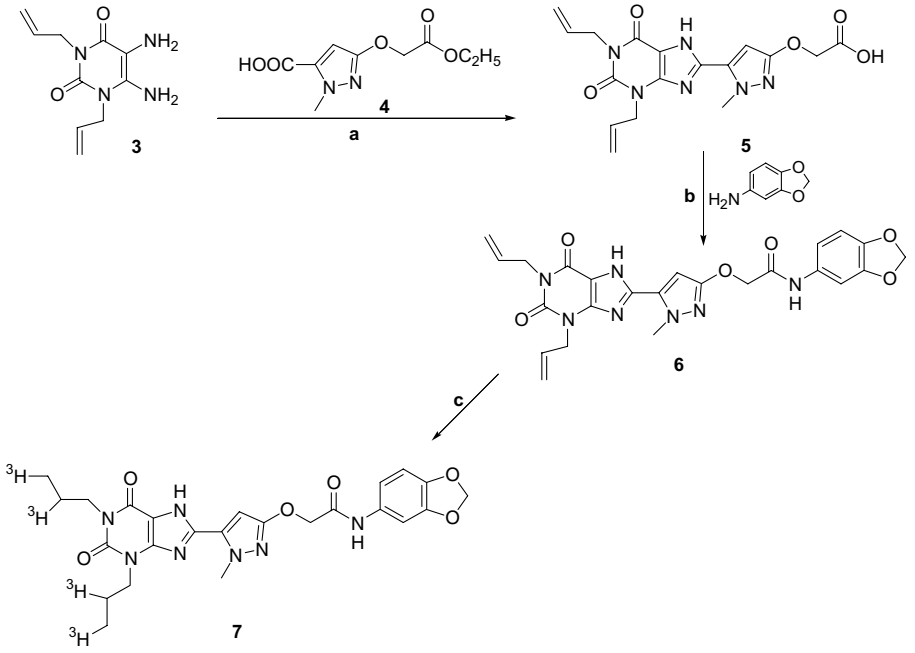
xanthines as potent and selective human A_{2B} adenosine receptor antagonists.[21] This study allowed us to identify the derivatives N-(3,4-dimethoxy-phenyl)-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide (**1**), and N-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yl-oxy]-acetamide (**2**) that showed high affinity at A_{2B} receptor subtype and very good selectivity versus the other adenosine receptors (Fig. 1).

Starting from these experimental observations, we focused our attention on the synthesis of an antagonist for A_{2B} adenosine receptors in labeled form. Our hypothesis was based on the introduction of an unsaturated chain on the N^1 and N^3 position of the xanthine nucleus that, after reduction with tritium gas, could afford the desired radioligand. In this case, we have synthesized the N-benzo[1,3]dioxol-5-yl-2-[5-(1,3-diallyl-2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yl-oxy]-acetamide **6** that, after reduction, afforded the labeled form (**7**) of compound **2**. This compound was found to be a selective, high-affinity radioligand useful for characterizing recombinant human A_{2B} receptors.

The desired compound **6** was prepared according to the general procedure for synthesis of the 8-pyrazole xanthine derivatives, as described recently.[21] 1,3-Diallyl-5,6-diaminouracil **3** was condensed with 5-ethoxycarbonyl-methoxy-2-methyl-2*H*-pyrazole-3-carboxylic acid **4** to give the 8-pyrazole derivative **5** that was transformed to amide **6**[22] in the presence of 3,4-(methylenedioxy)aniline (Scheme 1). [^{3}H]-MRE 2029-F20 was prepared by reduction of **6** with tritium gas in the presence of 10% Pd on charcoal in DMF. The labeled compound **7** was purified by high performance liquid chromatography (column: Ultrasphere ODS 250 × 4.6 mm; solvent A: water, solvent B: acetonitrile; gradient: 0–100% B over 20 min; flow rate: 1 mL/min; UV detection: 250 nm). The purified product was dissolved in ethanol and submitted for mass spectral analysis. The mass spectrum was consistent with the proposed structure **7** and a nonlabeled reference **2**. Specific activity determination (123 mCi) indicated a radiochemical purity of 97%.

In summary, the A_{2B} receptor-selective xanthine antagonist MRE2029-F20 was synthesized in tritiated form for use as a radioligand.

The expression of the human A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors in CHO cells has been previously described.[23] Human cloned adenosine receptor binding assays were performed according to the method as previously described.[24] Kinetic studies of [^{3}H]-MRE



Scheme 1. Reagents and conditions: (a) (i) 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDAC), CH_3OH, rt, (ii) NaOH (2.5 N), 70 °C, 2 h; (b) DMF, 1-hydroxybenzotrizole (HOBt), EDAC; (c) DMF, tritium gas, 10% Pd–C, 4 h.

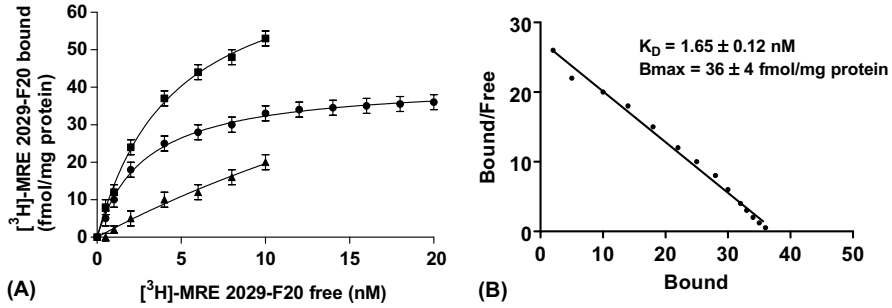


Figure 2. Saturation of [^{3}H]-MRE 2029-F20 binding to human A_{2B} adenosine receptors in CHO cells. The K_D value was 1.65 ± 0.10 nM and the B_{max} value of 36 ± 4 fmol/mg protein (A). The Scatchard plot of the same data is shown (B). Values are the means and vertical lines are the SE of the mean of four separate experiments performed in triplicate.

2029F20 were performed incubating membranes obtained by hA_{2B}CHO cells 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 ice-cold buffer four times. For the measurement of dissociation rate, the samples were incubated at 4 °C for 60 min, then 1 μM of the MRE 2029F20 was added to the mixture and the reaction was terminated at different times (from 5 to 150 min). Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass-fiber filters using a Micro-Mate 196 harvester (Packard Instrument Company). The filter bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20. Association and dissociation curves were fitted to a one-component model significantly better than to a two-component model ($P < 0.05$). The rate constants were: $K_{obs} = 0.064 \pm 0.005$/min and $K_{-1} = 0.031 \pm 0.003$/min. From the $K_{+1} = 0.0165 \pm 0.0023$/min/nM and the K_{-1} values, the apparent equilibrium dissociation constant (K_D) was estimated to be 1.88 nM. Saturation binding experiments of [^{3}H]-MRE 2029F20 (0.02–20 nM) to hA_{2B}CHO cell membranes were performed by incubating membranes (100 μg of protein/assay) for 60 min at 4 °C (Fig. 2A). The linearity of the Scatchard plot is indicative, in our experimental conditions, of the presence of a single class of binding sites with K_D value of 1.65 ± 0.10 nM and B_{max} value of 36 ± 4 fmol/mg protein (Fig. 2B). To verify the selectivity of this new ligand versus the A_1, A_{2A}, and A_3 adenosine receptor subtypes, competition experiments were performed using different concentrations of the unlabeled form of MRE 2029-F20. Briefly, in the inhibiton binding experiments to hA_1 CHO membranes [^{3}H]-1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]-DPCPX) were suspended in 50 mM Tris HCl buffer pH 7.4 with at least six to eight different concentrations of MRE 2029F20 and incubated for 120 min at 25 °C. Binding of [^{3}H]-4-(2-[7-amino-2-[furyl][1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylaminoethyl]phenol ([^{3}H]-ZM 241385) to hA_{2A}CHO membranes was performed using 50 mM Tris HCl buffer, 10 mM $MgCl_2$, pH 7.4, six to eight different concentrations of MRE 2029F20 for an incubation time of 60 min at 4 °C. Binding of [^{3}H]-5N-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl) pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*] pyrimidine ([^{3}H]-MRE 3008F20) to hA_3CHO membranes was performed using 50 mM Tris HCl buffer, 10 mM $MgCl_2$, pH 7.4, six to eight different concentrations of MRE 2029F20 for an incubation time of 120 min at 4 °C. MRE 2029-F20 shows low affinity for the human A_1 receptor ($K_i = 248 \pm 30$ nM) with no significant affinity for the human A_{2A} and A_3 adenosine receptors, revealing to be selective for the human A_{2B} adenosine receptors ($A_1/A_{2B} = 150$, $A_{2A}/A_{2B} > 606$, $A_3/A_{2B} > 606$). These results show that [^{3}H]-MRE 2029-F20 should be considered a new pharmacological tool to better detect human A_{2B} adenosine receptors.

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22. Characterization of compound **6**. Pale white solid, mp: 288–289 °C (dimethylformamide–H_2O); ^1H NMR (DMSO-d_6): δ 4.06 (s, 3H); 4.49 (m, 2H); 4.63 (m, 2H); 4.72 (s, 2H); 5.15 (m, 4H); 5.68 (m, 2H); 5.98 (s, 2H); 6.49 (s, 1H); 6.85 (d, 1H, $J = 8$ Hz); 6.99 (d, 1H, $J = 8$ Hz); 7.31 (s, 1H); 10.01 (s, 1H); 13.98 (br s, 1H).
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