Selective A_3 Adenosine Receptor Antagonists: Water-Soluble 3,5-Diacyl-1,2,4-trialkylpyridinium Salts and Their Oxidative Generation from Dihydropyridine Precursors

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A_3 adenosine receptor antagonists are sought for their potential antiinflammatory, antiasthmatic, and antiischemic properties. We have found that 3,5-diacyl-1,2,4-trialkyl-6-phenylpyridinium derivatives constitute a novel class of selective A_3 adenosine receptor antagonists. The structure−activity relationships of this class of antagonists, incorporating the 3-thioester, have been explored. The most potent analogue in this group was 2,4-diethyl-1-methyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenylpyridinium iodide (**11**), which had an equilibrium inhibition constant (K_i) value of 219 nM at human A_3 receptors (binding of [^{125}I]-AB-MECA (N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine)) expressed in Chinese hamster ovary (CHO) cells and >10 μM at rat brain A_1 and A_{2A} receptors and at recombinant human A_{2B} receptors. Compound **11** could be generated through oxidation of the corresponding 3,5-diacyl-1,2,4-trialkyl-6-phenyl-1,4-dihydropyridine, **24**, with iodine or in the presence of rat brain homogenates. A 6-cyclopentyl analogue was shown to increase affinity at human A_3 receptors upon oxidation from the 1-methyl-1,4-dihydropyridine analogue, **25**, to the corresponding pyridinium derivative, **23** (K_i 695 nM), suggesting a prodrug scheme. Homologation of the *N*-methylpyridinium derivatives to *N*-ethyl and *N*-propyl at the 1-position caused a progressive reduction in the affinity at A_3 receptors. Modifications of the alkyl groups at the 2-, 3-, 4-, and 5-positions failed to improve potency in binding at A_3 receptors. The pyridinium antagonists are not as potent as other recently reported, selective A_3 receptor antagonists; however, they display uniquely high water solubility (43 mM for **11**). Compound **11** antagonized the inhibition of adenylate cyclase elicited by IB-MECA in CHO cells expressing the human A_3 adenosine receptor, with a K_B value of 399 nM, and did not act as an agonist, demonstrating that the pyridinium salts are pure antagonists.

Introduction

The A_3 adenosine receptor occurs in the inflammatory/immune system,[1,2] the cardiovascular system,[3,4] the renal system,[5] and the central nervous system.[6,7] Activation of the receptor is coupled to the inhibition of adenylyl cyclase and the stimulation of phospholipase C. A_3 adenosine receptor antagonists are sought as potential antiinflammatory, antiasthmatic, or antiischemic agents.[1,2,10]

The classical adenosine antagonists, xanthines, while potent and/or selective for $A_1/A_{2A}/A_{2B}$ receptors, have not provided fruitful leads for A_3 receptor-selective antagonists.[11] Among the first selective antagonists reported for adenosine A_3 receptors were 1,4-dihydropyridine derivatives.[12] The substituents on the structures of common calcium channel blockers of this chemical class were modified to achieve A_3 receptor selectivity and eliminate binding to L-type calcium channels, thus the 1,4-dihydropyridine structure was used as a template for novel receptor ligands (Figure 1). The 4-phenylethynyl-6-phenyl-1,4-dihydropyridine (**1**)[13,14] was >1300-fold selective for human A_3 receptors versus other subtypes of adenosine receptors, and a dihydropyridine of this class was shown to be specific for adenosine receptors versus other receptors and ion channels.

The template approach was further extended with the report that not only could the substituent groups of the dihydropyridine be substituted with resulting increases in affinity but the dihydropyridine ring could be oxidized, leading to pyridine derivatives as novel A_3 receptor selective antagonists.[15] A 3-thioester group proved beneficial for affinity of pyridines at the A_3 receptor. There are also pronounced species differences in A_3 antagonist affinity,[16,17] and the pyridine antagonists tended to have higher affinity and selectivity at rat A_3 receptors than did other classes of antagonists.[15] For example, 2,4-diethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxylcarbonyl-6-phenylpyridine (**2**) was >100-fold selective for rat A_3 versus A_1 receptors. A three-dimensional quantitative structure−activity relationship (3D-QSAR) for 1,3-diacylpyridines as human A_3 receptor antagonists was obtained by applying comparative molecular field analysis (CoMFA)[18] and a previously reported

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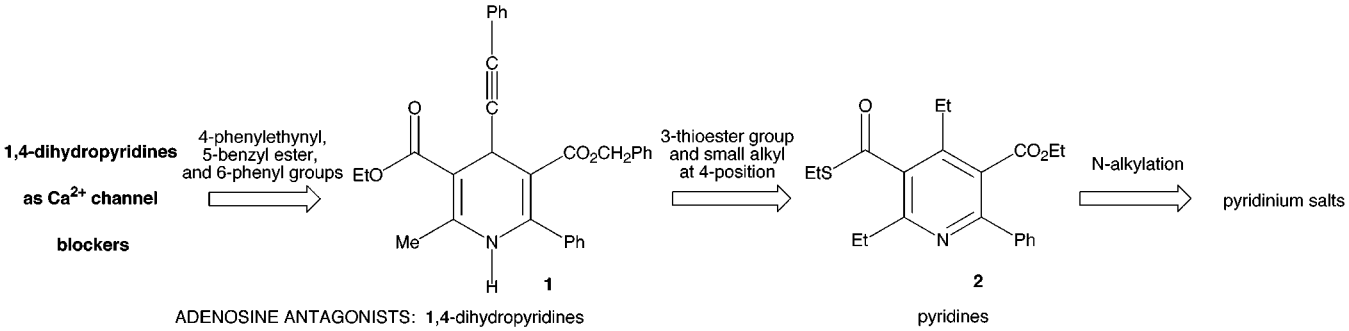


Figure 1. Sequential steps in the design of key A_3 adenosine receptor selective antagonists. In screening of chemical libraries for adenosine receptor affinity, 1,4-dihydropyridines used as L-type Ca^{2+} channel blockers were found to be weak, nonselective adenosine antagonists. Subsequently, derived 1,4-dihydropyridines (such as MRS 1191, **1**) and 3,5-diacylpyridines (such as **2**) were optimized for A_3 adenosine receptor affinity using a chemical template approach. The present study introduces pyridinium salts that are selective A_3 adenosine receptor antagonists of moderate potency and selectivity.

general A_3 receptor pharmacophore model.[19] A model of the entire transmembrane region of the human A_3 receptor, containing a docked pyridine reference ligand **2**, was reported.[18] Other antagonists for the human A_3 receptor have been described.[11,20,21]

In the present study, we report a conceptual extension of the template approach (Figure 1), which allows charge properties and lipophilicity to be altered. The pyridine ring may be quaternized as the 1-alkyl-pyridinium salts, resulting in decreased affinity at adenosine receptors compared to the pyridine derivatives such as **2**, but with retention of A_3 receptor selectivity. The pyridine antagonists of A_3 receptors have the deficiency of low water solubility, which is overcome in the present study involving permanently charged pyridinium ligands. Moreover, the corresponding 1-methyl dihydropyridine derivatives, which may be oxidized biochemically to the pyridinium antagonists, may be considered prodrugs. The oxidation occurs relatively rapidly in the presence of rat brain homogenates.

Results

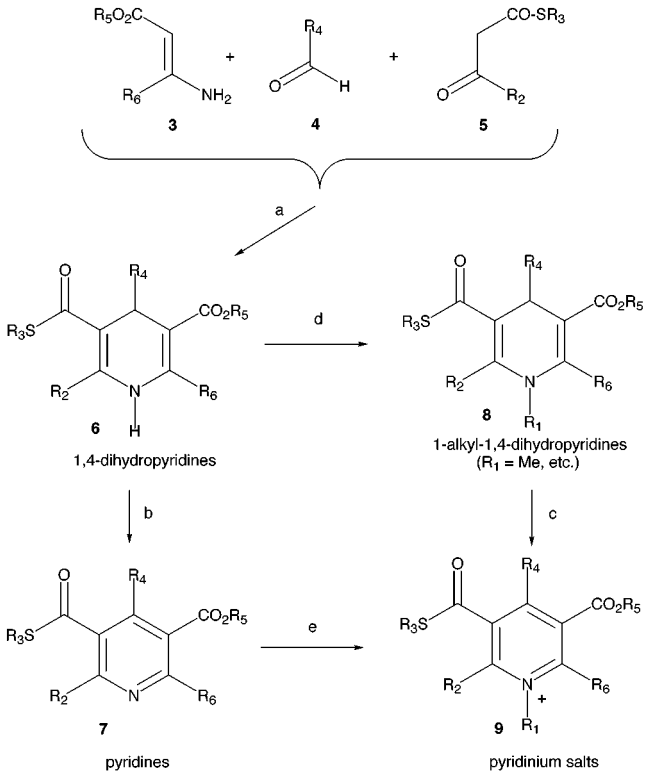
Synthesis. As in the previous studies,[15,18] pyridine analogues, **7**, were prepared from the corresponding dihydropyridines, **6** (Scheme 1), which were prepared through condensation of three components (**3**−**5**). The 3-thioester group, shown previously to enhance A_3 receptor affinity in the pyridine series,[15] was incorporated in the present set of pyridinium analogues. Two routes may be used to prepare the pyridinium salts, either the quaternization of **7** or the oxidation of the 1-alkyl-dihydropyridine derivative, **8**. Yields and characterization of the pyridinium salts synthesized and tested as A_3 receptor antagonists are described in Table 1. The affinities in radioligand binding assays[22−24] at adenosine receptors of the 3,5-diacylpyridinium derivatives prepared (**10**−**23**) are shown in Table 2.

The reduced precursors corresponding to **11** and **23**, the 1-methyl-1,4-dihydropyridine derivatives, **24** and **25**, respectively, were synthesized. Compounds **24** and **25** were prepared by the condensation of three components, **3**−**5** (Scheme 1), followed by alkylation of the dihydropyridine, **6**, using methyl iodide in THF.

Pharmacology. K_i values were determined in the binding of [^{125}I]AB-MECA (N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine)[24] to recombinant human A_3 adenosine receptors expressed in Chinese hamster ovary (CHO) cells. The pyridinium salts generally only weakly displaced binding at A_1 and A_{2A} receptors in rat brain membranes, using the radioligands [^{3}H]*R*-PIA[22] and [^{3}H]CGS 21680,[23] respectively.

Compound **11**, a 1-methyl-2,3,4,5-tetraethyl pyridinium analogue, was the most potent in the present set of compounds at displacing radioligand binding at human A_3 receptors (Table 2). A K_i value of 219 nM was determined; thus, the selectivity for human A_3 receptors versus rat A_1 and A_{2A} receptors was ~50-fold. In binding to rat A_3 receptors, a K_i value of 7.54 ± 1.07 μM was

Scheme 1. Synthesis of Substituted 1,4-Dihydropyridines Using the Hantzsch Reaction and Oxidation to Pyridine Derivatives[a,b]



[a] Structures **6**, **7**, and **9** are chemical families that include selective A_3 receptor antagonists, while structure **8** may be considered a precursor "prodrug" for in vivo conversion. This prodrug generates **9** under oxidative chemical or biochemical conditions. Compounds **24** and **25** are examples of general structure **8**, and compounds **10**−**23** are examples of general structure **9**. [b] Reagents: (a) EtOH, 90 °C, overnight; (b) tetrachlorobenzoquinone, THF; (c) I_2/nitromethane or brain homogenate; (d) NaH, THF, R_1-I, 0 °C; (e) nitromethane, R_1-I, 90 °C, 2 days.

Table 1. Characterization of Pyridinium (Compounds **10**−**23**) and Dihydropyridine (Compounds **24** and **25**) Derivatives

compd[a]	formula	analysis	yield (%)
10	$C_{21}H_{26}INO_3S\cdot0.85I_2$	C, H, N	52
11	$C_{22}H_{28}INO_3S\cdot0.55I_2$	C, H, N	49
12[b]	$C_{23}H_{30}INO_3S\cdot1.64H_2O$	C, H, N	31
13[c]	$C_{24}H_{32}INO_3S\cdot0.98H_2O$	C, H, N	25
14	$C_{22}H_{27}FINO_3S\cdot0.90I_2$	C, H, N	56
15	$C_{23}H_{30}INO_3S\cdot1.80I_2$	C, H, N	52
16	$C_{24}H_{32}INO_3S\cdot0.73I_2$	C, H, N	22
17	$C_{25}H_{33}FINO_3S\cdot1.80I_2$	C, H, N	48
18	$C_{25}H_{32}INO_4S_2\cdot0.75I_2$	C, H, N	49
19	$C_{30}H_{31}IN_2O_5S\cdot1.00I_2$	C, H, N	27
20	$C_{24}H_{32}INO_3S\cdot0.50I_2$	C, H, N	51
21	$C_{24}H_{30}INO_3S\cdot0.70I_2$	C, H, N	40
22	$C_{31}H_{38}INO_4S\cdot1.15I_2$	C, H, N	24
23	$C_{21}H_{32}INO_3S\cdot0.80I_2$	C, H, N	47
24[d]	$C_{22}H_{29}NO_3S$	C, H, N	71
25[d]	$C_{21}H_{33}NO_3S$	HRMS	34

[a] Unless otherwise noted, the R_1I used in the preparation of pyridinium salts (compounds **10**−**23**) is MeI. [b] The R_1I used is EtI. [c] The R_1I used is 1-PrI. [d] Compounds **24** and **25** are 1-methyl-1,4-dihydropyridines.

determined. The corresponding 2-methyl derivative, **10**, was slightly less potent at the three adenosine receptor subtypes. Homologation of the 1-position substituent to ethyl, **12**, and propyl, **13**, caused a progressive reduction in the affinity at A_3 receptors. Similarly, homologation at the 3-, 4-, and 5-positions and/or the introduction of fluorine atoms at the terminal carbons of the ester groups, i.e. compounds **14**−**17**, failed to improve potency in binding at A_3 receptors.

At the 4-position, introduction of a thioester group (cf. **18** vs **15**) had no effect on the A_3 receptor affinity, while steric bulk in the form of a phthaloylamino group, in compound **19**, increased affinity at A_1 and A_{2A} receptors, thus diminishing selectivity. Homologation, as in **20**, or introduction of steric bulk at the 2-position, as in **15**, only slightly diminished A_3 receptor affinity. Introduction of cycloalkyl groups at the 2- and 6- positions diminshed affinity by 6- and 3-fold, respectively, in compounds **21** and **23**.

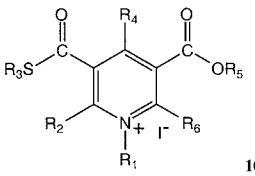
Introduction of a long chain at the 2-position, in **22**, did not decrease the human A_3 receptor affinity.

The affinity of **11** at recombinant human A_{2B} receptors expressed in human embryonic kidney (HEK-293) cells was measured. At 30 μM, the degree of displacement of specific binding of the radiolabeled antagonist [^{3}H]ZM241385[25] was only 40%. Thus, this pyridinium salt is selective in binding to human A_3 versus A_{2B} receptors.

Compound **11** effectively antagonized the effects of an agonist in a functional A_3 receptor assay, i.e. inhibition of adenylate cyclase in CHO cells expressing cloned human A_3 receptors.[8,14] In this functional assay (Figure 2), IB-MECA inhibited adenylate cyclase via human A_3 receptors with an IC_{50} of 41.4 ± 14.9 nM (n = 3). In the presence of 10 μM of **11**, the concentration response curve was shifted 26-fold to the right, with an IC_{50} of 1.08 ± 0.19 μM (n = 3). From a Schild analysis[30] a K_B value obtained for antagonism by **11** was 399 nM, i.e. approximately 1.8 times the K_i value obtained in binding to human A_3 receptors.

Pyridinium salt **11** could be generated through oxidation of the corresponding reduced precursor, 1-methyl-2,4-diethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenyl-1,4-dihydropyridine, **24**. The conversion of **24** to **11** by chemical means (iodine in nitromethane) and during incubation at 37 °C with rat brain membranes, to simulate in vivo conditions, was studied. The chemical conversion occurred readily, and the time course of the biochemical oxidation was recorded (Figure 3). At regular time points, aliquots were removed from the incubation mixture, extracted with ether, and both the

Table 2. Affinities of Pyridinium Derivatives in Radioligand Binding Assays at A_1, A_{2A}, and A_3 Receptors[a−d]



10 - 23, R_1 = Me, unless noted

						K_i (nM) or % displacement		
compd	R_2	R_3	R_4	R_5	R_6	rat A_1[a]	rat A_{2A}[b]	human A_3[c]
10	CH_3	CH_2CH_3	CH_2CH_3	CH_2CH_3	Ph	22 ± 12% (10^{-4})	20800 ± 9500	379 ± 94
11	CH_2CH_3	CH_2CH_3	CH_2CH_3	CH_2CH_3	Ph	31900 ± 8700	11600 ± 2900	219 ± 59
12 (R_1 = Et)	CH_2CH_3	CH_2CH_3	CH_2CH_3	CH_2CH_3	Ph	43700 ± 12400	25 ± 1% (10^{-4})	577 ± 64
13 (R_1 = n-Pr)	CH_2CH_3	CH_2CH_3	CH_2CH_3	CH_2CH_3	Ph	76900 ± 12700	28 ± 1% (10^{-4})	1350 ± 490
14	CH_2CH_3	CH_2CH_3	CH_2CH_3	CH_2CH_2F	Ph	30 ± 2% (10^{-4})	56600 ± 11200	364 ± 107
15	CH_2CH_3	CH_2CH_3	CH_2CH_3	$CH_2CH_2CH_3$	Ph	*d* (10^{-4})	18700 ± 6200	483 ± 154
16	CH_2CH_3	CH_2CH_3	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$	Ph	*d* (10^{-4})	25% (10^{-4})	2020 ± 1190
17	CH_2CH_3	$CH_2CH_2CH_2F$	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$	Ph	*d* (10^{-4})	7370 ± 1010	465 ± 99
18	CH_2CH_3	CH_2CH_3	$CH_2CH_2SCOCH_3$	$CH_2CH_2CH_3$	Ph	45 ± 3% (10^{-4})	53200 ± 23900	538 ± 106
19	CH_2CH_3	CH_2CH_3	CH_2CH_2N-Pth	CH_2CH_3	Ph	1530 ± 130	2150 ± 410	1250 ± 200
20	$(CH_2)_3CH_3$	CH_2CH_3	CH_2CH_3	CH_2CH_3	Ph	14500 ± 5900	6020 ± 1670	436 ± 152
21	cyclobutyl	CH_2CH_3	CH_2CH_3	CH_2CH_3	Ph	88000 ± 31700	33200 ± 7400	1410 ± 440
22	$CH_2CH_2OCH_2Ph$	CH_2CH_3	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$	Ph	21 ± 8% (10^{-4})	11900 ± 2700	348 ± 126
23	CH_2CH_3	CH_2CH_3	CH_2CH_3	CH_2CH_3	cyclopentyl	11500 ± 1800	7720 ± 2800	695 ± 198

[a] Displacement of specific [^{3}H]*R*-PIA binding in rat brain membranes, expressed as K_i ± SEM in μM (n = 3−5) or as a percentage of specific binding displaced at the indicated concentration (M). [b] Displacement of specific [^{3}H]CGS 21680 binding in rat striatal membranes, expressed as K_i ± SEM in μM (n = 3−6) or as a percentage of specific binding displaced at the indicated concentration (M). [c] Displacement of specific [^{125}I]AB-MECA binding at human A_3 receptors expressed in HEK cells, in membranes, expressed as K_i ± SEM in μM (n = 3−4). [d] Displacement of ≤10% of specific binding at the indicated concentration (M).

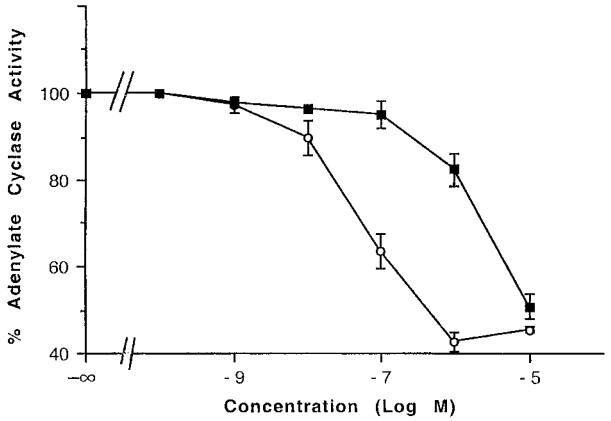


Figure 2. Inhibition of adenylate cyclase by N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine (IB-MECA) in membranes from Chinese hamster ovary (CHO) cells stably transfected with human A_3 receptors and its antagonism by the pyridinium derivative **11**. The assay was carried out as described in the Experimental Section in the presence of 5 μM forskolin, 20 μM Ro 20-1724, and 25 mM NaCl. Each data point is shown as mean ±SEM for three determinations. Responses are shown for agonist alone (○) or in combination with the A_3 adenosine antagonist **11** (10 μM, ■). IC_{50} values were 41.4 ± 14.9 nM (IB-MECA alone), 1.08 ± 0.19 μM (+ **11**).

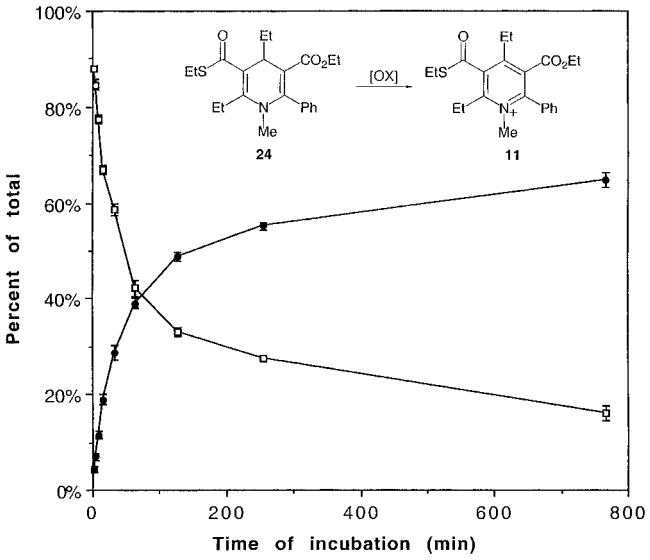


Figure 3. Biochemical oxidation of a 1-methyl-1,4-dihydropyridine derivative, **24**, as a prodrug for the selective A_3 receptor antagonist **11**. Concentrations of the prodrug (□) and pyridinium salt (●) are expressed as percent of total. Incubation was carried out at 37 °C. The initial concentration of **24** was 1.27 mM, and the pH was 7.4.

precursor and the pyridinium salt were assayed in the evaporated organic phase using HPLC. The oxidation occurred cleanly with a $t_{1/2}$ of approximately 47 min.

The 1-methyl dihydropyridine **24** was found to bind selectively to human A_3 adenosine receptors. At human A_3 receptors, the K_i value was 379 ± 122 nM (n = 4). The K_i value at rat A_1 receptors was 28.4 ± 2.5 μM (n = 3). At rat A_{2A} receptors, the percent displacement of specific radioligand binding was 48 ± 7% at 100 μM. Therefore, to demonstrate the prodrug principle, i.e. an inactive prodrug that could be converted to a selective adenosine antagonist, the corresponding 6-cyclopentyl dihydropyridine **25** was prepared. The 6-cyclopentyl analogue, 1-methyl-2,4-diethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-cyclopentyl-1,4-dihydropyridine (**25**), was shown to increase affinity at human A_3 receptors upon oxidation to the corresponding pyridinium salt (**23**). Compound **25** had a K_i value at human A_3 receptors of 6.40 ± 0.78 μM, while **23** was an order of magnitude more potent (K_i of 695 nM), suggesting a prodrug scheme. At rat A_1 receptors, both the precursor, compound **25** (34 ± 1% displacement at 100 μM), and the oxidized product, **23** (K_i of 11.5 μM), bound only weakly.

Discussion

The role of A_3 receptors in the central nervous system is under investigation. It appears that an A_3 receptor antagonist is cerebroprotective in a model of global ischemia in gerbils.[10] Furthermore, A_3 receptor antagonists may have a general function in inflammation and asthma.[1,2] Thus, any advance in the design of A_3 antagonists that may enhance the bioavailability or increase the specificity of the compound within the body is of potential therapeutic interest.

The pyridinium antagonists are not as potent as other recently reported selective A_3 receptor antagonists; however, they display uniquely high water solubility (43 mM for compound **11**) as well as the ability to be extracted readily into ether. Moreover, the present set of pyridinium antagonists provides a means for oxidative generation in vivo from prodrugs (1-methyl-1,4-dihydropyridine derivatives). In the present study, one reduced precursor, **25**, was found to be clearly less potent than the corresponding pyridinium salt, **23**, in binding to adenosine receptors, especially the A_3 subtype, and another precursor, **24**, was found to have the identical human A_3 receptor affinity before and after oxidation. Thus, depending on the structure of the pyridine substitutents, one can determine the degree to which the antagonist requires preactivation of a prodrug form in order to acheive antagonism of A_3 receptors.

A_3 antagonists have been proposed for use in inflammation.[1,2] In inflammation, oxidation in tissue may occur more readily due to the enhanced presence of various enzymes.[26] In ischemic tissue, reactive oxygen species are generated. Thus, it is conceivable that the activation of the prodrug may be enhanced in affected tissues, thus potentially providing site-selective activation of the A_3 antagonist.

The use of dihydropyridines as prodrugs has been thoroughly studied by Bodor and co-workers.[27,28] By the Bodor approach, an *N*-methyl dihydropyridine moiety is temporarily attached to a drug. Following passage across the blood−brain barrier, an oxidation occurs and the resultant 1-Me pyridinium derivative is restricted from leaving the brain. The pyridinium moiety gradually is cleaved from the active drug moiety in the brain compartment. In the present approach, the pyridinium species, itself, is the biologicallly active drug and thus is immediately available to act following oxidation. The in vivo properties of the 1-methyldihydropyridine and pyridinium derivatives in the present study, including the ability to cross biological membranes such as the blood−brain barrier, have yet to be explored.

Experimental Section

Chemical Synthesis. Materials. Iodomethane was purchased from Fluka (Buchs, Switzerland). Iodoethane and 1-iodopropane were purchased from Aldrich (Milwaukee, WI). PBS (1× pH 7.4) was purchased from Biofluids, Inc. (Rockville, MD). Starting 3,5-diacyl-2,4-dialkylpyridine and dihydropyridine derivatives were described previously.[15,18] All other materials were obtained from commercial sources.

Proton nuclear magnetic resonance spectroscopy was performed on a Varian GEMINI-300 spectrometer, and all spectra were obtained in $CDCl_3$. Chemical shifts (δ) relative to tetramethylsilane are given. Chemical-ionization (CI) mass spectrometry was performed with a Finnigan 4600 mass spectrometer, and electron-impact (EI) mass spectrometry was performed with a VG7070F mass spectrometer at 6 kV. Elemental analysis was performed by Galbraith Laboratories, Inc. (Knoxville, TN) and/or Atlantic Microlab, Inc. (Norcross, GA).

General Procedure for Preparation of Pyridinium Salt (10, 11, 14−23) by Quaternary Amination of 3,5-Diacyl-2,4-Dialkylpyridine Derivatives with Iodomethane (Scheme 1). A mixture of **2** (14 mg, 0.038 mmol) and iodomethane (59 mg, 0.38 mmol) in 2 mL of anhydrous nitromethane was sealed in a Pyrex tube and was heated at 80 °C for 2 days. After the mixture cooled to room temperature, the solvent and excess MeI were removed under reduced pressure to leave a yellow oil. It was applied to TLC separation [ethyl acetate:petroleum ether = 1:4 (v/v) for the first development; methanol:chloroform = 1:5 (v/v) for a second development and ethyl acetate: petroleum ether = 1:1 (v/v) for a third development] and 9.5 mg of the desired product (**11**) was afforded as a yellow solid (yield: 49%). If methanol or acetone was used as the solvent, the yield was much lower than with nitromethane.

HPLC results showed that **11** is free of the starting **2**. The mobile phase used for the analysis consisted of methanol, acetonitrile, and water (45:45:10). At a flow rate of 1.0 mL/min with a 4.6 × 250 mm (internal diameter) reverse-phase 300 Å C-18 column operated at ambient temperature, **11** had a retention time of 2.3 min (purity >99%). CHN analysis of **11**: Calcd for $C_{22}H_{28}INO_3S$: C, 51.47%; H, 5.50%; N, 2.73%. Found: C, 51.42%; H, 5.14%; N, 2.43%. HR-MS (FAB, m−b): Calcd for $C_{22}H_{28}NO_3S$ (M^+-I): 386.1790. Found: 386.1776. UV spectra was measured using a Beckman DU 640 spectrophotometer. In methanol at ambient temperature, **11** had λ_{max} = 203 nm, $\epsilon_{max} = 6.65 \times 10^4$ L mol^{-1} cm^{-1}; λ_{max} = 224 nm, $\epsilon_{max} = 3.30 \times 10^4$ L mol^{-1} cm^{-1}; λ_{max} = 288 nm, $\epsilon_{max} = 9.62 \times 10^3$ L mol^{-1} cm^{-1}.

The water solubility of **11** was measured by the following method. Deionized water (100 μL) was saturated with 5 mg of **11** with heating. After cooling to room temperature and the disappearance of turbidity, 50 μL of the clear supernatant was withdrawn and lyophilized to give 1.1 mg of **11**. The water solubility of **11** was calculated to be 42.8 mM at room temperature.

1-Methyl-2-methyl-4-ethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenylpyridinium Iodide (10). ^{1}H NMR δ: 0.88 (t, J = 6.9 Hz, 3 H), 1.26 (t, J = 7.5 Hz, 3 H), 1.33 (t, J = 7.5 Hz, 3 H), 2.69 (s, 3 H), 2.79 (q, J = 7.5 Hz, 2 H), 3.24 (q, J = 7.5 Hz, 2 H), 4.01 (q, J = 6.9 Hz, 2 H), 4.17 (s, 3 H), 7.49−7.53 (m, 3 H), 7.65−7.68 (m, 2 H). MS (CI/NH_3): m/z 500 (MH^+), 358 (MH^+-Me-I), 297 (MH^+-Me-I-SEt).

1-Methyl-2,4-diethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenylpyridinium Iodide (11). ^{1}H NMR δ: 0.86 (t, J = 7.2 Hz, 3 H), 1.32 (t, J = 7.8 Hz, 3 H), 1.41 (t, J = 7.8 Hz, 3 H), 1.44 (t, J = 7.8 Hz, 3 H), 2.84 (q, J = 7.8 Hz, 2 H), 3.22 (q, J = 7.8 Hz, 2 H), 3.44 (q, J = 7.8 Hz, 2 H), 3.98 (q, J = 7.2 Hz, 2 H), 4.22 (s, 3 H), 7.56−7.62 (m, 3 H), 7.72−7.75 (m, 2 H). MS (CI): m/z 514 (MH^+), 372 (MH^+-Me-I).

1-Methyl-2,4-diethyl-3-(ethylsulfanylcarbonyl)-5-(2-fluoroethyloxycarbonyl)-6-phenylpyridinium Iodide (14). ^{1}H NMR δ: 1.20 (t, J = 7.5 Hz, 3 H), 1.39 (t, J = 7.5 Hz, 3 H), 1.46 (t, J = 7.5 Hz, 3 H), 2.79 (q, J = 7.5 Hz, 2 H), 2.99 (q, J = 7.5 Hz, 2 H), 3.27 (q, J = 7.5 Hz, 2 H), 4.20 (s, 3 H), 4.28 (m, 2 H), 4.30−4.40 (m, 2 H), 7.47−7.50 (m, 3 H), 7.64−7.67 (m, 2 H). MS (CI/NH_3): m/z 390 (MH^+-Me-I).

1-Methyl-2-ethyl-4-ethyl-3-(ethylsulfanylcarbonyl)-5-propyloxycarbonyl-6-phenylpyridinium Iodide (15). ^{1}H NMR δ: 0.68 (t, J = 7.8 Hz, 3 H), 1.25 (t, J = 7.8 Hz, 3 H), 1.39 (m, 2 H), 1.49 (t, J = 7.8 Hz, 3 H), 1.57 (t, J = 7.8 Hz, 3 H), 2.97 (q, J = 7.8 Hz, 2 H), 3.28 (q, J = 7.8 Hz, 2 H), 3.38 (q, J = 7.8 Hz, 2 H), 4.07 (t, J = 6.9 Hz, 2 H), 4.21 (s, 3 H), 7.69−7.76 (m, 5 H). MS (CI/NH_3): m/z 386 (MH^+-Me-I).

1-Methyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-5-propyloxycarbonyl-6-phenylpyridinium Iodide (16). ^{1}H NMR δ: 0.67 (t, J = 7.8 Hz, 3 H), 1.04 (t, J = 7.8 Hz, 3 H), 1.40 (m, 2 H), 1.48 (t, J = 7.8 Hz, 3 H), 1.55 (t, J = 7.8 Hz, 3 H), 1.74 (m, 2 H), 2.87 (t, J = 7.8 Hz, 2 H), 3.27 (q, J = 7.8 Hz, 2 H), 3.42 (q, J = 7.8 Hz, 2 H), 4.05 (t, J = 7.8 Hz, 2 H), 4.20 (s, 3 H), 7.62−7.74 (m, 5 H). MS (CI/NH_3): m/z 558 (M^++NH_4), 525 (M^+-1-Me), 414 (M^+-I), 369 (M^+-1-I-Me-Et).

1-Methyl-2-ethyl-4-propyl-3-(3-fluoropropylsulfanylcarbonyl)-5-propyloxycarbonyl-6-phenylpyridinium Iodide (17). ^{1}H NMR δ: 0.68 (t, J = 7.8 Hz, 3 H), 1.04 (t, J = 7.8 Hz, 3 H), 1.41 (m, 2 H), 1.55 (t, J = 7.8 Hz, 3 H), 1.73 (m, 2 H), 2.16 (m, 2 H), 2.88 (m, 2 H), 3.35 (q, J = 7.8 Hz, 2 H), 3.41 (t, J = 6.9 Hz, 2 H), 4.06 (t, J = 6.9 Hz, 2 H), 4.22 (s, 3 H), 4.55 (t, J = 6.0 Hz, 1 H), 4.71 (t, J = 6.0 Hz, 1 H), 7.68−7.75 (m, 5 H). MS (CI/NH_3): m/z 432 (MH^+-Me-I).

1-Methyl-2-ethyl-4-(2-acetylthioethyl)-3-(ethylsulfanylcarbonyl)-5-propyloxycarbonyl-6-phenylpyridinium Iodide (18). ^{1}H NMR δ: 0.66 (t, J = 7.5 Hz, 3 H), 1.37 (m, 2 H), 1.39 (t, J = 7.8 Hz, 3 H), 1.45 (t, J = 7.2 Hz, 3 H), 2.35 (s, 3 H), 2.89−3.02 (m, 4 H), 3.11 (m, 2 H), 3.28 (q, J = 7.2 Hz, 2 H), 4.00 (t, J = 6.9 Hz, 2 H), 4.23 (s, 3 H), 7.53−7.55 (m, 3 H), 7.67−7.70 (m, 2 H). MS (CI/NH_3): m/z 460 (MH^+-Me-I).

1-Methyl-2-ethyl-4-(2-phthalimidoethyl)-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenylpyridinium Iodide (19). ^{1}H NMR δ: 0.94 (t, J = 6.9 Hz, 3 H), 1.34 (t, J = 7.2 Hz, 3 H), 1.48 (t, J = 7.2 Hz, 3 H), 2.94 (q, J = 6.9 Hz, 2 H), 3.15 (t, J = 7.8 Hz, 2 H), 3.24 (q, J = 7.2 Hz, 2 H), 4.01 (t, J = 7.8 Hz, 2 H), 4.21 (s, 3 H), 4.25 (q, J = 7.2 Hz, 2 H), 7.51 (m, 3 H), 7.65 (m, 2 H), 7.78 (m, 2 H), 7.89 (m, 2 H). MS (CI/NH_3): m/z 517 (MH^+-Me-I).

1-Methyl-2-butyl-4-ethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenylpyridinium Iodide (20). ^{1}H NMR δ: 0.90 (t, J = 7.5 Hz, 3 H), 1.04 (t, J = 7.5 Hz, 3 H), 1.29 (t, J = 7.5 Hz, 3 H), 1.34−1.43 (m, 2 H), 1.46 (t, J = 7.5 Hz, 3 H), 1.84 (m, 2 H), 2.76 (q, J = 7.5 Hz, 2 H), 2.88 (t, J = 7.5 Hz, 2 H), 3.16 (q, J = 7.5 Hz, 2 H), 4.05 (q, J = 7.5 Hz, 2 H), 4.26 (s, 3 H), 7.52−7.55 (m, 3 H), 7.69−7.72 (m, 2 H). MS (CI/NH_3): m/z 400 (MH^+-Me-I).

1-Methyl-2-cyclobutyl-4-ethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenylpyridinium Iodide (21). ^{1}H NMR δ: 0.99 (t, J = 7.5 Hz, 3 H), 1.29 (t, J = 7.5 Hz, 3 H), 1.45 (t, J = 7.5 Hz, 3 H), 1.88−1.97 (m, 1 H), 1.97−2.07 (m, 1 H), 2.18−2.32 (m, 2 H), 2.53−2.65 (m, 2 H), 2.71 (q, J = 7.5 Hz, 2 H), 3.13 (q, J = 7.5 Hz, 2 H), 3.81 (m, 1 H), 4.01 (q, J = 7.5 Hz, 2 H), 4.23 (s, 3 H), 7.54−7.56 (m, 3 H), 7.73−7.75 (m, 2 H). MS(CI/NH_3): m/z 398 (MH^+-Me-I).

1-Methyl-2-(2-benzyloxylethyl)-4-propyl-3-(ethylsulfanylcarbonyl)-5-propyloxycarbonyl-6-phenylpyridinium Iodide (22). ^{1}H NMR δ: 0.69 (t, J = 7.2 Hz, 3 H), 1.02 (t, J = 7.2 Hz, 3 H), 1.44 (t, J = 7.2 Hz, 3 H), 1.45 (m, 2 H), 1.68 (m, 2 H), 2.71 (m, 2 H), 3.17 (q, J = 7.2 Hz, 2 H), 3.24 (t, J = 7.2 Hz, 2 H), 3.99 (t, J = 7.2 Hz, 2 H), 4.02 (q, J = 7.2 Hz, 2 H), 4.25 (s, 3 H), 4.58 (s, 2 H), 7.29−7.35 (m, 5 H), 7.53−7.56 (m, 3 H), 7.68−7.71 (m, 2 H). MS (CI/NH_3): m/z 648 (MH^+), 506 (MH^+-Me-I), 445 (MH^+-Me-I-SEt).

1-Methyl-2,4-diethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-cyclopentylpyridinium Iodide (23). ^{1}H NMR δ: 1.10 (t, J = 7.5 Hz, 3 H), 1.32 (t, J = 7.5 Hz, 3 H), 1.41 (t, J = 7.5 Hz, 3 H), 1.44 (t, J = 7.5 Hz, 3 H), 1.66 (m, 2 H), 1.95 (m, 7 H), 2.62 (q, J = 7.5 Hz, 2 H), 2.81 (q, J = 7.5 Hz, 2 H), 3.97 (q, J = 7.5 Hz, 2 H), 4.28 (s, 3 H), 4.40 (q, J = 7.5 Hz, 2 H). MS(CI/NH_3): m/z 364 (MH^+-Me-I).

Preparation of Pyridinium Salt 12 by Quaternary Amination of 2,4-Diethyl-3-(ethylsulfanylcarbonyl)-5-

ethyloxycarbonyl-6-phenylpyridine (2) with Iodoethane (Scheme 1). A mixture of **2** (14 mg, 0.038 mmol) and iodoethane (59 mg, 0.38 mmol) in 2 mL of anhydrous nitromethane was sealed in a Pyrex tube and was heated at 90 °C for 2 days. After the mixture cooled to room temperature, the solvent and excess EtI were removed under reduced pressure to leave a yellow oil. It was applied to TLC separation [ethyl acetate:petroleum ether = 1:4 (v/v) for the first development; methanol:chloroform = 1:5 (v/v) for a second development and ethyl acetate:petroleum ether = 1:1 (v/v) for a third development], and 6 mg of the desired product (**12**) was afforded (yield: 31%).

1-Ethyl-2,4-diethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenylpyridinium Iodide (12). ^{1}H NMR δ: 0.89 (t, J = 7.5 Hz, 3 H), 1.28 (t, J = 7.5 Hz, 3 H), 1.39 (t, J = 7.5 Hz, 3 H), 1.44 (t, J = 7.2 Hz, 3 H), 2.79 (q, J = 7.5 Hz, 2 H), 2.94 (t, J = 7.5 Hz, 3 H), 3.11 (q, J = 7.5 Hz, 2 H), 3.34 (q, J = 7.5 Hz, 2 H), 4.02 (q, J = 7.2 Hz, 2 H), 5.20 (q, J = 7.5 Hz, 2 H), 7.52−7.58 (m, 3 H), 7.68−7.71 (m, 2 H). MS (CI/NH_3): m/z 372 (MH^+-Et-I).

Preparation of Pyridinium Salt 13 by Quaternary Amination of 2,4-Diethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenylpyridine (2) with 1-Iodopropane (Scheme 1). A mixture of **2** (14 mg, 0.038 mmol) and 1-iodopropane (65 mg, 0.38 mmol) in 2 mL of anhydrous nitromethane was sealed in a Pyrex tube and was heated at 100 °C for 2 days. After the mixture cooled to room temperature, the solvent and excess PrI were removed under reduced pressure to leave a yellow oil. It was applied to TLC separation [ethyl acetate:petroleum ether = 1:4 v/v for the first development then 1:1 for a second development], and 5 mg of the desired product (**13**) was afforded (yield: 25%).

1-Propyl-2,4-diethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenylpyridinium Iodide (13). ^{1}H NMR δ: 0.91 (m, J = 7.2 Hz, 6 H), 1.29 (t, J = 7.5 Hz, 3 H), 1.39 (t, J = 7.5 Hz, 3 H), 1.43 (t, J = 7.2 Hz, 3 H), 2.44 (t, J = 7.2 Hz, 3 H), 2.79 (q, J = 7.2 Hz, 2 H), 3.07 (q, J = 7.5 Hz, 2 H), 3.38 (q, J = 7.2 Hz, 2 H), 4.01 (q, J = 7.2 Hz, 2 H), 4.29 (q, J = 7.2 Hz, 2 H), 5.22 (q, J = 7.2 Hz, 2 H), 7.51−7.54 (m, 3 H), 7.68−7.71 (m, 2 H). MS (CI/NH_3): m/z 372 (MH^+-Pr-I).

General Procedure for Preparation of 1-Methyl Dihydropyridines (24 and 25) by Alkylation of 3,5-Diacyl-2,4-dialkyl-1,4-dihydropyridine Derivatives with Iodomethane (Scheme 1). A solution of the appropriate DHP (2,4-diethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenyl-1,4-dihydropyridine, 15 mg, 0.04 mmol) in 2 mL of anhydrous THF was treated with NaH (60%, 2 mg, 0.08 mmol) at room temperature under stirring for 5 min. Then iodomethane (28 mg, 0.2 mmol) was added, and the reaction mixture was stirred for another 10 min (monitor by TLC). At completion the reaction mixture was applied to TLC separation [ethyl acetate:petroleum ether = 1:19 v/v], and 11 mg of **24** was obtained (yield: 71%).

1-Methyl-2,4-diethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-phenyl-1,4-dihydropyridine (24). ^{1}H NMR δ: 0.85 (t, J = 7.2 Hz, 3 H), 0.93 (t, J = 7.2 Hz, 3 H), 1.20 (t, J = 7.2 Hz, 3 H), 1.29 (t, J = 7.2 Hz, 3 H), 2.74 (q, J = 7.2 Hz, 2 H), 2.85 (s, 3 H), 2.92 (q, J = 7.2 Hz, 2 H), 3.07 (q, J = 7.2 Hz, 2 H), 3.87 (q, J = 7.2 Hz, 2 H), 4.02 (t, J = 7.2 Hz, 1 H), 7.20 (m, 2 H), 7.38−7.41 (m, 3 H). MS (CI/NH_3): m/z 388 (MH^+).

1-Methyl-2,4-diethyl-3-(ethylsulfanylcarbonyl)-5-ethyloxycarbonyl-6-cyclopentyl-1,4-dihydropyridine (25). ^{1}H NMR δ: 0.78 (t, J = 7.8 Hz, 3 H), 1.11 (t, J = 7.8 Hz, 3 H), 1.28 (t, J = 7.8 Hz, 3 H), 1.32 (t, J = 7.8 Hz, 3 H), 1.46 (m, 2H), 1.60−1.78 (m, 7H), 2.10 (m, 2 H), 2.60−2.67 (m, 1 H), 2.89 (q, J = 7.8 Hz, 2 H), 3.01−3.13 (m, 1 H), 3.15 (s, 3 H), 4.10 (t, J = 6.0 Hz, 1 H), 4.13−4.26 (m, 2 H). MS (CI/NH_3): m/z 380 (MH^+), 318 (M^+-SEt, base).

Chemical Transformation of 24 to 11 through Oxidation with Iodine (Scheme 1). A solution of **24** (5 mg, 0.013 mmol) in 0.5 mL of dry nitromethane was treated with iodine (10 mg, 0.040 mmol) at room temperature with stirring for 1 day (monitor by TLC). At completion the reaction mixture was applied to TLC separation [ethyl acetate:petroleum ether = 1:4 v/v for the first development then 1:1 for a second development], and 2 mg of a yellow solid was obtained (yield: 30%), with ^{1}H NMR and MS data consistent with those of compound **11**.

Oxidation of a 1-Methyl-1,4-dihydropyridine Derivative (24) in the Presence of Rat Brain Homogenate.[27,28] The rat brain homogenate was prepared by the following method. One rat (1 kg) was killed, and the brain (weighing 2.26 g) was removed and homogenized in 12 mL of PBS (Biofluids, Inc., 1× pH 7.4). The homogenate was centrifuged at 12000*g* rpm, and the supernatant was used. Then 5 mg of **24** dissolved in 0.2 mL of DMSO was mixed with 10 mL of brain homogenate (initial concentration of 1.27 mM), which was previously equilibrated to 37 °C in a water bath incubator, and shaking was continued at that temperature. Aliquots of 500 μL were withdrawn at 2, 4, 8, 16, 32, 64, 128, 256, and 768 min (2× each) from the test medium, added immediately to 3 mL of ice-cold ethyl ether, shaken vigorously, and placed in a freezer. When all samples had been collected, the ether layer of each sample was separated. After evaporation of the ether, each residue was dissolved in 200 μL of methanol, filtered through Whatman No. 1 filter paper, and analyzed by HPLC.

HPLC Method for the Study of Oxidation of a 1-Methyl-1,4-dihydropyridine Derivative (24) in the Presence of Rat Brain Homogenate. The chromatographic analysis was performed on a Hewlett-Packard Series 1100 HPLC system consisting of a G1311A QuatPump, G1315A DAD, G1322A Degasser, G1328A Manlnj, and HP Kayak PC workstations operated at HP ChemStation. A 4.6 × 250 mm (internal diameter) reverse-phase 300 Å C-18 column (Whatman, Inc.), operated at ambient temperature, was used for all analyses. The mobile phase used for the analysis consisted of methanol, acetonitrile, and water (45:45:10). At a flow rate of 1.0 mL/min, **24** had a retention time of 4.4 min, while its oxidation product had a retention time of 2.3 min. The mean value (in percentage of total) of two samples at each time point was calculated and plotted against time of incubation.

Pharmacology. Adenylate Cyclase Assay. Adenylate cyclase assays were performed with membranes prepared from Chinese hamster ovary (CHO) cells stably expressing either the human A_1 receptor or human A_3 receptor by the method of Salomon et al.,[29] as described previously with the following modifications.[14] 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724, 20 μM, Calbiochem, San Diego, CA) was employed to inhibit phosphodiesterases rather than papaverine, and the NaCl concentration in the assay was 25 mM. Membranes were pretreated with 2 units/mL adenosine deaminase, and the antagonist **11** (10 μM) at 30 °C for 5 min prior to initiation of the adenylate cyclase assay. Adenylate cyclase was stimulated with forskolin (1 μM). Concentration−response data for the inhibition of adenylate cyclase activity by IB-MECA (human A_3 receptor) were obtained. Maximal inhibition of adenylate cyclase by IB-MECA at the human A_3 receptor correlated to ∼60% of total stimulation, respectively. IC_{50} values were calculated using InPlot (GraphPad, San Diego, CA). K_B values were calculated as described.[30]

Radioligand Binding Studies. Binding of [^{3}H]*R*-N^6-phenylisopropyladenosine ([^{3}H]*R*-PIA) to A_1 receptors from rat cerebral cortex membranes and of [^{3}H]-2-[4-[(2-carboxyethyl)-phenyl]ethylamino]-5′-*N*-ethylcarbamoyladenosine ([^{3}H]CGS 21680) to A_{2A} receptors from rat striatal membranes was performed as described previously.[22,23] Adenosine deaminase (3 units/mL) was present during the preparation of the brain membranes, in a preincubation of 30 min at 30 °C, and during the incubation with the radioligands. Nonspecific binding was determined in the presence of 10 μM (A_1 receptors) or 20 μM (A_{2A} receptors) 2-chloroadenosine.

Binding of [^{125}I]N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine ([^{125}I]AB-MECA)[24] to membranes prepared from human embryonic kidney (HEK-293) cells stably expressing the human A_3 receptor[16] or clone HS-21a (Receptor Biology, Inc., Beltsville, MD) or to membranes prepared from Chinese

hamster ovary (CHO) cells stably expressing the rat A_3 receptor[8] was performed as described at 4 °C.[24] The assay medium consisted of a buffer containing 10 mM Mg^{2+}, 50 mM Tris, 3 units/mL adenosine deaminase, and 1 mM EDTA, at pH 8.0 (4 °C). The glass incubation tubes contained 100 μL of the membrane suspension (0.3 mg protein/mL, stored at −80 °C in the same buffer), 50 μL of [^{125}I]AB-MECA (final concentration 0.3 nM), and 50 μL of a solution of the proposed antagonist. Nonspecific binding was determined in the presence of 100 μM N^6-phenylisopropyladenosine (NECA).

All nonradioactive compounds were initially dissolved in DMSO and diluted with buffer to the final concentration, where the amount of DMSO never exceeded 1%.

Incubations were terminated by rapid filtration over Whatman GF/B filters, using a Brandell cell harvester (Brandell, Gaithersburg, MD). The tubes were rinsed three times with 3 mL of buffer each.

At least five different concentrations of competitor, spanning 3 orders of magnitude adjusted appropriately for the IC_{50} of each compound, were used. IC_{50} values, calculated with the nonlinear regression method implemented in the InPlot program (Graph-PAD, San Diego, CA), were converted to apparent K_i values using the Cheng−Prusoff equation[31] and K_d values of 1.0 nM ([^{3}H]*R*-PIA); 15.5 nM ([^{3}H]CGS 21680); 0.59 and 1.46 nM ([^{125}I]AB-MECA at human and rat A_3 receptors, respectively).

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