Interaction of 1,4-Dihydropyridine and Pyridine Derivatives with Adenosine Receptors: Selectivity for A_3 Receptors

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1,4-Dihydropyridine and pyridine derivatives bound to three subtypes of adenosine receptors in the micromolar range. Affinity was determined in radioligand binding assays at rat brain A_1 and A_{2A} receptors using [^{3}H]-(*R*)-PIA [[^{3}H]-(*R*)-N^6-(phenylisopropyl)adenosine] and [^{3}H]CGS 21680 [[^{3}H]-2-[[4-(2-carboxyethyl)phenyl]ethylamino]-5′-(*N*-ethylcarbamoyl)adenosine], respectively. Affinity was determined at cloned human and rat A_3 receptors using [^{125}I]AB-MECA [N^6-(4-amino-3-iodobenzyl)-5′-(*N*-methylcarbamoyl)adenosine]. Structure−activity analysis at adenosine receptors indicated that sterically bulky groups at the 4-, 5-, and 6-positions are tolerated. (*R,S*)-Nicardipine, **12**, displayed K_i values of 19.6 and 63.8 μM at rat A_1 and A_{2A} receptors, respectively, and 3.25 μM at human A_3 receptors. Similarly, (*R*)-niguldipine, **14**, displayed K_i values of 41.3 and 1.90 μM at A_1 and A_3 receptors, respectively, and was inactive at A_{2A} receptors. A preference for the *R*- vs the *S*-enantiomer was observed for several dihydropyridines at adenosine receptors, in contrast with the selectivity at L-type Ca^{2+} channels. A 4-*trans*-β-styryl derivative, **24**, with a K_i value of 0.670 μM at A_3 receptors, was 24-fold selective vs A_1 receptors (K_i = 16.1 μM) and 74-fold vs A_{2A} receptors (K_i = 49.3 μM). The affinity of **24** at L-type Ca^{2+} channels, measured in rat brain membranes using [^{3}H]isradipine, indicated a K_i value of 0.694 μM, and the compound is thus nonselective between A_3 receptors and L-type Ca^{2+} channels. Inclusion of a 6-phenyl group enhanced A_3 receptor selectivity: Compound **28** (MRS1097; 3,5-diethyl 2-methyl-6-phenyl-4-(*trans*-2-phenylvinyl)-1,4(*R,S*)-dihydro-pyridine-3,5-dicarboxylate) was 55-fold selective vs A_1 receptors, 44-fold selective vs A_{2A} receptors, and over 1000-fold selective vs L-type Ca^{2+} channels. In addition, compound **28** attenuated the A_3 agonist-elicited inhibitory effect on adenylyl cyclase. Furthermore, whereas nicardipine, **12**, displaced radioligand from the Na^+-independent adenosine transporter with an apparent affinity of 5.36 ± 1.51 μM, compound **28** displaced less than 10% of total binding at a concentration of 100 μM. Pyridine derivatives, when bearing a 4-alkyl but not a 4-phenyl group, maintained affinity for adenosine receptors. These findings indicate that the dihydropyridines may provide leads for the development of novel, selective A_3 adenosine antagonists.

The 1,4-dihydropyridines have been developed extensively as potent blockers and activators of L-type calcium channels.[1] A number of these channel blockers, such as nifedipine (Figure 1, **9**) and nicardipine (Figure 1, **12**), are used therapeutically in the treatment of cardiovascular disorders, especially hypertension and coronary heart disease.[2]

Dihydropyridines appear to be "privileged structures" in medicinal chemistry and pharmacology, *i.e.*, they display affinity for many diverse binding sites.[3] This adaptability of dihydropyridines has been utilized to optimize affinity in binding to α_{1a}-adrenergic receptors (*e.g.*, the antagonist SNAP 5089, Figure 1),[4] to platelet activating factor (PAF, 1-*O*-hexadecyl/octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine) receptors,[5] and at other receptor targets.[6] Thus, by careful structural modification, it has been possible to select for affinity at sites other than Ca^{2+} channels. For example, a dihydropyridine derivative, 3-ethyl 5-[2-(phenylthio)-ethyl] 2,4,6-trimethyl-1,4-dihydropyridine-3,5-dicarboxylate (Figure 1, **4**), has been found to inhibit PAF binding with an affinity of 69 nM, while the same derivative was inactive as a calcium channel blocker.[5] Several dihydropyridines were also found to bind to A_1 adenosine receptors in rat brain.[7,8] In the present study we have used the 1,4-dihydropyridine nucleus as a template for probing structure−activity relationships (SAR) at several subtypes of central adenosine receptors (A_1, A_{2A}, and A_3), in an effort to design new selective antagonists. The classical (A_1 and A_{2A}) adenosine receptor antagonists are xanthines,[9] but it would be desirable to expand the list of diverse chemical structures known to bind to adenosine receptors. There is already a large number of non-xanthine antagonists known for the A_1 receptor.[9−13] Although selective agents have been reported for both A_1 and A_{2A} subtypes of adenosine receptors,[9] the development of ligands for the recently discovered A_3 receptor is lagging behind the other subtypes.[14] Activation of the A_3 receptor results in hypotension and promotion of release of inflammatory mediators from mast cells.[15] A_3 adenosine receptor antagonists are being sought as potential antiasthmatic,[15] antiinflammatory,[15] and cerebroprotective agents.[16] There may also be an involvement of A_3 receptors in cancer[17] and apoptosis.[18] We have introduced the first selective agonists for the A_3 adenosine receptor subtype, including N^6-(3-iodobenzyl)-5′-(*N*-

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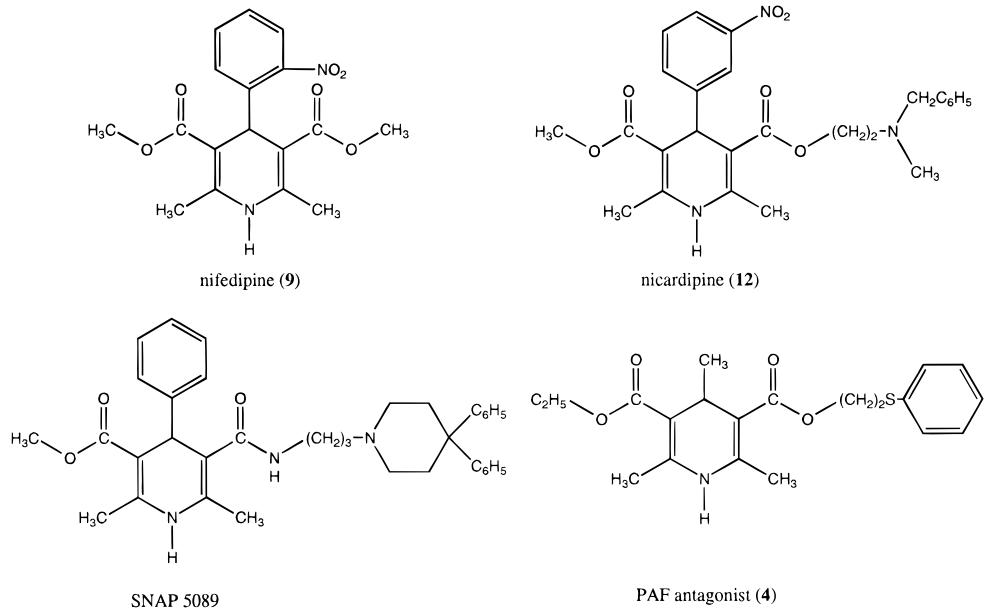


Figure 1. Structures of 1,4-dihydropyridines as potent calcium channel antagonists (nifedipine, **9**, and nicardipine, **12**) or as ligands at other receptor sites (SNAP 5089, an adrenoceptor antagonist, and 3-ethyl 5-[2-(phenylthio)ethyl] 2,4,6-trimethyl-1,4-dihydropyridine-3,5-dicarboxylate, **4**, a PAF antagonist).

methylcarbamoyl)adenosine (IB-MECA),[19,20] but studies of the SAR of xanthines at this subtype have thus far failed to identify principles of achieving selectivity.[21] Furthermore, at A_3 receptors the affinity of xanthines is generally much weaker than at the A_1 or A_{2A} subtypes,[21,22] and considerable species variability in their affinity has been noted.[23−25] A screen of >100 cyclic compounds of diverse structure has provided several unexplored leads for A_3 selectivity, but potencies were generally low.[11] A recent study has identified additional natural product leads having moderate affinity, but the degree of selectivity was still minimal.[13] Thus, the design of potent and selective A_3 antagonists has remained an unfulfilled challenge.[14]

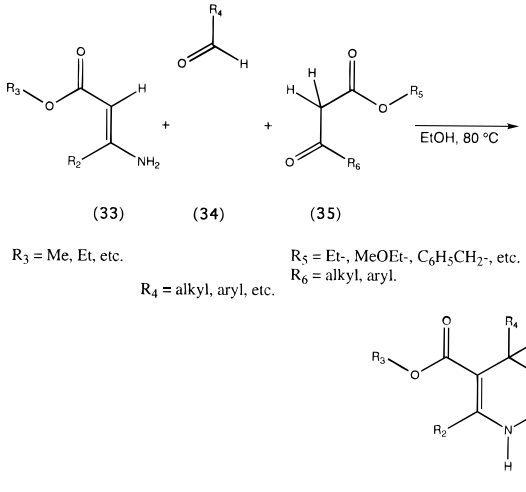
Results

Synthesis. The structures of the 1,4-dihydropyridines and related derivatives tested for affinity in radioligand binding assays at adenosine receptors are shown in Table 1. Some of the derivatives, *i.e.*, both well-known Ca^{2+} channel ligands (nifedipine, **9**; nitrendipine, **10**; nicardipine, **12**; nimodipine, **13**; niguldipine, **14** and **15**; Bay K 8644, **18** and **19**) and a PAF antagonist (**4**),[5] were obtained from commercial sources. Other compounds were synthesized using standard methodology (Scheme 1),[26−28] in fair yield, as reported in Table 2. The synthesis consisted of the Hantzsch condensation of a 3-amino-2-butenoate ester, **33a**,**b**, an aldehyde, **34a**−**o**, and a 3-ketopropionate ester derivative, **35a**−**e**, that were dissolved in ethanol and heated to 100 °C. In order to obtain substitution at the 4-position, the aldehyde component, **34a**−**o**, was varied. Substitution at the 6-position was achieved by varying the 3-ketopropionate ester component, **35a**−**e** (Scheme 1).

For compound **26**, the precursor butyl β-keto ester (**35e**) was prepared from the condensation of the enolate lithium salt of ethyl acetate and valeryl chloride.[28]

Good yields of the 6-phenyl-1,4-dihydropyridines were obtained using a 72 h reaction time.

Scheme 1. General Procedure for the Synthesis of 1,4-Dihydropyridine Derivatives



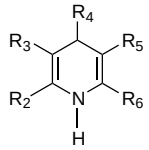
Oxidation of 1,4-dihydropyridines (**1** and **27**) to the corresponding pyridine derivatives (**29** and **30**, respectively) was carried out using tetrachloro-1,4-benzoquinone (chloranil, **37**) in tetrahydrofuran (Scheme 2).[29]

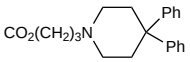
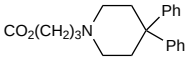
Most of the dihydropyridines examined, except for two pairs of pure enantiomers, **14** and **15**, **18** and **19**, and the nonchiral compounds **9** and **11**, were racemic mixtures at position C-4.

Binding at Adenosine Receptors. K_i values at A_1 and A_{2A} receptors were determined in radioligand binding assays in rat brain membranes vs [^{3}H]-(*R*)-PIA [[^{3}H]-(*R*)-N^6-(phenylisopropyl)adenosine] or [^{3}H]CGS 21680 [[^{3}H]-2-[[4-[(2-carboxyethyl)phenyl]ethylamino]-5′-(*N*-ethylcarbamoyl)adenosine], respectively.[30,31] Affinity at cloned human A_3 receptors expressed in HEK-293 cells[23] was determined using [^{125}I]AB-MECA [N^6-(4-amino-3-[^{125}I]iodobenzyl)-5′-(*N*-methylcarbamoyl)-adenosine].[13,32]

SAR analysis at adenosine receptors indicated that sterically bulky groups are tolerated at the 4-, 5-, and 6-positions (Table 1). R_5 was varied from ethoxycarbo-

Table 1. Affinities of Dihydropyridine and Pyridine Derivatives in Radioligand Binding Assays at A_1, A_{2A}, and A_3 Receptors[a-d]



						K_i (mM) or % inhibition[c]		
compd	R_2	R_3	R_4	R_5	R_6	rA_1	rA_{2A}	hA_3
1	CH_3	CO_2CH_3	CH_3	$CO_2CH_2CH_3$	CH_3	32.6 ± 6.3	46.1 ± 6.8	32.3 ± 5.1
2	CH_3	CO_2CH_3	CH_3	$CO_2(CH_2)_2OCH_3$	CH_3	49.2 ± 0.7	37 ± 18% (10^{-4})	62.3 ± 16.7
3	CH_3	CO_2CH_3	CH_3	CO_2CH_2Ph	CH_3	6.45 ± 1.47	9.72 ± 0.63	2.78 ± 0.89
4	CH_3	$CO_2CH_2CH_3$	CH_3	$CO_2(CH_2)_2SPh$	CH_3	6.50 ± 0.47	7.10 ± 2.46	5.56 ± 1.36
5	CH_3	CO_2CH_3	CH_2CH_3	$CO_2CH_2CH_3$	CH_3	7.52 ± 2.79	9.56 ± 2.69	13.6 ± 2.0
6	CH_3	CO_2CH_3	$(CH_2)_2CH_3$	$CO_2CH_2CH_3$	CH_3	8.17 ± 1.58	11.5 ± 3.8	6.51 ± 0.74
7	CH_3	CO_2CH_3	$CH_2CHCH_3(CH_2)_2$-$CH{=}C(CH_3)_2$ (*R,S*)	$CO_2CH_2CH_3$	CH_3	9.10 ± 2.90	23.1 ± 8.6	7.90 ± 0.88
8	CH_3	CO_2CH_3	Ph	$CO_2CH_2CH_3$	CH_3	11.0 ± 1.6	2.74 ± 0.85	12.0 ± 3.3
9 (nifedipine)	CH_3	CO_2CH_3	2-NO_2Ph	CO_2CH_3	CH_3	2.89 ± 0.23	18.2 ± 2.51	8.29 ± 2.41
10 (nitrendipine)	CH_3	CO_2CH_3	3-NO_2Ph	$CO_2CH_2CH_3$	CH_3	8.96 ± 2.06	23.0 ± 3.7	8.30 ± 1.41
11	CH_3	$CO_2CH_2CH_3$	3-NO_2Ph	$CO_2CH_2CH_3$	CH_3	3.34 ± 2.17	18.2 ± 7.9	2.51 ± 0.15
12 (nicardipine)	CH_3	CO_2CH_3	3-NO_2Ph	$CO_2CH_2CH_2N(CH_3)CH_2Ph$	CH_3	19.6 ± 1.9	63.8 ± 4.2	3.25 ± 0.26
13 (nimodipine)	CH_3	$CO_2CH(CH_3)_2$	3-NO_2Ph	$CO_2CH_2CH_2OCH_3$	CH_3	20.1 ± 1.7	44.3 ± 14.4	8.47 ± 2.75
14 ((*R*)-niguldipine)	CH_3	CO_2CH_3	3-NO_2Ph		CH_3	41.3 ± 3.5	*d* (10^{-4})	1.90 ± 0.40
15 ((*S*)-niguldipine)	CH_3	CO_2CH_3	3-NO_2Ph		CH_3	*d* (10^{-4})	*d* (10^{-4})	2.80 ± 0.35
16	CH_3	CO_2CH_3	4-NO_2Ph	$CO_2CH_2CH_3$	CH_3	37 ± 14% (10^{-4})	35.6 ± 1.9	5.90 ± 1.65
17	CH_3	CO_2CH_3	2-CF_3Ph	$CO_2CH_2CH_3$	CH_3	6.68 ± 2.37	20.7 ± 2.8	11.6 ± 1.7
18 ((*R*)-BayK8644)	CH_3	CO_2CH_3	2-CF_3Ph	NO_2	CH_3	0.785 ± 0.113	35.1 ± 10.1	2.77 ± 0.34
19 ((*S*)-BayK8644)	CH_3	CO_2CH_3	2-CF_3Ph	NO_2	CH_3	6.66 ± 1.89	86.3 ± 23.4	23.5 ± 0.6
20	CH_3	CO_2CH_3	4-CH_3OPh	$CO_2CH_2CH_3$	CH_3	2.75 ± 0.35	12.7 ± 3.8	4.10 ± 0.14
21	CH_3	CO_2CH_3	3-CH_3O-4-OHPh	$CO_2CH_2CH_3$	CH_3	51.0 ± 3.7	56.8 ± 1.9	32.1 ± 9.2
22	CH_3	CO_2CH_3	3,4-OCH_2OPh	$CO_2CH_2CH_3$	CH_3	3.66 ± 0.61	5.27 ± 1.97	4.58 ± 1.11
23	CH_3	CO_2CH_3	$PhCH_2CH_2$	$CO_2CH_2CH_3$	CH_3	8.81 ± 0.92	6.71 ± 2.06	2.30 ± 0.70
24	CH_3	CO_2CH_3	Ph-CH=CH-(*trans*)	$CO_2CH_2CH_3$	CH_3	16.1 ± 0.5	49.3 ± 12.5	0.670 ± 0.195
25	CH_3	CO_2CH_3	Ph-C≡C-	$CO_2CH_2CH_3$	CH_3	5.39 ± 0.33	38.3 ± 7.9	0.940 ± 0.070
26	CH_3	$CO_2CH_2CH_3$	CH_3	$CO_2CH_2CH_3$	$(CH_2)_3CH_3$	10.8 ± 3.52	38.0 ± 10.6	47.1 ± 10.8
27	CH_3	$CO_2CH_2CH_3$	CH_3	$CO_2CH_2CH_3$	Ph	25.9 ± 7.3	35.9 ± 15.3	7.24 ± 2.13
28 (MRS1097)	CH_3	$CO_2CH_2CH_3$	Ph-CH=CH-(*trans*)	$CO_2CH_2CH_3$	Ph	5.93 ± 0.27	4.77 ± 0.29	0.108 ± 0.012
29	CH_3	CO_2CH_3	CH_3	$CO_2CH_2CH_3$	CH_3	6.95 ± 2.66	8.96 ± 0.93	29.5 ± 2.1
30	CH_3	$CO_2CH_2CH_3$	CH_3	$CO_2CH_2CH_3$	Ph	7.41 ± 1.29	28.4 ± 9.1	4.47 ± 0.46
31	CH_3	CO_2CH_3	2-NO_2Ph	CO_2CH_3	CH_3	*d* (10^{-4})	*d* (10^{-4})	*d* (10^{-4})
32	H	H	4-CH_3OPh	H	H	44.5 ± 1.0	71 ± 29	*d* (10^{-4})

[a] Displacement of specific [^{3}H]-(*R*)-PIA binding in rat brain membranes, expressed as K_i ± SEM in μM (n = 3−5). [b] Displacement of specific [^{3}H]CGS 21680 binding in rat striatal membranes, expressed as K_i ± SEM in μM (n = 3−6). [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 = 2−3), or as a percentage of specific binding displaced at 10 μM. [d] Displacement of ≤ 10% of specific binding at the indicated concentration. nd, not determined.

nyl to larger ester groups in a homologous series (**1** vs **2**, **3**, and **4**), all having a methyl group at the 4-position, resulting in a general enhancement of affinity at all three adenosine receptor subtypes. Compound **4** is a potent PAF−acether antagonist[5] and not a calcium channel antagonist; thus there is an uncoupling of the SAR in this series for interaction with both PAF and adenosine receptors on the one hand and L-type calcium channels on the other hand.

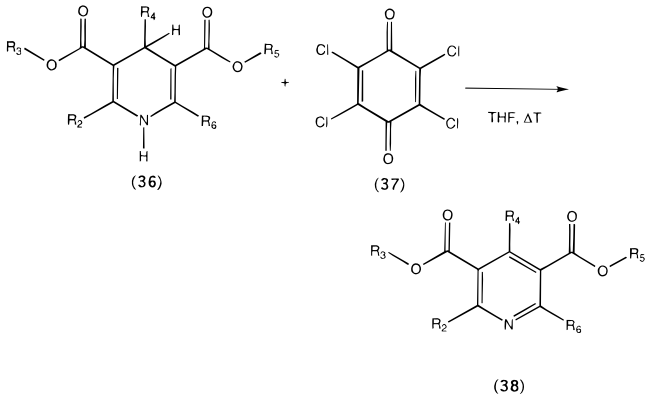
Another means of enhancing adenosine receptor affinity (relative to compound **1**) was to enlarge the R_4 substituent. In the series of ethyl, *n*-propyl, and even larger alkyl substituents at the 4-position (**5**, **6**, and **7**) it was demonstrated that steric bulk at this position is tolerated in adenosine receptor binding and favored at the A_3 subtype and not detrimental at the A_1 subtype. The affinity at the A_{2A} subtype is generally decreased with increasing chain length of the alkyl substituent. Consecutively, 4-aryl substituents, typical of the potent Ca^{2+} channel blockers in clinical use, were also examined. In general, the affinity and receptor subtype selectivity of 4-aryl analogues was highly dependent on the substituents of the phenyl ring. For example, the unsubstituted 4-phenyl compound **8** was more potent (4-fold) at A_{2A} receptors than at either A_1 or A_3 receptors. Of the nitrophenyl derivatives, compound **10**, bearing an *o*-nitro group, was 3-fold selective for A_1 vs A_3, and 6-fold for A_1 vs A_{2A} receptors, whereas the *m*-nitro derivative **10** was about equipotent at A_1 and A_3 receptors, but 3-fold less potent at A_{2A} receptors than at either A_1 or A_3 receptors. To complete the series, we also synthesized the *p*-nitro-substituted compound **16**, which displayed a >17-fold selectivity for A_3 receptors vs A_1 receptors and 6-fold selectivity for A_3 receptors vs A_{2A} receptors. In addition, we prepared compounds with different substituents. Compound **17**, bearing a *o*-trifluoromethyl group behaved similarly to the *o*-nitro-substituted compound **10** and was 2-fold selective for A_1 vs A_3 receptors. The *p*-methoxy derivative **20** was almost equipotent at A_1 (K_i = 2.75 μM), and A_3 receptors (K_i = 4.10 μM), but slightly less potent at A_{2A} receptors (K_i = 12.7 μM). Affinity at A_3 receptors did not appear

Table 2. Characterization of Dihydropyridine and Pyridine Derivatives

no.	T_m (°C)	formula	MS	analysis	yield (%)	purification
1	125−126	$C_{13}H_{19}NO_4$	253 (CI)	C,H,N	98.2	1; MeOH
2	88−89	$C_{14}H_{21}NO_5 \cdot 0.25H_2O$	283 (CI)	C,H,N	53.0	2; TLC
3	oil	$C_{18}H_{21}NO_4 \cdot 0.50H_2O$	315 (CI)	C,H,N	70.5	2; TLC
5	83−84	$C_{14}H_{21}NO_4$	267 (CI)	C,H,N	50.5	1; MeOH
6	95−96	$C_{15}H_{23}NO_4$	281 (CI)	H,N[b]	73.3	1; MeOH
7	glass	$C_{21}H_{33}NO_4$	363 (CI)	*c*	33.4	2; TLC
8	121−122	$C_{18}H_{21}NO_4$	315 (CI)	C,H,N	78.6	1; MeOH
11	165−166	$C_{19}H_{22}N_2O_6$	374 (EI)	C,H,N	35.5	1; EtOAc
16	151−152	$C_{18}H_{20}N_2O_6$	360 (CI)	C,H,N	68.1	1; MeOH
17	114−115	$C_{19}H_{20}F_3NO_4$	383 (CI)	C,H[d]	45.6	2; TLC
20	112−113	$C_{19}H_{23}NO_5 \cdot 0.21EtOAc$	345 (CI)	C,H,N	61.8	2; TLC
21	190	$C_{19}H_{23}NO_6$	361 (CI)	C,H,N	79.6	1; MeOH
22	oil	$C_{19}H_{21}NO_6 \cdot 0.32EtOAc$	359 (CI)	C,H,N	63.0	2; TLC
23	87−88	$C_{20}H_{25}NO_4$	343 (CI)	*e*	71.9	2; TLC
24	135−136	$C_{20}H_{23}NO_4$	341 (CI)	C,H,N	87.9	1; MeOH
25	176−177	$C_{20}H_{21}NO_4$	339 (EI)	C,H,N	43.3	1; EtOH
26	oil	$C_{17}H_{27}NO_4$	309 (CI)	C,H,N	58.8	2; TLC
27	123−124	$C_{19}H_{23}NO_4$	329 (CI)	C,H,N	53.0	1; pe 35−60
28	oil	$C_{26}H_{27}NO_4$	417 (CI)	C,H,N	34.7	2; column
29	oil	$C_{13}H_{17}NO_4$	251 (EI)	*f*	57.9	2; TLC
30	oil	$C_{19}H_{21}NO_4 \cdot 0.20EtOH$	327 (CI)	C,H,N	48.9	2; TLC

[a]Purification was achieved by either (1) (re)crystallization from the solvent specified or (2) chromatography by the specified method, using EtOAc/pe, 20:80 (v/v), eluent. MeOH, methanol; EtOH, ethanol; EtOAc, ethyl acetate; pe, petroleum ether 35−60 °C fraction; TLC, preparative thin layer chromatography, silica 60, 1000 μm layer thickness; column, preparative column chromatography, silica 60, 220−440 mesh. [b] **6** ($C_{15}H_{23}NO_4$) H, N; C: calcd, 64.03; found, 64.75. EI: calcd, 281.1627; found, 281.1630. [c] **7** ($C_{21}H_{33}NO_4$). This compound decomposes upon prolonged standing. Biological data and mass spectrometry data were obtained with the freshly purified compound, but extensive drying and shipping proved detrimental for the elemental analysis. C, H, N: calcd, 69.39, 9.15, 3.85; found, 61.65, 7.27, 4.70. EI: calcd, 363.2410; found, 363.2398. [d] **17** ($C_{19}H_{20}F_3NO_4$) C, H; N: calcd, 3.65; found, 4.17. EI: calcd, 383.1344; found, 383.1346. [e] **23** ($C_{20}H_{25}NO_4$) EI: calcd, 343.1784; found, 343.1791. [f] **29** ($C_{13}H_{17}NO_4$) EI: calcd, 251.1158; found, 251.1162.

Scheme 2. General Procedure for the Oxidation of 1,4-Dihydropyridine Derivatives (Compounds **29** and **30**)



to be a direct function of electron density of the phenyl substituents; *i.e.* compounds containing an electron-withdrawing *p*-nitro group (**16**) and an electron-donating *p*-methoxy group (**20**) were equipotent. Remarkably, the introduction of a hydroxy substituent on the *para* position of the 4-phenyl ring (in addition to a *m*-methoxy substituent) led to a general decrease in affinity (**21**; K_i = 51.0 μM at A_1, 56.8 μM at A_{2A}, and 32.1 μM at A_3 receptors). Thus, steric factors surrounding the phenyl ring may be more important than electronic factors. The piperonal derivative **22** had a generally increased affinity at A_1 and A_3 adenosine receptor subtypes (vs the unsubstituted phenyl compound **8**), but did not display subtype selectivity.

The relatively high affinity of a 4-aryl group larger than phenyl (**22**), an arylalkyl group (**23**), and dehydro analogues thereof (**24**, **25**) further indicated a bulk tolerance at this position. The 4-*trans*-β-styryl derivative, **24**, was particularly potent (K_i = 0.670 μM) and selective (24- and 74- fold vs rat A_1 and A_{2A}, respectively) at human A_3 receptors. The phenylacetylenic analogue, **25**, was nearly as potent at A_3 receptors, but not as selective as **24** for A_3 vs A_1 receptors (only 6-fold).

Substitution at the 3- and 5-positions of the dihydropyridine moiety also had a large effect on affinity at adenosine receptors. Changing an asymmetrical mixed methyl and ethyl ester of nitrendipine, **10**, to the corresponding symmetrical diethyl ester, **11**, resulted in a 3.3-fold enhancement of A_3 affinity. At A_1 and A_{2A} receptors a similar effect was observed, and subtype selectivity was therefore not affected. Thus, compound **11** displayed K_i values of 3.34 and 18.2 μM at rat A_1 and A_{2A} receptors, respectively, and 2.51 μM at human A_3 receptors. Nicardipine,[33] **12**, differing from nitrendipine,[34] **10**, in the presence of a sterically bulky ester group at the 5-position, displayed a moderate enhancement of affinity at human A_3 receptors of 2.6-fold, while at rat A_1 and A_{2A} receptors affinity was diminished 2−3 fold. Even larger ester substituents (*i.e.*, **14**, and **15**) were well tolerated at human A_3 receptors, while affinity at A_1 and A_{2A} receptors was drastically diminished.

Affinities of enantiomeric pairs, *i.e.*, of Bay K 8644 (**18** and **19**, *R* and *S*, a Ca^{2+} channel blocker and activator, respectively) and the Ca^{2+} channel blocker niguldipine (**14** and **15**, *R* and *S*, respectively), indicated a general preference for the *R*- over the *S*-enantiomer at all of the receptor subtypes. This is in contrast to the affinity at L-type calcium channels [(*R*)-(−)-niguldipine, K_i = 8.1 nM; (*S*)-(+)-niguldipine, K_i = 0.18 nM; or 45-fold stereoselectivity][35] and at α_{1a} adrenoceptors [(*R*)-(−)-niguldipine, K_i = 4.7 nM; (*S*)-(+)-niguldipine, K_i = 0.16 nM; or 29-fold stereoselectivity],[4] at which the (*S*)-(+)-enantiomer is preferred. Both niguldipine enantiomers tended particularly towards selectivity for the A_3 subtype (vs A_1 and A_{2A} receptors, but not vs Ca^{2+} channels). Thus, (*R*)-niguldipine, **14**, displayed K_i values of 41.3 and >100 μM at rat A_1 and A_{2A} receptors, respectively, and 1.90 μM at human A_3 receptors.

Table 3. Inhibition of Specific Binding of [^{3}H]Isradipine Binding at L-Type Calcium Channels by Various Dihydropyridine and Pyridine Derivatives in Rat Brain Membranes and the Selectivity Ratio vs Affinity at Cloned Human A_3 Receptors

compd	K_i (rCa^{2+})[a]	ratio of K_i (rCa^{2+})/K_i (hA_3)
3	1.17 ± 0.08	0.42
14	0.0597 ± 0.0001	0.031
23	0.910 ± 0.207	0.40
24	0.694 ± 0.165	1.04
25	23 ± 3% (10^{-4})	>106
27	17 ± 5% (10^{-4})	>14
28	<10% (10^{-4})	>1000
30	<10% (10^{-5})	>2.2

[a] Expressed in μM as K_i ± SEM or percent displacement of specific binding at the indicated concentration (M) for three determinations, each performed in duplicate. Rat brain membranes (ca. 100 μg of protein/tube) were incubated for 1 h at 25 °C with 0.1 nM [^{3}H]isradipine and varying concentrations of the dihydropyridine or pyridine derivative in a 50 mM Tris buffer, pH 7.4 in a total volume of 0.5 mL. Nonspecific binding was determined in the presence of 10 μM nitrendipine. K_i values were calculated using the Cheng−Prusoff equation[43] assuming a K_d value of 0.13 nM for [^{3}H]isradipine.

At the 6-position, *n*-butyl substitution (**26**) was tolerated, and phenyl substitution (**27**) enhanced A_3 adenosine receptor binding (4.5-fold vs **1**), and lead to slight subtype selectivity. Combination of 6-phenyl and 4-*trans*-β-styryl substituents greatly enhanced both affinity and selectivity. Consequently, compound **28** was 55-fold selective for A_3 vs A_1 receptors, and 44-fold selective for A_3 vs A_{2A} receptors. Two other blockers of L-type Ca^{2+} channels, verapamil and diltiazem, which are structurally unrelated to the dihydropyridines, failed to bind appreciably to adenosine receptors.

Pyridine derivatives maintained affinity for adenosine receptors, particularly when bearing a 4-alkyl group, *e.g.*, the oxidized compound **29**, displayed a K_i value of 29.5 μM at human A_3 receptors, whereas the related dihydropyridine **1** displayed a K_i value of 32.3 μM at the same receptor subtype. Similarly, a comparison of the 6-phenyldihydropyridine **27** and the corresponding pyridine **30** shows only minor differences in affinity. In contrast, the nifedipine metabolite **31** no longer bound to adenosine receptors. The distantly related pyridine derivative **32** demonstrated that affinity of 4-arylpyridine analogues for adenosine receptors may be rescued to some extent by careful manipulation of the other substituents.

Binding at L-Type Calcium Channels. Affinity at L-type Ca^{2+} channels, measured in rat brain membranes using [^{3}H]isradipine[36] (Table 3), indicated a K_i value of 0.694 ± 0.165 μM for the styryl derivative **24** (cf. 0.670 μM at human A_3 receptors, and therefore not selective). In the same assay, (*R*)-niguldipine, **14**, had a K_i value of 59.7 ± 0.1 nM (cf. 8.1 nM at guinea pig skeletal muscle[35] and 1.90 μM at human A_3 receptors, and therefore at least 32-fold selective for Ca^{2+} channels). Compound **30** at a concentration of 10 μM (K_i = 4.47 μM at A_3 receptors) or **28** (K_i = 0.108 μM at human A_3 receptors, and therefore at least 1000-fold A_3 selective vs Ca^{2+} channels) at a concentration of 100 μM displaced <10% of specific binding. Slightly more potent in Ca^{2+} channel binding were **27** (17% displacement at 100 μM; K_i = 7.24 μM at human A_3 receptors, and thus >14-fold A_3 selective) and **25** (23% displacement at 100 μM; K_i = 0.94 μM at human A_3 receptors, and thus >100-fold A_3 selective). The 5-benzyl ester **3** (K_i = 1.17 ± 0.08 μM at Ca^{2+} channels and 2.78 ± 0.89 μM at A_3 receptors) and the 4-(2-phenylethyl) analogue **23** (K_i = 0.910 ± 0.207 μM at Ca^{2+} channels and 2.30 ± 0.70 μM at A_3 receptors) were about 2.5-fold selective for Ca^{2+} channels compared to human A_3 receptors.

Table 4. Effects of Dihydropyridine Derivatives at a Concentration of 10 μM on the Inhibition of Adenylyl Cyclase Elicited by the A_3 Agonist IB-MECA

	% inhibition[a]			
compd	IB-MECA (10^{-7} M)	*n*	IB-MECA (10^{-6} M)	*n*
control	31.4 ± 4.0	5	40.2 ± 3.3	4
12	7.94 ± 3.15[b]	5	27.2 ± 8.5	4
18	19.4 ± 0.6[b]	2	27.8 ± 7.8	2

[a] Assayed in membranes from CHO cells stably expressing the cloned rat A_3 receptor using a previously reported method in the presence of 1 μM forskolin.[15,28] [b] Statistically significant (p < 0.02) vs the control value.

Functional Assays at A_3-Adenosine Receptors. We examined the antagonist properties of two dihydropyridine derivatives in a functional assay utilizing cloned rat A_3 adenosine receptors expressed in CHO cells. Forskolin-stimulated adenylyl cyclase was inhibited by IB-MECA [N^6-(3-iodobenzyl)-5′-(*N*-methylcarbamoyl)adenosine; 10^{-9}−10^{-4} M) in CHO cells transfected with rat A_3 adenosine receptors (maximum degree of inhibition was 40−50%).[22] The concentration of IB-MECA used to test prospective antagonists was between 10 nM and 1.0 μM, *i.e.*, respectively 0.1- and 10-fold the IC_{50} of rat A_3 receptor mediated inhibition of adenylyl cyclase. Both nicardipine, **12**, and (*R*)-Bay K 8644, **18**, at a concentration of 10 μM showed a significant (p < 0.02) effect on the agonist-mediated inhibition of adenylyl cyclase (Table 4). The inhibition of adenylyl cyclase induced by 10^{-6} M IB-MECA (40.2 ± 3.3%) was attenuated to 27.2 ± 8.5% by 10 μM nicardipine, **12**, and the inhibition of adenylyl cyclase activity caused by 10^{-7} M IB-MECA was nearly completely reversed. A similar, but not as extensive, effect could be observed for (*R*)-Bay K 8644, **18**, even though the affinity of these compounds at human A_3 adenosine receptors was virtually identical. Compound **28** at a concentration of 50 μM attenuated the IB-MECA induced inhibition of adenylyl cyclase activity at all concentrations tested (Figure 2). This suggests that compound **28** is not only an effective displacer of the A_3-selective radioligand [^{125}I]AB-MECA but also an effective functional antagonist at this receptor subtype.

Interaction of 1,4-Dihydropyridines with the NBI-Sensitive Nucleoside Transporter Protein. It was shown previously that the nucleoside uptake inhibitor NBI (*S*-(4-nitrobenzyl)-6-thioinosine) binds to 1,4-dihydropyridine ([^{3}H]nimodipine) binding sites on human red blood cell membranes.[37] We therefore investigated the possibility that 1,4-dihydropyridines may bind to the NBI-sensitive nucleoside transporter protein. Both the commercial Ca^{2+} channel blocker nicardipine, **12**, and the A_3 adenosine receptor selective ligand **24** displaced [^{3}H]NBI from its binding site in rat forebrain membranes, with K_i values of 5.36 ± 1.51 and 1.20 ± 0.11 μM, respectively. In contrast, the A_3-selective antagonist **28** displaced less than 10% of specific binding at a concentration of 100 μM. Compound **28** is therefore >900-fold selective for A_3 receptors compared to the NBI-sensitive nucleoside transporter.

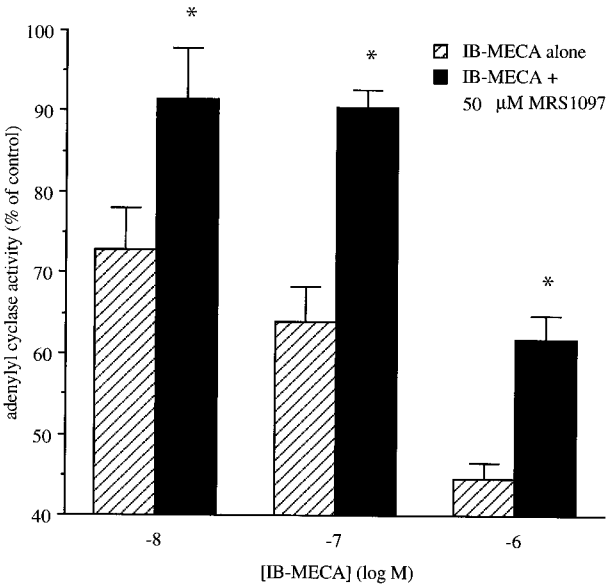


Figure 2. Attenuation of A_3 agonist elicited inhibition of forskolin stimulated adenylyl cyclase activity via cloned rat A_3 adenosine receptors. Adenylyl cyclase activity was determined in membranes from CHO cells stably expressing the rat A_3 receptor, as reported previously.[28] Adenylyl cyclase activity was stimulated with 1 μM forskolin, and this stimulation was inhibited by the A_3 selective agonist IB-MECA (shaded bars). Addition of 50 μM MRS1097, **28**, attenuated the IB-MECA induced inhibition of adenylyl cyclase activity (closed bars). The asterisk (*) denotes a statistically significant effect ($p < 0.02$) vs the control experiment.

Affinity at Other Binding Sites. Since compound **28** was the most potent and (A_3 adenosine receptor) selective compound of the series, its affinity at non-adenosine receptor binding sites was examined in a battery of radioligand binding assays (NovaScreen, Division of Oceanix Biosciences, Hanover, MD).[38] At a concentration of 10^{-5} M, there was no significant (0 ± 30%) displacement of radioligand from adrenergic (α_1, α_2, β), cholinergic (nicotinic and muscarinic M_1, M_2, M_3), dopaminergic (D_1 and D_2), histaminergic (H_1 and H_2), serotoninergic (5-HT_1, 5-HT_2, and 5-HT_3), C5a complement, central benzodiazepine (RO 151788), $GABA_A$ (muscimol), $GABA_B$ (baclofen), NMDA (kainate, quisqualate, and phencyclidine), glycine (strychnine sensitive and insensitive), σ (MK-801), angiotensin (AT-II), vasopressin V_1, neuropeptide Y, cholecystokinin (CCK-B), neurotensin, somatostatin, ANF1, and EGF receptors. There was also no significant displacement of binding of radioligand from second messenger sites (forskolin, phorbol ester, and inositol trisphosphate) and ion channels (N-type calcium channels, chloride channels, and low conductance potassium channels). Significant displacement of radioligand was observed for the CCK-A (40%) and VIP (45%) binding sites, but the (estimated) IC_{50} for these compounds was still higher than 10 μM.

The Effect of Species Differences on Ligand Selectivity. Ordinarily, a comparison of A_1, A_{2A}, and A_3 affinities within one species would be preferred. We chose the human A_3 receptor for our study instead of the rat A_3 receptor for the following reasons: Whereas the affinity of many ligands varies slightly between rat and human A_1 and A_{2A} adenosine receptors, the affinity of most known adenosine receptor antagonists is only marginal at rat A_3 receptors.[11,12,21,25] The human A_3 receptor, being more susceptible to displacement of a selective radioligand by competing compounds, therefore allowed for a better comparison between those compounds.[25] The comparison was justified by the similarity of affinity of dihydropyridines at rat and human non-A_3 adenosine receptors. For example, the calcium channel blocker nifedipine (**9**) displayed a K_i value of 5.6 ± 0.3 μM at human A_{2A} adenosine receptors (unpublished data) and a K_i value of 18 ± 3 μM at rat A_{2A} receptors (this manuscript), but the affinities of 1,4-dihydropyridines at rat A_3 receptors differed considerably from their affinity at human A_3 receptors (Table 5). Least affected by the species difference was (*R*)-Bay K 8644 (**18**) with K_i values of 2.77 ± 0.34 and 6.20 ± 2.12 μM at human and rat A_3 receptors, respectively. Thus, the species selectivity ratio for (*R*)-Bay K 8644 (**18**) is only 2.2-fold, but this value increases to 38.6-fold for our most potent and selective compound (**28**), which displayed a K_i value of 4.16 ± 1.55 μM at rat A_3 receptors. Consequently, the receptor subtype selectivity of the test compounds for A_3 vs either A_1 or A_{2A} adenosine receptors was disproportionately affected (Table 5).

Table 5. Affinity of and Selectivity Ratio for Selected Dihydropyridine Derivatives at Rat A_3 vs Human A_3, Rat A_1, and Rat A_{2A} Adenosine Receptors, Respectively

		selectivity ratio		
compd	K_i (μM)[a]	rA_3/hA_3	rA_1/rA_3	rA_{2A}/rA_3
12	11.0 ± 7.3	3.38	1.78	5.80
18	6.20 ± 2.12	2.24	0.13	5.66
24	12.1 ± 2.6	18.1	1.33	4.07
28	4.16 ± 1.55	38.6	1.43	1.15

[a] Assayed in membranes from CHO cells stably expressing the cloned rat A_3 receptor using [^{125}I]-N^6-(4-amino-3-iodobenzyl)-5′-(*N*-methylcarbamoyl)adenosine, as described previously.[13,28]

Discussion

In the present study, we have demonstrated that structural modification and careful examination of the SAR of 1,4-dihydropyridines resulted in the development of one of the first A_3 adenosine receptor-selective non-xanthine ligands. Compound **28** [3,5-diethyl 2-methyl-6-phenyl-4-(*trans*-2-phenylvinyl)-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate; MRS1097] displayed a good (submicromolar) affinity in a radioligand binding assay (K_i = 0.108 ± 0.012 μM). In addition, we have shown that dihydropyridines can be effective in attenuating the IB-MECA elicited inhibition of adenylyl cyclase in CHO cells expressing the cloned rat A_3 adenosine receptor. Compound **28** was 55-fold selective vs A_1 receptors, 44-fold selective vs A_{2A} receptors, and over 1000-fold selective vs L-type Ca^{2+} channels.

It has been suggested that 1,4-dihydropyridines may bind to the nucleoside transporter protein of human red blood cells and human brain.[37,43] We therefore measured the ability of some 1,4-dihydropyridines to displace [^{3}H](nitrobenzyl)thioinosine ([^{3}H]NBI) from the Na^+-independent adenosine transporter in rat forebrain. Whereas the commercial compound nicardipine (**12**) and the novel compound MRS1045 (**24**) displaced [^{3}H]NBI with affinities similar to their affinity at human A_3 receptors (5.36 ± 1.51 and 1.20 ± 0.11 μM, respectively), the most selective compound of this series (MRS1097, **28**) displaced less than 10% of total [^{3}H]NBI binding at a concentration of 10^{-4} M.

In this study we have shown that the affinity of dihydropyridines can be optimized selectively for adenosine receptors vs L-type calcium channels and for A_3 vs A_1 and A_{2A} receptors. Hu *et al*. previously found no effect of nifedipine binding on A_1 receptor mediated adenylyl cyclase inhibition,[7] but current work with the more recently cloned A_3 receptor[22] demonstrates that IB-MECA-induced inhibition of adenylyl cyclase was attenuated by several compounds of the 1,4-dihydropyridine class (Table 4). Preliminary tests show that both nicardipine, **12**, and (*R*)-Bay K 8644, **18** (both Ca^{2+} channel blockers), functionally antagonize the inhibitory effects of an A_3 selective agonist on adenylyl cyclase. Furthermore, we have shown that MRS1097, **28**, significantly ($p < 0.02$) attenuates the effects of IB-MECA on adenylyl cyclase activity (Figure 2). Further pharmacological characterization will be required to determine if this is due to a competitive interaction with adenosine A_3 receptors or at some other stage of the functional cascade. Thus, we have discovered dihydropyridines as a promising lead in the search for A_3 adenosine receptor antagonists.

L-type calcium channel blockers such as nimodipine, **13**, have been examined as cerebroprotective agents in experimental models of neurotoxicity in cell culture and *in vivo*.[39] Adenosine agonists and antagonists have also been evaluated in models of brain ischemia. It has also been postulated that an adenosine A_3 receptor antagonist would have cerebroprotective properties, based on the *in vivo* effects of the A_3 receptor agonist IB-MECA administered to gerbils in a chronic regimen.[16] Thus, a compound that would display a dual action *in vivo* might be even more useful in treating stroke than either an L-type calcium channel blocker or A_3 receptor antagonist alone. Therefore, some of the compounds found in the present study to have affinity at both sites may be clinically useful as combined therapeutic agents.

Experimental Section

Materials. (*R*)-(+)- and (*S*)-(−)-Bay K 8644, (*R*)-(−)- and (*S*)-(+)-niguldipine, nicardipine, nifedipine, nimodipine, and oxidized nifedipine were purchased from Research Biochemicals International (Natick, MA). Ethyl acetoacetate (**35a**), acetaldehyde (**34a**), propionaldehyde (**34b**), butyraldehyde (**34c**), benzaldehyde (**34d**), anisaldehyde (**34e**), vanillin (**34f**), methyl 3-aminocrotonate (**33a**), and *trans*-cinnamaldehyde (**34g**) were obtained from Fluka (Ronkonoma, NY). *o*-Nitrobenzaldehyde (**34h**), *m*-nitrobenzaldehyde (**34i**), *p*-nitrobenzaldehyde (**34j**), ethyl 3-aminocrotonate (**33b**), phenylpropargylaldehyde (**34k**), α,α,α-trifloro-*o*-tolualdehyde (**34l**), benzyl acetoacetate (**35b**), 2-methoxyethyl acetoacetate (**35c**), piperonal (**34m**), tetrachloro-1,4-benzoquinone (**37**), valeryl chloride, and ethyl benzoylacetate (**35d**) were from Aldrich (St. Louis, MO). Hydrocinnamaldehyde (**34n**) was from Eastman (Rochester, NY), and (±)-citronellal (**34o**) was from ICN (Plainview, NY). Compound **4** was from Tocris-Cookson (St. Louis, MO), and (*S*)-(4-nitrobenzyl)-6-thioguanosine was from Sigma (St. Louis, MO). [^{3}H]PN200,110 (isradipine) was from DuPont NEN (Boston, MA). Compound **32** was prepared according to the literature.[40] All other materials were obtained from commercial sources.

Synthesis. Proton nuclear magnetic resonance spectroscopy was performed on a Varian GEMINI-300 spectrometer, and spectra were taken in DMSO-d_6 or $CHCl_3$-*d*. Chemical-ionization (CI) mass spectrometry was performed with a Finnigan 4600 mass spectrometer, and electron-impact (EI) mass spectrometry with a VG7070F mass spectrometer at 6 kV. Elemental analysis was performed by Atlantic Microlab Inc. (Norcross, GA). All melting points were determined with a Unimelt capillary melting point apparatus (Arthur H. Thomas Co., Philadelphia, PA) and were uncorrected.

General Procedure for Preparation of 1,4-Dihydropyridine-3,5-dicarboxylate Esters (36; 1−3, 5−8, 11, 16, 17, 20−28). Equimolar amounts (0.5 mmol) of the appropriate 3-amino-2-butenoate ester (**33a**,**b**), aldehyde (**34a**−**o**), and 3-ketopropionate ester (**35a**−**e**) derivative were dissolved in 5 mL of absolute ethanol. The solution was sealed in a glass tube and heated to 100 °C for at least 24 h, and at most 72 h. The solvent was then evaporated, and products were purified either by crystallization, column chromatography (silica 60; 220−440 mesh; Fluka, CH; 20% ethyl acetate−80% petroleum ether 35−60) or preparative TLC (silica 60; 1000 μm; Analtech, DE; 20% ethyl acetate−80% petroleum ether 35−60). From the moment the reactants were sealed into the glass tube, all procedures were performed under nitrogen and low-light conditions to prevent oxidation of the products. The products were shown to be homogeneous by TLC.

3-Methyl 5-ethyl 2,4,6-trimethyl-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (1): ^{1}H NMR ($CHCl_3$-*d*) δ 0.98 (d, 3H, 4-CH_3, J = 6.7 Hz), 1.31 (t, 3H, 5-methyl, J = 7.2 Hz), 2.28 (s, 6H, 2- and 6-CH_3), 3.73 (s, 3H, 3-methyl), 3.83 (q, 1H, H-4, J = 6.4 Hz); 4.13−4.26 (m, 2H, 5-methylene), 5.51 (wide, 1H, H-1).

3-Methyl 5-(2-methoxyethyl) 2,4,6-trimethyl-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (2): ^{1}H NMR ($CHCl_3$-*d*) δ 0.99 (d, 3H, 4-CH_3, J = 6.7 Hz), 2.28 (s, 6H, 2- and 6-CH_3), 3.41 (s, 3H, 5-methoxy), 3.66 (t, 2H, 5-(2-methylene), J = 4.9 Hz), 3.73 (s, 3H, 3-methyl), 3.85 (q, 1H, H-4, J = 6.5 Hz), 4.22−4.38 (m, 2H, 5-(1-methylene)), 5.54 (wide, 1H, H-1).

3-Methyl 5-benzyl 2,4,6-trimethyl-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (3): ^{1}H NMR ($CHCl_3$-*d*) δ 0.98 (d, 3H, 4-CH_3, J = 5.0 Hz), 2.28 (s, 6H, 2- and 6-CH_3), 3.72 (s, 3H, 3-methyl), 3.89 (q, 1H, H-4, J = 6.5 Hz), 5.21 (q, 2H, 5-methylene, J = 14.8 Hz), 5.54 (wide, 1H, H-1), 7.30−7.41 (m, 5H, 5-phenyl).

3-Methyl 5-ethyl 2,6-dimethyl-4-ethyl-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (5): ^{1}H NMR (DMSO-d_6) δ 0.63 (t, 3H, 4-methyl, J = 7.4 Hz), 1.18 (t, 3H, 5-methyl, J = 7.1 Hz), 2.19 (s, 6H, 2- and 6-CH_3), 3.58 (s, 3H, 3-methyl), 3.73 (t, 1H, H-4, J = 5.4 Hz), 4.00−4.13 (m, 4H, 4- and 5-methylene).

3-Methyl 5-ethyl 2,6-dimethyl-4-propyl-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (6): ^{1}H NMR (DMSO-d_6) δ 0.77 (t, 3H, 4-methyl, J = 6.7 Hz), 1.12 (wide, 4H, 4a- and 4b-methylene), 1.19 (t, 3H, 5-methyl, J = 7.2 Hz), 2.19 (s, 6H, 2- and 6-CH_3), 3.59 (s, 3H, 3-methyl), 3.76 (t, 1H, H-4, J = 5.2 Hz), 4.07 (q, 2H, 5-methylene, J = 6.6 Hz).

3-Methyl 5-ethyl 2,6-dimethyl-4-(2(*R,S*),6-dimethylhexen-5-yl)-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (7): ^{1}H NMR ($CHCl_3$-*d*) δ 0.91 (double d, 3H, 4-(2-CH_3), J_1 = 5.8 Hz, J_2 = 24.0 Hz), 1.30 (m, 7H), 1.63 (d, 6H, 4-(6- and 7-CH_3), J = 25.4 Hz), 2.28 (d, 6H, 2- and 6- CH_3, J = 7.8 Hz), 3.71 (s, 3H, 3-methyl), 3.97 (t, 1H, H-4, J = 7.2 Hz), 4.11−4.24 (m, 2H, 5-methylene), 5.07 (t, 1H, 4-(2-methyne), J = 6.5 Hz), 5.71 (wide, 1H, H-1).

3-Methyl 5-ethyl 2,6-dimethyl-4-phenyl-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (8): ^{1}H NMR (DMSO-d_6) δ 1.11 (t, 3H, 5-methyl, J = 6.4 Hz), 2.23 (s, 6H, 2- and 6-CH_3), 3.51 (s, 3H, 3-methyl), 3.97 (m, 2H, 5-methylene), 4.84 (s, 1H, H-4), 7.08−7.20 (m, 5H, 4-phenyl).

3,5-Diethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (11): ^{1}H NMR ($CHCl_3$-*d*) δ 1.23 (t, 6H, 3- and 5-methyl, J = 6.9 Hz), 2.38 (s, 6H, 2- and 6-CH_3), 4.02−4.18 (m, 4H, 3- and 5-methylene), 5.10 (s, 1H, H-4), 5.68 (wide, 1H, H-1), 7.38 (t, 1H, H-5′, J = 8.0 Hz), 7.65 (d, 1H, H-6′, J = 7.8 Hz), 8.02 (d, 1H, H-4′, J = 7.4 Hz), 8.14 (s, 1H, H-2′).

3-Methyl 5-ethyl 2,6-dimethyl-4-(4-nitrophenyl)-1,4-(*R,S*)-dihydropyridine-3,5-dicarboxylate (16): ^{1}H NMR (DMSO-d_6) δ 1.11 (t, 3H, 5-methyl, J = 7.2 Hz), 2.26 (s, 6H, 2- and 6-CH_3), 3.52 (s, 3H, 3-methyl), 4.00 (m, 2H, 5-methylene), 4.96 (s, 1H, H-1), 7.39 (d, 2H, H-2′ and H-6′, J = 8.7 Hz), 8.09 (d, 2H, H-3′ and H-5′, J = 8.7 Hz).

3-Methyl 5-ethyl 2,6-dimethyl-4-(2-α,α,α-trifluoromethylphenyl)-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate

(17): ^{1}H NMR (DMSO-d_6) δ 1.05 (m, 3H, 5-methyl), 2.21 (s, 6H, 2- and 6-CH_3), 3.44 (s, 3H, 3-methyl), 3.84−4.10 (m, 2H, 5-methylene), 5.38 (s, 1H, H-4), 7.31 (t, 1H, J = 7.3 Hz), 7.51 (m, 3H, 4-phenyl).

3-Methyl 5-ethyl 2,6-dimethyl-4-(4-methoxyphenyl)-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (20): ^{1}H NMR ($CHCl_3$-*d*) δ 1.23 (t, 3H, 5-methyl, J = 7.8 Hz), 2.34 (s, 6H, 2- and 6-CH_3), 3.65 (s, 3H, 4′-OCH_3), 3.76 (s, 3H, 3-methyl), 4.07−4.14 (m, 2H, 5-methylene), 4.94 (s, 1H, H-4), 5.57 (wide, 1H, H-1), 6.76 (d, 2H, H-2′ and H-6′, J = 8.5 Hz), 7.20 (d, 2H, H-3′ and H-5′, J = 8.6 Hz).

3-Methyl 5-ethyl 2,6-dimethyl-4-(4-hydroxy-3-methoxyphenyl)-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (21): ^{1}H NMR (DMSO-d_6) δ 1.13 (t, 3H, 5-methyl, J = 7.4 Hz), 2.21 (s, 6H, 2- and 6-CH_3), 3.52 (s, 3H, 3′-OCH_3), 3.66 (s, 3H, 3-methyl), 3.96−4.01 (m, 2H, 5-methylene), 4.74 (s, 1H, H-4), 6.49 (t, 1H, H-6′, J = 3.9 Hz), 6.58 (d, 1H, H-5′, J = 7.9 Hz), 6.66 (s, 1H, H-2′).

3-Methyl 5-ethyl 2,6-dimethyl-4-[3,4-(methylenedioxy)phenyl]-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (22): ^{1}H NMR ($CHCl_3$-*d*) δ 1.24 (t, 3H, 5-methyl, J = 7.3 Hz), 2.33 (s, 6H, 2- and 6-CH_3), 3.66 (s, 3H, 3-methyl), 3.73 (s, 1H, H-4), 4.09−4.22 (m, 2H, 5-methylene), 4.92 (s, 2H, 3′,4′-methylenedioxy), 5.57 (wide, 1H, H-1), 5.89 (s, 1H, H-2′), 6.66 (d, 1H, H-5′, J = 8.1 Hz), 6.75 (d, 1H, H-6′, J = 7.9 Hz).

3-Methyl 5-ethyl 2,6-dimethyl-4-(2-phenyl)ethyl-1,4-(*R,S*)-dihydropyridine-3,5-dicarboxylate (23): ^{1}H NMR ($CHCl_3$-*d*) δ 1.25−1.55 (m, 3H, 5-methyl), 1.63−1.71 (m, 2H, 4-(α-methylene)), 2.31 (s, 6H, 2- and 6-CH_3), 2.51−2.57 (m, 2H, 4-(β-methylene)), 3.73 (s, 3H, 3-methyl), 4.05 (t, 1H, H-4, J = 5.6 Hz), 4.12−4.27 (m, 2H, 5-methylene), 5.58 (wide, 1H, H-1), 7.15 (d, 2H, H-2′ and H-6′, J = 6.4 Hz), 7.24 (t, 3H, H-3′, and H-4′ and H-5′, J = 6.8 Hz).

3-Methyl 5-ethyl 2,6-dimethyl-4-(*trans*-2-phenylvinyl)-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (24): ^{1}H NMR ($CHCl_3$-*d*) δ 1.31 (t, 3H, 5-methyl, J = 7.1 Hz), 2.34 (s, 6H, 2- and 6-CH_3), 3.74 (s, 3H, 3-methyl), 4.14−4.28 (m, 2H, 5-methylene), 4.63 (d, 1H, H-4, J = 5.4 Hz), 5.60 (wide, 1H, H-1), 6.19 (t, 1H, 4-(H-1 vinylidene), J = 6.0 Hz), 7.18 (d, 1H, 4-(H-2 vinylidene), J = 6.6 Hz), 7.24−7.34 (m, 5H, 4-phenyl).

3-Methyl 5-ethyl 2,6-dimethyl-4-(phenylethynyl)-1,4-(*R,S*)-dihydropyridine-3,5-dicarboxylate (25): ^{1}H NMR ($CHCl_3$-*d*) δ 1.35 (t, 3H, 5-methyl, J = 7.1 Hz), 2.36 (s, 6H, 2- and 6-CH_3), 3.80 (s, 3H, 3-methyl), 4.23−4.31 (m, 2H, 5-methylene), 4.99 (s, 1H, H-4), 5.71 (wide, 1H, H-1), 7.24 (t, 3H, H-3′, and H-4′, and H-5′, J = 3.2 Hz), 7.36 (d, 2H, H-2′ and H-6′, J = 3.6 Hz).

3,5-Diethyl 2,4-dimethyl-6-butyl-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (26): ^{1}H NMR ($CHCl_3$-*d*) δ 0.92−0.98 (m, overlap, 6H, 6-(4-CH_3) and 4-CH_3, J = 7.0 Hz), 1.27 (t, 6H, 3- and 5-methyl, J = 7.0 Hz), 1.51 (m, 4H, 6-(2- and 3-CH_2)), 2.30 (s, 3H, 2-CH_3), 2.52−2.76 (m, 2H, 6-(1-CH_2)), 3.85 (q, 1H, H-4, J = 7.0 Hz), 4.18 (m, 4H, 3- and 5-methylene), 5.53 (wide, 1H, H-1).

3,5-Diethyl 2,4-dimethyl-6-phenyl-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (27): ^{1}H NMR ($CHCl_3$-*d*) δ 0.89 (t, 3H, 3-methyl, J = 7.4 Hz), 1.14 (d, 3H, 5-methyl, J = 7.4 Hz), 1.31 (t, 3H, J = 7.0 Hz), 2.3 (s, 3H, CH_3), 3.94 (m, 3H), 4.23 (m, 2H), 7.28−7.41 (m, 5H, C_6H_5).

3,5-Diethyl 2-methyl-4-(*trans*-2-phenylvinyl)-6-phenyl-1,4(*R,S*)-dihydropyridine-3,5-dicarboxylate (28): ^{1}H NMR ($CHCl_3$-*d*) δ 0.92 (t, 3H, 5-methyl, J = 7.0 Hz), 1.32 (t, 3H, 3-methyl, J = 7.0 Hz), 2.37 (s, 3H, 2-CH_3), 3.94 (q, 3H, 5-methylene, J = 6.7 Hz), 4.22, (q, 3H, 3-methylene, J = 6.7 Hz), 4.76 (d, 1H, H-4, J = 6.2 Hz), 5.78 (wide, 1H, H-1), 6.30−6.38 (m, 2H, CH=CH), 7.20−7.44 (m, 10H, 2 x C_6H_5).

General Procedure for Oxidation of 1,4-Dihydropyridine-3,5-dicarboxylate Esters (38; 29, 30). Equimolar amounts (0.25 mmol) of the 1,4-dihydropyridine-3,5-dicarboxylate ester (**36**; **1**, **27**) and tetrachloro-1,4-benzoquinone (**37**) in tetrahydrofuran (2 mL) were mixed and refluxed for up to 4 h. The solvent was then evaporated, and products were purified by preparative TLC (silica 60; 1000 μm; Analtech, DE; 20% ethyl acetate−80% petroleum ether 35−60).

3-Methyl 5-ethyl 2,4,6-trimethylpyridine-3,5-dicarboxylate (29): ^{1}H NMR (DMSO-d_6) δ 1.27 (t, 3H, 5-methyl, J 6.9 Hz), 2.16 (s, 3H, 4-CH_3), 2.39 (s, 6H, 2- and 6-CH_3), 3.87 (s, 3H, 3-methyl), 4.36 (q, 2H, 5-methylene, J = 7.4 Hz).

3,5-Diethyl 2,4-dimethyl-6-phenyl-pyridine-3,5-dicarboxylate (30): ^{1}H NMR ($CHCl_3$-*d*) δ 1.00 (t, 3H, 3-methyl, J = 7.4 Hz), 1.43 (t, 3H, 5-methyl, J = 7.5 Hz), 2.37 (s, 3H,4-CH_3), 2.62 (s, 3H, 2-CH_3), 4.10 (q, 2H, 3-methylene, J = 7.4 Hz), 4.47 (q, 2H, 5-methylene, J = 7.4 Hz), 7.40−7.58 (m, 5H, C_6H_5).

Ethyl Valerylacetate (35e). A solution of *n*-butyllithium (6.3 mL, 1.6 M in hexane) was slowly added to a solution of *N*-isopropylcyclohexylamine (1.41 g, 10 mmol) in dry THF (20 mL) at −5 °C. The mixture was stirred for 15 min and then cooled to −78 °C. Ethyl acetate (440 mg, 5 mmol) was added dropwise over a period of 5 min, followed by valeryl chloride (600 mg, 5 mmol). The reaction mixture was allowed to stir an additional 10 min, at which point it was quenched with 5 mL of 20% HCl in water. The organic layer was separated off, and the aqueous layer was extracted with 10 mL of diethyl ether twice. The combined organic phases were washed with a saturated sodium bicarbonate solution (10 mL × 2) and brine (5 mL × 2) and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue purified by column chromatography (silica gel 60, eluted with ethyl acetate/petroleum ether, 1:9) to yield ethyl valerylacetate (772 mg, 90%): ^{1}H NMR ($CHCl_3$-*d*) δ 0.90 (t, 3H, CH_3, J = 7.0 Hz), 1.20−1.65 (m, 7H, CH_3 and 2 × CH_2), 2.30 (t, 2H, $COCH_2$, J = 7.0 Hz), 2.65 (s, 2H, $COCH_2CO$), 4.15 (q, 2H, $COOCH_2$, J = 7.0 Hz); MS (EI) 85 $[CH_3(CH_2)_3CO]^+$, 172 (base), 57 $[CH_3(CH_2)_3]^+$. The ethyl valerylacetate (**35e**) was then used in the procedure described above for the preparation of compound **26**.

Pharmacology: Radioligand Binding Studies. Binding of [^{3}H]-(*R*)-N^6-(phenylisopropyl)adenosine ([^{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*-(ethylcarbamoyl)-adenosine ([^{3}H]CGS 21680) to A_{2A} receptors from rat striatal membranes was performed as described previously.[30,31] 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.

Binding of [^{125}I]-N^6-(4-amino-3-iodobenzyl)-5′-(*N*-methylcarbamoyl)adenosine ([^{125}I]AB-MECA) to membranes prepared from HEK-293 cells stably expressing the human A_3 receptor (Receptor Biology, Inc., Baltimore, MD) or to membranes prepared from CHO cells stably expressing the rat A_3 receptor was performed as described.[13,32] The assay medium consisted of buffer containing 50 mM Tris, 10 mM $MgCl_2$, and 1 mM EDTA at pH 8.0. The glass incubation tubes contained 100 μL of the membrane suspension (0.3 mg of 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-(phenylisopropyl)adenosine (*R*-PIA).

Binding of [^{3}H]isradipine to rat cerebral cortex membranes was performed essentially as described in Moody et al.[36] Briefly, an aliquot of a membrane suspension corresponding to 100 μg of protein was incubated in a total volume of 0.5 mL of Tris buffer (50 mM, pH 7.4, 25 °C) for 1 h in the presence of 0.1 nM [^{3}H]isradipine and varying concentrations of the dihydropyridine or pyridine derivatives. Nonspecific binding was determined in the presence of 10 μM nitrendipine.

All nonradioactive compounds were initially dissolved in DMSO and diluted with buffer to the final concentration, where the amount of DMSO never exceeded 2%.

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[41] and K_d values of 1.0, 14, and 0.13 nM for [^{3}H]-(*R*)-PIA, [^{3}H]CGS 21680, and

[^{3}H]isradipine, respectively, and 0.59 nM for binding of [^{125}I]-AB-MECA at human A_3 receptors, respectively.

Inhibition of Adenylyl Cyclase Activity. Adenylyl cyclase activity was determined in membranes from CHO cells stably expressing the rat A_3 receptor, prepared as reported previously.[32] [α-^{32}P]ATP was added to a membrane suspension containing 1 μM forskolin to stimulate adenylyl cyclase. The reaction was terminated by addition of a stop buffer containing 20 000 cpm/mL [^{3}H]cyclic AMP. Cyclic AMP was isolated by consecutive chromatography over columns of Dowex 50 ion exchange resin and alumina. Maximal stimulation of adenylyl cyclase activity was obtained in the presence of 1 μM forskolin (circa 6−8-fold), and maximal inhibition of forskolin-stimulated adenylyl cyclase activity (40−50% residual activity from the stimulated level) was obtained in the presence of 10 μM IB-MECA. Statistical significance was calculated in a *t*-test, with 4 degrees of freedom at a significance level of 98%.

Interaction of 1,4-Dihydropyridines with the NBI-Sensitive Nucleoside Transporter Protein. Competition of 1,4-dihydropyridines for binding of [^{3}H]NBI to the NBI-sensitive nucleoside transporter protein was carried out by the slightly modified procedure of Marangos et al.[42] Rat forebrain membranes were incubated for 30 min at 23 °C with 0.7 nM [^{3}H]NBI and varying concentrations of competitor in Tris buffer (50 mM, pH 7.4) in a total of 500 μL. Then 5 μM (*S*)-(4-nitrobenzyl)-6-thioguanosine was added to determine non-specific binding. Nonspecific binding did not exceed 15% of total binding. A K_d value of 0.15 nM was used for the calculation of the K_i values.[42]

Abbreviations: [^{125}I]AB-MECA, [^{125}I]-N^6-(4-amino-3-iodobenzyl)-5′-(*N*-methylcarbamoyl)adenosine; Bay K 8644, 3-methyl 2,6-dimethyl-5-nitro-4-(2-(α,α,α-trifluoromethyl)phenyl)-1,4-dihydropyridine-3-carboxylate; CGS 21680, 2-[[4-(2-carboxyethyl)phenyl]ethylamino]-5′-(*N*-ethylcarbamoyl)-adenosine; CHA, N^6-cyclohexyladenosine; CHO cells, Chinese hamster ovary cells; DMSO, dimethyl sulfoxide; HEK cells, human embryonic kidney cells; IB-MECA, N^6-(3-iodobenzyl)-5′-(*N*-methylcarbamoyl)adenosine; K_i, equilibrium inhibition constant; NBI, *S*-(4-nitrobenzyl)-6-thioinosine; NECA, (*N*-ethylcarbamoyl)adenosine; PAF, 1-*O*-hexadecyl/octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine, platelet activating factor; (*R*)-PIA, (*R*)-N^6-(phenylisopropyl)adenosine; SAR, structure−activity relationship; Tris, tris(hydroxymethyl)aminomethane; XAC, 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine, xanthine amine congener.

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