Journal of Medicinal Chemistry Article

J. Med. Chem. **2009,** *52,* 7669–7677 **7669**
DOI: 10.1021/jm900538v

Nucleoside-5′-monophosphates as Prodrugs of Adenosine A_{2A} Receptor Agonists Activated by ecto-5′-Nucleotidase†

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Received April 27, 2009

Prodrugs of adenosine A_{2A} receptor agonists were developed that are activated by ecto-5′-nucleotidase (ecto-5′-NT, CD73). Because ecto-5′-NT is upregulated in inflamed tissue, the A_{2A} agonists are expected to be released from their prodrug form at the sites of inflammation. 2-(Ar)alkyl-substituted AMP derivatives were synthesized and investigated. Certain 2-substituted AMP derivatives, including 2-hexylthio-AMP, 2-cyclopentylthio-AMP, 2-cyclohexylmethylthio-AMP, and 2-cyclohexylethylthio-AMP were accepted as substrates by ecto-5′-NT and readily converted to the corresponding 2-substituted adenosine derivatives. The 2-cyclohexylethylthio substitution was a good compromise between the requirements of the ecto-5′-NT and the adenosine A_{2A} receptor. The corresponding AMP derivative (**12g**) was a similarly good substrate as AMP itself, while the resulting adenosine derivative (**11g**) was a relatively potent A_{2A} agonist (radioligand binding to rat brain striatal membranes: K_i = 372 nM; inhibition of anti-CD3/anti-CD28-induced IFN-γ release in mouse CD4+ cells: EC_{50} = 50 nM). Compound **11g** was released from **12g** by incubation with CD4+ cells isolated from wild-type mice but only to a much smaller extent by cells from ecto-5′-NT knockout mice. Compound **12g** will be a new lead structure for the development of more potent and selective ecto-5′-NT-activated prodrugs of selective anti-inflammatory A_{2A} receptor agonists.

Introduction

Adenosine (**1**, Figure 1) is a naturally occurring nucleoside that plays an important role in physiological and pathophysiological processes through its action on G protein-coupled adenosine receptors (P1 receptors).[1] There are four subtypes of adenosine receptors that have been cloned and characterized: A_1, A_{2A}, A_{2B}, and A_3. The A_1 and A_3 receptors are known to couple to adenylate cyclase in an inhibitory manner via G_i protein, whereas A_{2A} and A_{2B} receptor subtypes stimulate the enzyme via G_s protein. In addition, coupling to other second messenger systems, such as calcium or potassium ion channels or phospholipase C, has been described.[1,2] Recently, the first crystal structure of an adenosine receptor, the human A_{2A} receptor, in complex with the high affinity antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]-triazin-5-ylamino]ethyl)phenol (ZM241385), was obtained in 2.6 Å resolution.[3] Adenosine A_{2A} receptors mediate the potent immunosuppressive and hypotensive effects of adenosine.[4] They are highly expressed in cells of the immune system, e.g., in spleen, thymus, and leukocytes, as well as on smooth muscle cells, endothelium, and blood platelets.[5] Furthermore, a high density of A_{2A} receptor expression is found in the brain in the caudate-putamen, nucleus accumbens, and olfactory tubercle.[5] One of the main therapeutic potentials of adenosine A_{2A} receptor agonists is due to their anti-inflammatory and immunosuppressive effects.[6] On the other hand, A_{2A} receptor agonists are potent vasodilators and are applied as diagnostics for pharmacologic stress testing in the heart.[7] Further potential therapeutic applications of A_{2A} agonists are the treatment of psychosis and Huntington's disease.[8]

The main approach for discovering A_{2A}-selective agonists has been the modification of adenosine itself.[9,10] Most of the potent and selective adenosine derivatives are modified in the 2-position of the adenine moiety and/or in the 5′-position of the riboside as shown in Figure 1. N^6,2-Disubstitution and substitution in position 8 may also lead to potent A_{2A} agonists. Typical variations include the introduction of an amide moiety at the 5′-position, a tetrazole ring at the 4′-position of the ribose ring, and large substituents at the 2-position of the purine moiety. Typical 2-substituents contain a cyclic structure (aromatic or cycloalkyl) or a simple lipophilic alkyl chain and a three-membered linker often containing an electron-rich function (e.g., O, NH, triple bond) attached to the purine ring (Figure 1). Additional features, such as bulky substituents at N^6 or alkylamino substitution at the 8-position of the purine moiety, have been described.[8−10] 2-(Ar)alkylthioadenosine derivatives have also been investigated as A_{2A} receptor agonists,[11,12] and some derivatives,

†Contribution to celebrate the 100th anniversary of the Division of Medicinal Chemistry of the American Chemical Society.

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Published on Web 07/06/2009

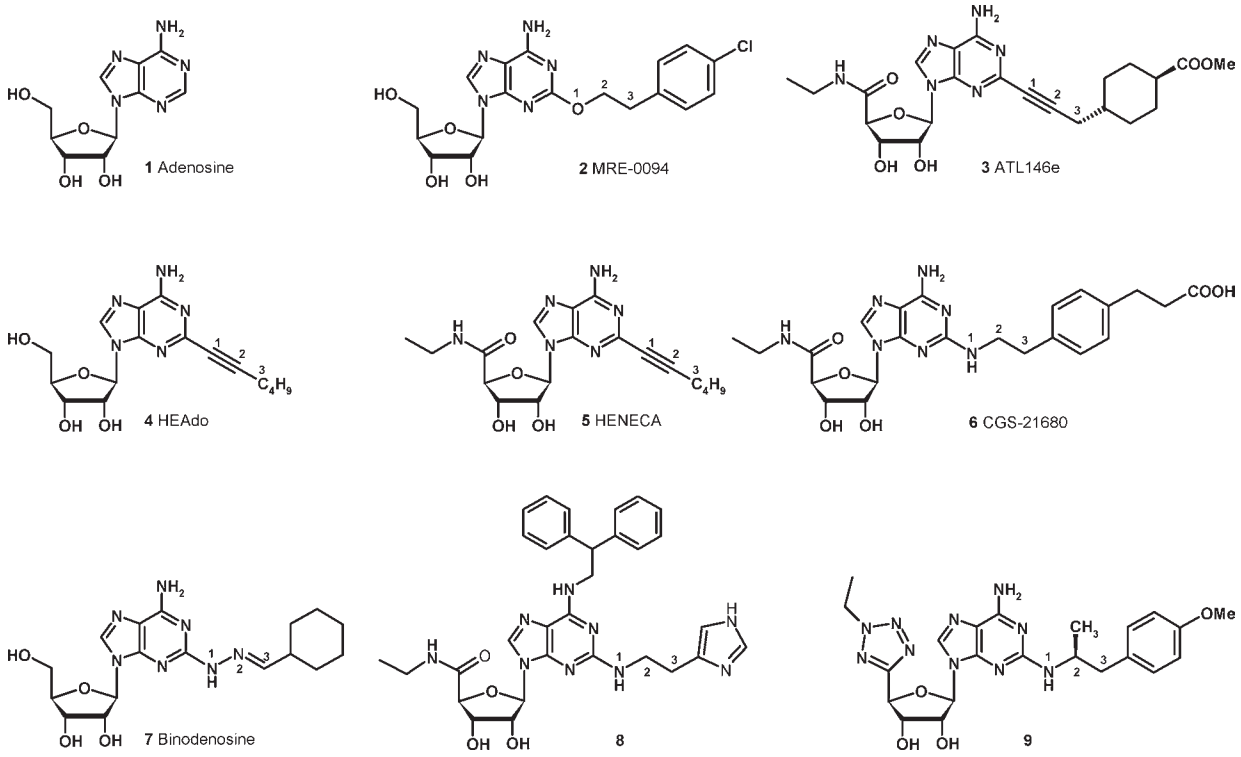


Figure 1. Structures of some potent and selective adenosine A_{2A} receptor agonists.

e.g., 2-(phenylethylthio)adenosine, were found to be potent A_{2A} receptor agonists.[12]

A major limitation in the use of A_{2A} adenosine receptor agonists when applied systemically as anti-inflammatory agents is their potent hypotensive activity.[8,13] In an attempt to dissociate the vasodilatory from the anti-inflammatory activity we have synthesized in this study phosphorylated A_{2A} adenosine receptor agonists which require ecto-5′-nucleotidase (ecto-5′-NT, CD73,[a] EC 3.1.3.5) to be converted into the active drug.

Dephosphorylation of extracellular AMP to adenosine is physiologically mediated by ecto-5′-NT, which is the last step in the enzymatic chain that catalyzes the breakdown of extracellular ATP to adenosine.[1] ecto-5′-NT is attached via a glycosylphosphatidylinositol anchor to the plasma membrane, where it catalyzes the hydrolysis of nucleoside 5′-monophosphates such as AMP, GMP, and UMP to the corresponding nucleosides. The enzyme can also be cleaved off from its anchor and retains its catalytic activity in the soluble form. The main physiological function of ecto-5′-NT is the hydrolysis of extracellular AMP formed by the degradation of the P2 receptor agonists ATP and ADP by other ecto-nucleotidases. Thus, the enzyme generates adenosine, which can act on P1 (adenosine) receptors.[14] ecto-5′-NT is localized on the outer surface of endothelial cells of large and small arteries. Hypoxia enhances the expression of ecto-5′-NT,[15] and ecto-5′-NT is generally up-regulated at the site of inflammation. It has been shown that ecto-5′-NT is up-regulated on the neointima after wire-induced injury in the carotid artery.[16] In addition, ecto-5′-NT is highly up-regulated in the plaque of ApoE mouse, a well established model of arteriosclerosis.[17] In addition to hydrolysis by ecto-5′-NT, AMP can also be dephosphorylated by alkaline phosphatase. While the K_m value for the ecto-5′-NT is about 14 μM, the corresponding value for alkaline phosphatase is more than 30-fold higher (441 μM).[18,19] In addition, at physiological pH, the AMP-hydrolyzing activity of alkaline phosphatase is considerably lower than that of ecto-5′-NT.[20]

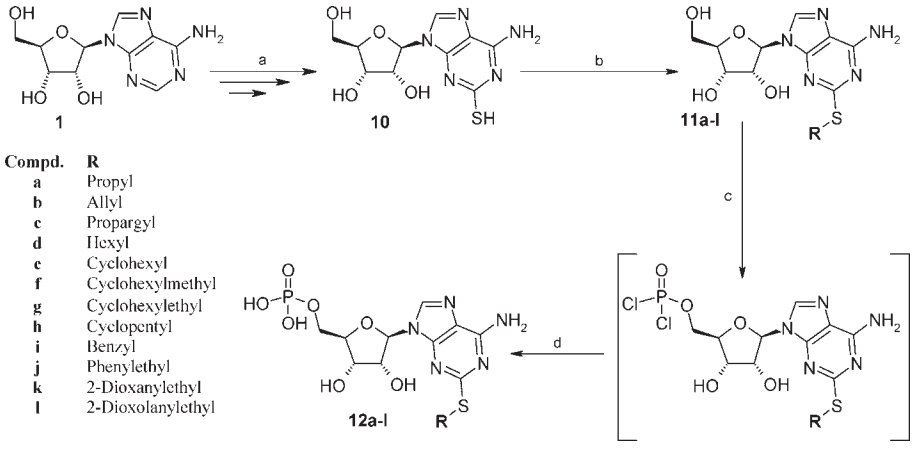
The goal of the present study was to develop prodrugs of adenosine A_{2A} receptor agonists, which as such are biologically inactive and require for activity conversion by ecto-5′-NT to the corresponding drugs. The rate of activation can be expected to be high in tissues with a high expression of the enzyme, e.g., inflamed tissue. This might allow high drug levels in pathological tissues and thereby would result in a drug targeting effect, increasing effects (anti-inflammation) at the desired site of actions while decreasing unwanted side effects (vasodilation).

Results and Discussion

As target structures, we selected 2-(ar)alkylthio-substituted AMP derivatives. They allow for a broad variation of the 2-substituent in order to study structure−activity relationships of this class of compounds as potential substrates of ecto-5′-NT. Furthermore, they permit study of the affinity and selectivity of the corresponding nucleosides for adenosine A_{2A} receptors. Certain 2-substituted AMP derivatives have previously been found to be agonists or antagonists at P2Y receptor subtypes, but their potency was low (in the micromolar range).[21a−21c] Therefore, this should not limit the feasibility of the designed prodrug concept.

[a] Abbreviations: CCPA, 2-chloro-N^6-cyclopentyladenosine; CE, capillary electrophoresis; CD73, cluster of differentiation 73; [^{3}H]-CGS21680, [^{3}H]2-(4-(2-carboxymethyl)phenyl)ethylamino-5′-*N*-ethylcarboxamidoadenosine; CHO, Chinese hamster ovary; ecto-5′-NT, ecto-5′-nucleotidase; ESI, electrospray ionization; DEAE, diethylaminoethyl; IFN-γ, interferon-γ; MSX-2, 3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine; NECA, *N*-ethyl-carboxamidoadenosine; [^{3}H]PSB-11, [^{3}H]8-ethyl-4-methyl-2-phenyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]purin-5-one; TCR, T cell receptor; TEAB, triethylammonium hydrogencarbonate buffer; Treg, regulatory T-cells.

Scheme 1. Synthesis of Nucleoside Monophosphates[a]



[a] Reagents and conditions: (a) three steps: (1) H_2O_2, CH_3COOH; (2) 5-N aq NaOH; (3) CS_2, MeOH, H_2O, 120 °C autoclave, 5 h. (b) R-Br, H_2O/NaOH, rt, 3−7 h. (c) $(OCH_3)_3PO$, $POCl_3$, 1,8-bis-(dimethylamino)naphthaline, 5 h, 0−4 °C. (d) Triethylammonium hydrogenbicarbonate buffer pH 7.4−7.6, rt, 1 h.

Syntheses. 2-Thioadenosine (**10**) was synthesized starting from adenosine (**1**) according to published procedures.[22] Oxidation with hydrogen peroxide in acetic acid yielded the N1-oxide. Subsequent ring-opening using sodium hydroxide, followed by treatment with a mixture of carbon disulfide, methanol, and water (50 : 175 : 25) at 120 °C in an autoclave, gave 2-thioadenosine (**10**).[22] 2-Alkylated derivatives **11a**−**l** were obtained by reaction of **10** with alkyl or arylalkyl halogenides in the presence of sodium methoxide in DMF or NaOH in water[11,23] (Scheme 1; for details see Supporting Information).

The 2-substituted adenosine derivatives **11a**−**l** were subjected to phosphorylation according to the Ludwig procedure[24] with minor modifications (Scheme 1): The lyophilized nucleosides **11a**−**l** were dissolved in trimethylphosphate and reacted with phosphorus oxychloride in the presence of 1,8-bis-(dimethylamino)naphthaline ("proton sponge") to yield the reactive 5′-dichlorophosphate intermediates. Hydrolysis with triethylammonium hydrogen bicarbonate buffer yielded the desired nucleoside monophosphates **12a**−**l**.

The nucleotides **12a**−**l** were purified by anion exchange chromatography on Sephadex diethylaminoethyl (DEAE) A-25 gel using a fast protein liquid chromatography (FPLC) apparatus by applying a linear gradient (0−100%, pure water → 0.5 M aqueous triethylammonium hydrogencarbonate buffer). The neutral impurities (e.g., nucleosides) eluted first, followed by the monophosphates. The products were further purified by high performance liquid chromatography (HPLC) on reverse-phase C18 material in order to remove inorganic phosphates and buffer components.

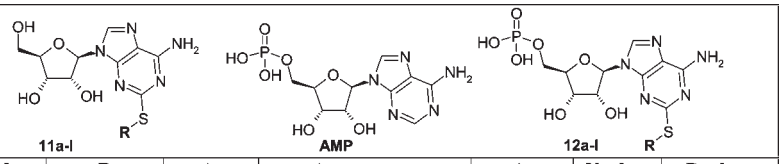
The structures of the synthesized nucleosides and nucleotides were confirmed by ^{1}H and ^{13}C NMR spectroscopy, in addition to HPLC analysis coupled to electrospray ionization mass spectrometry (LC/ESI-MS) performed in both positive and negative mode. The nucleotides were additionally investigated by ^{31}P NMR spectroscopy.

Biological Activity. The 2-substituted (ar)alkylthioadenosine derivatives were investigated in radioligand binding studies at rat brain striatal adenosine A_{2A} receptors using the agonist radioligand [^{3}H]CGS21680. Most compounds were additionally tested versus the A_{2A} antagonist radioligand [^{3}H]MSX-2 in order to assess whether the compounds act as agonists or antagonists. Agonists show a markedly lower affinity versus the antagonist radioligand than versus the agonist radioligand.[25] Selectivity versus other adenosine receptor subtypes was assessed by performing radioligand binding studies at rat brain cortical membranes (A_1) using [^{3}H]CCPA as a radioligand and at membrane preparations of human recombinant A_3 receptors expressed in Chinese hamster ovary (CHO) cells using [^{3}H]PSB-11 as a radioligand. A capillary electrophoresis-based enzyme assay[14] was used to investigate the properties of the nucleoside monophosphates as substrates of ecto-5′-NT. Catalytically active recombinant soluble glutathione-*S*-transferase/rat ecto-5′-NT fusion protein was expressed in insect cells with the baculovirus system and purified by affinity chromatography using agarose-coupled glutathione as previously described.[26] Furthermore, selected compounds were investigated for their potency to inhibit interferon-γ (IFN-γ) release induced by anti-CD3/anti-CD28 antibodies in CD4+ T-cells purified from the spleen of C57/BL6 mice (wild-type, and ecto-5′-NT knockout, respectively) by magnetic beads.

Adenosine Receptor Affinity. All 2-alkylthio- or arylalkylthio-substituted adenosine derivatives investigated showed higher affinity at A_{2A} receptors when determined versus the agonist radioligand [^{3}H]CGS21680 than versus the antagonist radioligand [^{3}H]MSX-2 (see Table 1). The differences were 3- to 14-fold. This means that all compounds had a higher affinity to the (active) agonist-labeled conformation than to the (inactive) antagonist-labeled conformation, indicating that they are all agonists rather than antagonists at A_{2A} receptors.

The determined structure−activity relationships confirmed previous observations with 2-substituted adenosine derivatives. Small alkyl groups linked by a thioether group to the 2-position of adenosine resulted in only weak A_{2A} receptor agonists with K_i values in the low micromolar range (**11a**,**b**,**c**). An increase in the chain length from propyl (**11a**) to hexyl (**11d**) increased A_{2A} affinity by almost 3-fold (**11d**, K_i = 542 nM). However, **11d** was even somewhat more potent at the A_1 receptor (K_i = 256 nM). A cyclohexyl residue (in **11e**) instead of *n*-hexyl (**11d**) led to a 5-fold reduction in affinity. The introduction of a methylene or, even better, an ethylene spacer between the cyclohexyl ring and the thioether group led to an increase in A_{2A} affinity (2-(cyclohexylmethylthio) adenosine, **11f**, K_i = 709 nM; 2-(cyclohexylethylthio)adenosine, **11g**, K_i = 372 nM). A cyclopentyl analogue was slightly

Table 1. Adenosine Receptor Affinities of Nucleosides and Percent Dephosphorylation of Corresponding Nucleotides (5′-Monophosphates) by ecto-5′-Nucleotidase in Comparison with the Physiological Substrate AMP



Nucleoside	R	A_1 receptor rat brain cortical membranes vs. [^{3}H]CCPA[a] K_i ± SEM (nM) (n = 3)	A_{2A} receptor rat brain striatal membranes vs. [^{3}H]CGS-21680[a] K_i ± SEM (nM) (n = 3)	A_{2A} receptor rat brain striatal membranes vs. [^{3}H]MSX-2[b] K_i ± SEM (nM)[c] (n = 3)	A_3 receptor human vs. [^{3}H]PSB-11[b] K_i ± SEM (nM)[c] (n = 3)	Nucleotide	Dephosphorylation (%)[d] by rat ecto-5′-NT (n = 3)
						AMP	100 ± 1
11a	Propyl	1,270 ± 230	1,350 ± 390	n.d.	n.d.	**12a**	47 ± 1
11b	Allyl	532 ± 25	1,520 ± 280	15,200 ± 455	6,640 ± 787	**12b**	0
11c	Propargyl	555 ± 73	2,900 ± 500	14,200 ± 2,200	6,990 ± 738	**12c**	0
11d	Hexyl	256 ± 11	542 ± 120	2,940 ± 359	2,320 ± 134	**12d**	84 ± 3
11e	Cyclohexyl	139 ± 5	2,480 ± 655	10,600 ± 1,630	≥ 10,000	**12e**	28 ± 1
11f	Cyclohexylmethyl	498 ± 95	709 ± 123	4,130 ± 82	1,720 ± 167	**12f**	71 ± 1
11g	Cyclohexylethyl	400 ± 3	372 ± 57	945 ± 84	3,640 ± 719	**12g**	96 ± 1
11h	Cyclopentyl	994 ± 112	793 ± 150	7,650 ± 142	10,400 ± 924	**12h**	71 ± 4
11i	Benzyl	927 ± 57 (304)[e]	606 ± 183 (1,510)[e]	8,560 ± 280	120 ± 15 (68)[e]	**12i**	0
11j	Phenylethyl	180 ± 14 (99)[e]	18.5 ± 1.9 (85)[e]	249 ± 32	1,810 ± 588	**12j**	0
11k	2-Dioxanylethyl	454 ± 79	1,100 ± 30	n.d.	1,330 ± 530	**12k**	67 ± 3
11l	2-Dioxolanylethyl	1,140 ± 34	1,160 ± 28	n.d.	3,450 ± 530	**12l**	104 ± 6

[a] Agonist radioligand. [b] Antagonist radioligand. [c] Affinities of the agonists will be underestimated because an antagonist radioligand was used for the determination. [d] Percentage of dephosphorylation of nucleoside monophosphates was calculated with respect to the dephosphorylation of AMP, set at 100%. The peak areas of the substrate peak (nucleoside monophosphate) and the product peak (nucleoside) were measured and quantified in the same

less potent (**11h**, K_i = 793 nM). Oxygen-containing ring analogues of **11g** (**11k**, 2-(1,4-dioxanyl) instead of cyclohexyl) and **11l**, 2-(1,3-dioxolanyl), led to a decrease in A_{2A} affinity.

As previously reported, the introduction of a phenyl ring increased A_{2A} affinity, the most potent A_{2A} agonist of the present series being 2-(phenylethylthio)adenosine (**11j**, K_i rat A_{2A}=18.7 nM) exhibiting 10-fold selectivity vs the rat A_1 receptor. The same compound had previously been investigated by Volpini et al.[12] at the human adenosine receptor subtypes, where it was somewhat less potent and less selective for the A_{2A} receptor, indicating species differences between rat and human adenosine receptors (see Table 1). All compounds, except for 2-(benzylthio)adenosine (**11i**, K_i A_3 = 120 nM), showed only moderate to low affinity for the adenosine A_3 receptor subtype. Affinity for the adenosine A_{2B} receptor subtype was not determined since Volpini et al. had shown that A_{2B} affinity cannot be expected for such structures.[12]

Hydrolysis of Nucleoside Monophosphates by Purified ecto-5′-Nucleotidase. As a next step, we investigated the properties of the corresponding 2-substituted AMP derivatives as substrates of rat ecto-5′-NT. The percentage of dephosphorylation of nucleoside monophosphates was calculated with respect to the dephosphorylation of AMP, set at 100%. The enzyme assays were performed in reaction vials, and after the reaction was stopped, substrate and product were separated by capillary electrophoresis and quantified by their UV absorbance at 232 nm. The results are collected in Table 1, compounds **12a**−**l**.

Short, unsaturated substituents, such as in 2-(allylthio)-AMP (**12b**) and in 2-(propargylthio)-AMP (**12c**), were not tolerated by the enzyme. Similarly, aromatic residues as in 2-(benzylthio)-AMP (**12i**) and in 2-(phenylethylthio)-AMP (**12j**) prevented dephosphorylation of the nucleotides by ecto-5′-NT. Saturated alkyl or cycloalkyl residues linked via a thioether bridge to the 2-position of AMP as in compounds **12a** (propyl, 47% dephosphorylation), **12d** (hexyl, 84%), **12h** (cyclopentyl, 71%), and **12e** (cyclohexyl, 28%) were tolerated by the enzyme. It is surprising that a longer chain (hexyl) was better accepted than a small residue (propyl), indicating that the ecto-5′-NT possesses a lipophilic

binding pocket in that area. Even longer and bulkier cyclohexylmethylthio- or cyclohexylethylthio-AMP derivatives (**12f**, **12g**) turned out to be good substrates for ecto-5′-NT. In contrast to the adenosine A_{2A} receptor, the enzyme tolerated oxygen-containing rings as in dioxanylethylthio- and dioxolanylethylthio-AMP derivatives **12k** and **12l**.

These results showed that the SARs of 2-substituted adenosine derivatives at adenosine A_{2A} receptors and those of corresponding 2-substituted AMP derivatives as substrates of ecto-5′-NT were very different. The best compromise for both targets was the cyclohexylethylthio substituent. The respective nucleotide **12g** was an excellent substrate of ecto-5′-NT, almost as good as AMP, while the corresponding nucleoside **11g** was still a quite potent adenosine A_{2A} receptor agonist (K_i=372 nM) although not selective versus the A_1 adenosine receptor subtype. Nevertheless, we selected these derivatives for further proof-of-principle studies.

Interferon γ Production in T Cells. One selected nucleotide (**12g**) and the corresponding nucleoside (**11g**) were investigated in murine T cells for their inhibitory effect on T cell receptor (TCR)-mediated IFN-γ production. To this end, CD4+ T-cells were purified from the spleen of C57/BL6 mice by magnetic beads and were stimulated with anti-CD3 and anti-CD28 for 18 h. Measurement of IFN-γ production as determined by ELISA in the supernatant was 2463 ± 600 pg/mL (n = 4). No IFN-γ could be detected in unstimulated cell. As shown in Figure 2, cyclohexylethyadenosine (**11g**) potently inhibited the IFN-γ formation. The EC_{50} was 50.0 ± 21.4 nM, which is in the same range as the K_i value determined in the receptor assay (372 ± 57 nM). The dose−response curve for the phosphorylated derivative (**12g**) was shifted to the right by about 2 orders of magnitude (EC_{50} 5.70 ± 2.33 μM).

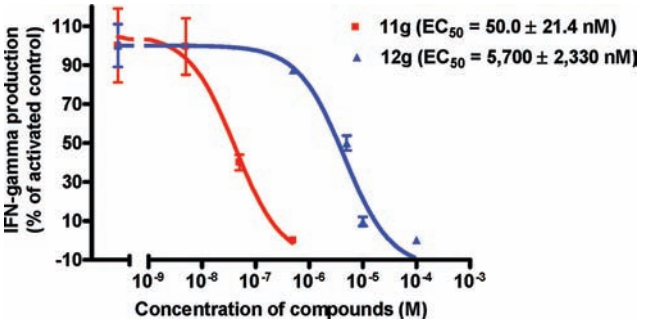


Figure 2. Effect of 2-(cyclohexylethylthio)adenosine (**11g**, n = 2) and of 2-(cyclohexylethylthio)-AMP (**12g**, n=3) on TCR-mediated IFN-γ production in murine CD4+ T cells. Data show means ± SD.

The degree of hydrolysis of the prodrug **12g** was measured at the end of the experiment. For comparison, degradation of the prodrug by Treg cells isolated from ecto-5′-NT knockout mice was assessed under the same conditions. The concentrations of prodrug **12g** and of released nucleoside **11g** were determined by LC/MS with electrospray ionization (ESI). Three different starting concentrations of prodrug **12g** were investigated. A linear correlation between the initial concentration of prodrug **12g** added and the amount of drug **11g** formed during the incubation was observed in cells from wild-type as well as from knockout mice (see Figure 3).

From the results given in Table 2, it is evident that during an incubation period of 18 h, 10.8−15.0% of prodrug **12g** was dephosphorylated to the corresponding nucleoside **11g** by CD4+ T-cells isolated from wild-type mice. In CD4+ T-cells from knockout mice, the degree of hydrolysis was much smaller (2.7−5.3% hydrolysis), indicating that ecto-5′-NT but not the alkaline phosphatase was mainly responsible for converting the phosphate prodrug **12g** to the drug **11g**.

Purified CD4+ T-cells consist of T-effector and regulatory T-cells (Treg). Recently, it has been shown that only Treg possesses ecto-5′-NT, which is a novel marker enzyme besides CD39 and FoxP3 for these cells.[27] The ability of nucleotide **12g** to inhibit IFN-γ formation will depend on the following critical factors: First, on the activity of ecto-5′-NT, in our experiments the fraction of Treg cells in purified CD4+ T-cells was only about 20%. Furthermore, the nucleotide was added together with stimulatory antibodies and the accumulation of the nucleoside occurred linearly over time. Consistent with this, preincubation of cells with the nucleotide **12g** considerably shifted the dose−response curve to the left (unpublished observation). Second, the distribution space into which the nucleoside is formed is of importance because this space determines the steady-state concentration of the active drug. In our cell experiments, the ratio of cell to supernatant fluid space was about 4×10^5 cells in 200 μL volume, which translates into a volume ratio of 1:2800, which in the case of Treg increases to 1:13000. Consequently, at a given cellular ecto-5′-NT activity, the effective concentration of the nucleoside will increase faster over time when the cell/fluid volume ratio is low. Under pathological conditions, e.g., in an inflamed tissue with infiltrated immune cells such as Treg cells and monocytes, all of which express ecto-5′-NT, the distribution space is the

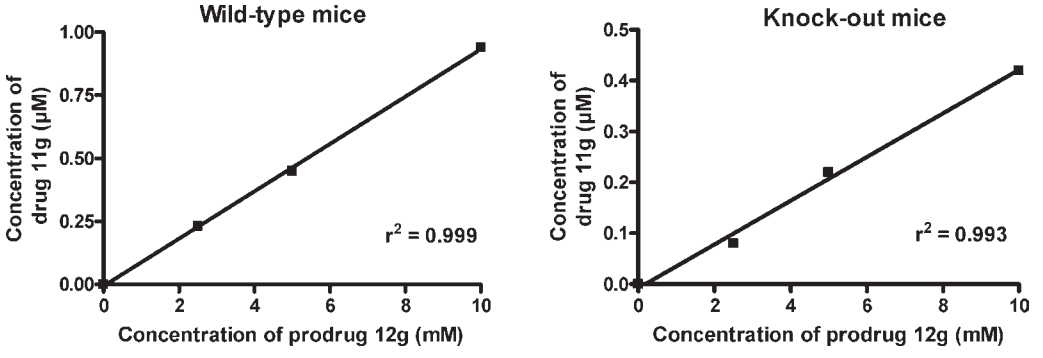


Figure 3. Correlation between the concentration of added prodrug **12g** (mM) and the produced drug **11g** (μM): the results showed that there was a linear relationship between the concentration of the prodrug **12g** and the released drug **11g** in CD4+ T-cells from wild-type and ecto-5′-NT knockout mice.

Table 2. Quantitative Determination of the Nucleoside (**11g**) and Nucleotide (**12g**) Concentrations by LC-MS after Incubation with CD4+ Cells from Wild-Type and ecto-5′-NT Knockout Mice

	LC/MS detection of prodrug **12g**		LC/MS detection of nucleoside **11g**	
initial concentration of nucleotide **12g** (mM)	conc. of **12g** (μM)	% of **12g** relative to **11g**	conc. of **11g** (μM)	% of **11g** relative to **12g**
		Wild-Type Mice		
10	5.33	85.0	0.94	15.0
5	3.05	87.2	0.45	12.8
2.5	1.90	89.2	0.23	10.8
		Knockout Mice		
10	7.46	94.7	0.42	5.3
5	4.47	95.3	0.22	4.7
2.5	2.84	97.3	0.08	2.7

interstitial space only, which is relatively small. Therefore, the curves shown in Figure 2 functionally may reflect the other extreme: the situation in a blood perfused vessel. Here the vascular distribution space of the nucleoside is rather large. Because ecto-5′-NT is expressed only on the vascular endothelium and circulating immune cells,[28] this would lead to substantial dilution. Consequently, the plasma concentrations of the dephosphorylated purine derivative are expected to be lower than those of its precursor, which may result in reduced vasodilatory side effects.

It should be noted that the prodrug can also be degraded by alkaline phosphatase. Given the K_m value of the alkaline phosphatase to be substantially higher than that of ecto-5′-NT, it is expected that at low substrate concentrations hydrolysis by ecto-5′-NT will dominate. Little is known, however, on the relative expression of both enzymes in various target tissues. In support for the therapeutic concept proposed here is that Treg cells only express negligible levels of alkaline phosphatase (unpublished observation), which is consistent with data given in Table 2. Another enzyme, the membrane-bound splice variant of prostatic acid phosphatase (TM-PAP), has recently been shown to carry ecto-5′-nucleotidase activity.[29] This enzyme is widely expressed in mouse tissues such as prostate lobes, salivary gland, thymus, lung, kidney, brain, spleen, and thyroid, and future experiments need to define its physiological implications.[29b]

Conclusions

2-(Cyclohexylethylthio)-AMP (**12g**) has been identified as an excellent substrate of ecto-5′-NT. It is dephosphorylated by ecto-5′-NT at a similar rate as AMP to the corresponding adenosine derivative **11g**, an adenosine receptor agonist with submicromolar affinity (K_i = 372 nM) for the A_{2A} receptor. Immune cells such as monocytes/macrophages, Treg cells, and neutrophils express both ecto-5′-NT and adenosine A_{2A} receptors at high density and thereby may be the preferential target for immunosuppression by the proposed prodrug concept. AMP derivative **12g** will serve as a new lead compound for the development of novel prodrugs of anti-inflammatory adenosine A_{2A} receptor agonists, which may be preferably activated in inflamed tissues with high expression of ecto-5′-NT. Further studies will be aimed at improving the A_{2A} affinity and selectivity while keeping the ecto-5′-NT substrate properties; this includes testing of these novel compounds under pathological conditions in the intact animal.

Experimental Section

All commercially available reagents were obtained from various producers (Acros, Aldrich, Fluka, Merck, and Sigma) and used without further purification. Solvents were used without additional purification or drying unless otherwise noted. The reactions were monitored by thin layer chromatography (TLC) using aluminum sheets with silica gel 60 F_{254} (Merck). Column chromatography was carried out with silica gel 0.060−0.200 mm, pore diameter ca. 6 nm. Mass spectra were recorded on an API 2000 (Applied Biosystems, Darmstadt, Germany) mass spectrometer (turbo ion spray ion source) coupled with an HPLC system (Agilent 1100) using a Phenomenex Luna 3 μ C18 column. ^{1}H-, ^{31}P-, and ^{13}C NMR spectra were performed on a Bruker Avance 500 MHz spectrometer. DMSO-d_6, MeOD-d_4, or D_2O were used as solvents as indicated below. ^{31}P NMR spectra were recorded at rt; orthophosphoric acid (85%) was used as an external standard. Shifts are given in ppm relative to the external standard (^{31}P NMR) or relative to the remaining protons of the deuterated solvents used as internal standard (^{1}H, ^{13}C). Purity of the prepared nucleosides was checked by TLC on silica gel 60 F_{254} (Merck) aluminum plates, using dichloromethane:methanol (9:1) as the mobile phase. Purity of the prepared nucleotides was confirmed by HPLC by dissolving 1 mg/mL in H_2O:MeOH = 1:1, containing 2 mM ammonium acetate. A sample of 10 μL was injected into an HPLC instrument (Agilent 1100) using a Phenomenex Luna 3 μ C18 column. Elution was performed with a gradient of water: methanol (containing 2 mM ammonium acetate) from 90:10 to 0:100 for 30 min at a flow rate of 250 μL/min, starting the gradient after 10 min. UV absorption was detected from 220 to 300 nm using a diode array detector. The purity of the nucleotide samples was checked in a second chromatography system using capillary electrophoresis (CE, for details see Supporting Information). The purity of the products was generally ≥95%.

General Procedure for the Synthesis of 2-Thioadenosine Derivatives. 2-Thioadenosine[22] (1 mmol) was dissolved in 20 mL of water (or water:ethanol; 1:1), and 5 mL of sodium hydroxide (0.5 N) was added to the reaction mixture, followed by the addition of (ar)alkyl halide (1.2 mmol) 10 min later. The reaction mixture was stirred for 3−7 h at rt, and the completion of the reaction was determined by TLC (CH_2Cl_2:MeOH = 9:1). The crude product was extracted by ethyl acetate, and the organic phase was separated, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The crude product was crystallized from ethanol and, in some cases, the product was further purified by silica gel column chromatography (CH_2Cl_2: MeOH = 9:1) to afford the pure product.

General Procedure for Phosphorylation. Preparation of Triethylammonium Hydrogen Carbonate Buffer (TEAB). A 1 M solution of TEAB was prepared by bubbling CO_2 through a 1 M triethylamine solution in water at 0−4 °C for several hours (pH approximately 7.4−7.6).[30]

General Procedure for the Preparation of Monophosphates by Phosphorylation of Nucleosides (or Analogues). Lyophilized nucleosides (1 mmol) were dissolved in 5 mL of trimethyl phosphate (dried over 10 Å molecular sieve). The mixture was stirred at rt under argon and then cooled to 4 °C. Dry 1,8-bis-(dimethylamino)naphthaline (proton sponge, 0.32 g, 1.5 mmol)

was added, followed by 0.20 g (1.3 mmol) of $POCl_3$ 5 min later. After 5 h of stirring at 0−4 °C, the mixture was poured into a cold 0.5 M aqueous TEAB solution (10 mL, pH 7.5) and stirred at 0−4 °C for several min. The solution was allowed to reach rt upon stirring and then left standing for 1 h. Trimethylphosphate was extracted with *tert*.-butylmethyl ether, and the aqueous solution was lyophilized to yield glassy colorless oils. The reactions were controlled by TLC using a freshly prepared solvent system (2-propanol:NH_4OH (25% of ammonia in water):water = 6:3:1). TLC plates were dried before UV absorption was detected, and the plates were subsequently sprayed with a phosphate reagent.[30,31]

Purification of Nucleotides Using Ion Exchange Chromatography. Nucleoside 5′-monophosphates were purified by ion exchange chromatography on an FPLC instrument (AKTA FPLC, from Amersham Biosciences) with an XK 26 mm/20 cm length column (Pharmacia) using Sephadex DEAE A-25 gel HCO_3^--form swelled in a 1 M solution of TEAB at 4 °C. After equilibration of the column with deionized water, the crude product was dissolved in 2 mL of aqueous triethylammonium hydrogen carbonate buffer. The column was washed with deionized water, followed by a solvent gradient of water:TEAB 1 M buffer from 0 to 100% using approximately 500 mL of solvent to elute the monophosphates. Fractions were collected and appropriate fractions pooled, diluted in water, and lyophilized.

Purification of Monophosphates Using Preparative HPLC. Lyophilized nucleoside 5′-monophosphates were dissolved in 5 mL of deionized water and injected into an RP-HPLC column (Knauer 20 mm ID, Eurospher-100 C18). The column was eluted with a solvent gradient of 0−50% of acetonitrile in 50 mM aq NH_4HCO_3 buffer for 40 min at a flow rate of 5 mL/min. The UV absorption was detected at 254 nm. Fractions were collected and appropriate fractions pooled, diluted with water, and lyophilized several times to remove the NH_4HCO_3 buffer, yielding the products as white powders. Compounds **11a**−**f**, **h**−**j** and **12a**−**f**, **h**−**i** were synthesized essentially as previously described; for details, see Supporting Information.

2-(Cyclohexylethylthio)adenosine (11g). ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.87−1.60 (m, 11H), 1.61−1.74 (m, 2H), 3.0−3.14 (m, 2H), 3.50−3.64 (m, 2H), 3.90 (q, 1H, J = 3.99 Hz), 4.11 (t, 1H, J = 4.25 Hz), 4.59 (t, 1H, J = 5.35 Hz), 4.98 (t, 1H, J = 5.51 Hz), 5.10 (m, 1H), 5.36 (m, 1H), 5.80 (d, 1H, J = 5.99 Hz), 7.28 (s, 2H), 8.19 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 25.89, 26.26, 27.94, 32.46, 36.61, 36.82, 61.75, 70.60, 73.31, 85.57, 87.42, 117.05, 138.86, 150.31, 155.63, 163.94. LC/ESI-MS: negative mode 408 ([M − H]$^-$), positive mode 410 ([M + H]$^+$).

2-(Dioxanylethylthio)adenosine (11k). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.31−1.34 (m, 1H), 1.85−1.88 (m, 3H), 3.01−3.12 (m, 2H), 3.50−3.64 (m, 2H), 3.67−3.72 (m, 2H), 3.90 (q, 1H, J = 3.67 Hz), 3.97−4.01 (m, 2H), 4.11 (q, 1H, J = 2.41 Hz), 4.59 (t, 1H, J = 5.51 Hz), 4.66 (t, 1H, J = 5.20 Hz), 4.98 (m, 1H), 5.26 (m, 2H), 5.79 (d, 1H, J = 5.99 Hz), 7.28 (s, 2H), 8.20 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 25.22, 25.51, 34.69, 61.76, 66.20, 70.60, 73.31, 85.60, 87.51, 100.40, 117.10, 138.97, 150.25, 155.67, 163.58. LC/ESI-MS: negative mode 412 ([M − H]$^-$), positive mode 414 ([M + H]$^+$).

2-(Dioxolanylethylthio)adenosine (11l). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.03−1.07 (m, 2H), 3.04−3.14 (m, 2H), 3.37−3.43 (m, 2H), 3.50−3.63 (m, 2H), 3.77−3.79 (m, 1H), 3.89−3.92 (m, 2H), 4.11 (q, 1H, J = 2.41 Hz), 4.59 (t, 1H, J = 5.51 Hz), 4.93 (t, 1H, J = 4.88 Hz), 5.01 (m, 1H), 5.26 (m, 2H), 5.80 (d, 1H, J = 5.99 Hz), 7.31 (s, 2H), 8.20 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 25.21, 33.58, 61.74, 64.34, 70.60, 73.37, 85.60, 87.44, 102.71, 117.09, 138.92, 150.27, 155.68, 163.55. LC/ESI-MS: negative mode 398 ([M − H]$^-$), positive mode 400 ([M + H]$^+$).

2-(Cyclohexylethylthio)adenosine-5′-monophosphate (12g). ^{1}H NMR (500 MHz, D_2O) δ 0.92−1.67 (m, 11H), 1.73−1.75 (m, 2H), 3.28−3.36 (m, 2H), 3.99 (m, 2H), 4.33 (m, 1H), 4.50 (m, 1H), 4.70 (m, 1H), 6.10 (d, 1H, J = 5.67 Hz), 8.45 (s, 1H). ^{31}P NMR (202 MHz, D_2O) δ 0.51 (s). ^{13}C NMR (125 MHz, D_2O) δ 27.71, 28.02, 29.86, 34.65, 38.61, 38.70, 66.16, 72.75, 76.49, 86.18, 88.85, 117.05, 140.15, 150.25, 156.91, 168.44. LC/ESI-MS: negative mode 488 ([M − H]$^-$), positive mode 490 ([M + H]$^+$).

2-(Phenylethylthio)adenosine-5′-monophosphate (12j). ^{1}H NMR (500 MHz, MeOD) δ 3.05−3.08 (t, 2H, J = 7.72 Hz), 3.38−3.49 (m, 2H), 3.76−3.90 (m, 2H), 4.17 (q, 1H, J = 3.25 Hz), 3.35 (q, 1H, J = 4.34 Hz), 4.75 (t, 1H, J = 4.75 Hz), 6.03 (d, 1H, J = 5.67 Hz), 7.20−7.36 (m, 5H), 8.24 (s, 1H). ^{31}P NMR (202 MHz, MeOD) δ 0.54 (s). ^{13}C NMR (125 MHz, MeOD) δ 33.84, 37.52, 63.51, 72.58, 75.70, 87.72, 90.65, 123.02, 127.56−130.88, 140.77, 150.25, 157.19, 166.92. LC/ESI-MS: negative mode 482 ([M−H]$^-$), positive mode 484 ([M + H]$^+$).

2-(Dioxanylethylthio)adenosine-5′-monophosphate (12k). ^{1}H NMR (500 MHz, D_2O) δ 1.39−1.4 (m, 2H), 2.12−2.15 (m, 2H), 3.02−3.05 (t, 2H, J = 7.40 Hz), 3.78−3.83 (m, 2H), 3.86 (m, 1H), 3.97 (m, 2H), 4.06−4.10 (m, 2H), 4.32 (m, 1H), 4.47 (t, 1H, J = 4.57 Hz), 4.66 (m, 1H), 6.11 (d, 1H, J = 5.99 Hz), 8.44 (s, 1H). ^{31}P NMR (202 MHz, D_2O) δ 2.77 (s). ^{13}C NMR (125 MHz, DMSO-d_6) δ 27.94, 28.38, 37.27, 55.65, 69.94, 73.55, 77.15, 87.42, 89.64, 104.19, 119.20, 142.24, 153.23, 156.83, 167.67. LC/ESI-MS: negative mode 492 ([M − H]$^-$), positive mode 494 ([M + H]$^+$).

2-(Dioxolanylethylthio)adenosine-5′-monophosphate (12l). ^{1}H NMR (500 MHz, D_2O) δ 2.12−2.16 (m, 2H), 3.04 (t, 2H, J = 7.40 Hz), 3.91−3.93 (m, 2H), 3.96−3.3.97 (m, 2H), 4.02−4.03 (m, 2H), 4.31 (m, 1H), 4.47 (t, 1H, J = 4.72 Hz), 4.65 (m, 1H), 5.07 (t, 1H, J = 4.57 Hz), 6.10 (d, 1H, J = 5.67 Hz), 8.42 (s, 1H). ^{31}P NMR (202 MHz, D_2O) δ 2.72 (s). ^{13}C NMR (125 MHz, DMSO-d_6) δ 28.09, 35.74, 66.44, 67.58, 73.55, 77.10, 87.36, 89.68, 105.93, 119.16, 142.20, 153.22, 158.20, 167.68. LC/ESI-MS: negative mode 478 ([M − H]$^-$), positive mode 480 ([M + H]$^+$).

Adenosine Receptor Binding Assays. Chemicals. Tris(hydroxymethyl)aminomethan (Tris-buffer) was obtained from Acros Organics (Leverkusen, Germany); DMSO was from Fluka (Switzerland), HCl was from Merck, and HAT supplement from Gibco. [^{3}H]CGS21680 ([^{3}H]2-(4-(2-carboxymethyl)phenyl)ethylamino)-5′-*N*-ethylcarboxamidoadenosine, 45 Ci/mmol)[32] was purchased from PerkinElmer Life Sciences (USA). [^{3}H]CCPA ([^{3}H]2-chloro-N^6-cyclopentyladenosine (48.6 Ci/mmol),[33] [^{3}H]MSX-2 ([^{3}H]3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine, 84 Ci/mmol),[25] and [^{3}H]PSB-11 ([^{3}H]8-ethyl-4-methyl-2-phenyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]-purin-5-one (53 Ci/mmol))[34] were custom-labeled by Amersham from suitable precursors that were synthesized as previously described. All other chemical reagents, cell culture materials, and adenosine receptor ligands were obtained from Sigma.

Receptor−Radioligand Binding Studies. Rat brain cortical membrane preparations were used as a source for A_1, and rat brain striatal membrane preparations as a source for A_{2A} receptors as previously described.[35,36] Membrane preparations of Chinese hamster ovary (CHO) cells expressing the human A_3 receptors were used for A_3 assays.[33] Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO); the final concentration of DMSO was 2.5%. The radioligand concentrations and incubation times (incubation at rt) were as follows: [^{3}H]CCPA, 1.0 nM (rat A_1), incubation for 90 min; [^{3}H]CGS21680, 10.0 nM (rat A_{2A}), incubation for 60 min; [^{3}H]-MSX-2, 1.0 nM (rat A_{2A}), incubation for 30 min; [^{3}H]PSB-11, 1.0 nM (human A_3), incubation for 60 min. About 30−50 μg of protein /vial were used in the assays. Membranes were preincubated for 10−15 min with 0.12 IU/mL of adenosine deaminase in order to remove endogenous adenosine. Binding assays were performed essentially as previously described.[35,36] Curves were determined using 6−7 different concentrations of test compounds spanning 3 orders of magnitude. At least two to three separate experiments were performed, each in duplicate (human receptors) or triplicate (rat receptors).

Recombinant Expression of Rat ecto-5′-Nucleotidase. Catalytically active recombinant soluble glutathione-*S*-transferase/ecto-5′-nucleotidase fusion protein was expressed in insect cells using the baculovirus system and purified by affinity chromatography using agarose-coupled reduced glutathione as described previously.[26]

ecto-5′-Nucleotidase Assays. Preparation of Standard Solutions. Compounds were dissolved in DMSO to obtain 5.0 mM stock solutions. These were further diluted to obtain 1 mM solutions in assay buffer (10 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 1 mM $CaCl_2$). The 1 mM solutions were further diluted in the same buffer as required for the standard calibration curves and the enzyme assays.

Capillary Enzymatic Reaction. CE separations were carried out using a P/ACE MDQ system (Beckman Coulter Instruments, Fullerton, CA) equipped with a DAD detection system. The electrophoretic separations were carried out using an eCAP fused-silica capillary [30 cm (20 cm effective length), ×75 μm internal diameter (I.D.), ×375 μm outside diameter (O.D.) obtained from Beckman Coulter]. The following conditions were applied: $T = 25$ °C, $\lambda_{max} = 232$ nm, voltage = 15 kV, running buffer 40 mM sodium borate buffer, pH 9.1. The capillary was washed with 0.1 M NaOH for 2 min, deionized water for 1 min, and running buffer for 1 min before each injection. Injections were made by applying 0.1 psi of pressure to the sample solution for 30 s. The amount of adenosine or other nucleosides formed was determined. The CE instrument was fully controlled through a personal computer, which operated with the analysis software 32 KARAT obtained from Beckman Coulter. Electropherograms were evaluated using the same software.

Investigation of ecto-5′-Nucleotidase Substrates by Capillary Electrophoresis. Assays were carried out at 37 °C in a final volume of 100 μL. The reaction mixture contained 500 μM of the nucleoside 5′-monophosphate dissolved in reaction buffer (10 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 1 mM $CaCl_2$). The reactions were initiated by adding 10 μL of the appropriately diluted enzyme and were then allowed to proceed at 37 °C for 20 min. The reaction was stopped by heating the mixture at 99 °C for 5 min. Aliquots of 50 μL of the reaction mixture were transferred to mini CE vials and injected into the CE instrument under the conditions described above. The absorbance at 232 nm was monitored continuously, and the nucleoside concentrations were determined from the area under each absorbance peak. The experiments were repeated twice with triplicate injections. Control experiments were performed in the absence of substrate and in the absence of enzyme in order to take into account the spontaneous hydrolysis of nucleoside 5′-monophosphates under the experimental conditions, which always amounted to less than 1%. All of the nucleosides were injected into the capillary electrophoresis under the same assay conditions at 500 μM concentrations to confirm the migration times of the nucleoside peaks observed after enzymatic hydrolysis of the nucleoside monophosphates. For the quantification of the dephosphorylation products, the nucleosides, we applied the internal normalization method. Thus, the individual peak areas reflected directly the relative content of a corresponding analyte. This was possible because starting compound (nucleoside monophosphate) and product (nucleoside) exhibit the same molar absorption coefficient; the phosphate residue does not show any UV absorption at the wavelength used for quantification (232 nm). This method is also completely independent of the amount of substrate injected into the capillary, which represents 100% at zero reaction time.

T-lymphocyte Isolation and Activation. C57/Bl6 male mice (8−12 wk old) were obtained from the central animal facility of the Heinrich-Heine-University (Düsseldorf, Germany). They were fed a standard chow diet and received tap water ad libitum. Mice were sacrificed, and the spleens were removed. Splenocytes were passed through a 70 μm nylon cell strainer (BD Bioscience) and collected in phosphate-buffered saline (PBS). Red blood cells were removed with lysing buffer (ammonium chloride), and CD4+ T lymphocytes were isolated by magnetic cell sorting with CD4 MicroBeads (Miltenyi). After resuspending purified cells in Panserin 413 (Pan), cells were activated by incubation in 96-well plates coated with 0.4 μg/mL anti-CD3 mAb and 2.5 μg/mL anti-CD28 (BD Bioscience) for 18 h. Supernatant was used for the analysis of IFN-γ and hydrolysis of AMP derivative **12g**.

Measurement of IFN-γ. IFN-γ concentrations in supernatants of CD4+ cell cultures were measured by ELISA according to the manufacturer's protocol (R&D Systems).

Quantitative Determination by LC-MS. Samples (10, 5, or 2.5 mM) were injected (10 μL) into an HPLC instrument (Agilent 1100) equipped with a Phenomenex Luna 3 μ C18 column. Elution was performed with a gradient of water:methanol (containing 2 mM ammonium acetate) from 90:10 to 0:100 for 20 min at a flow rate of 250 μL/min, followed by 100% methanol for 35 min. The compounds were detected by MS/MS multireaction monitoring (MS/MS-MRM) in the negative mode with an electrospray ion source (ESI) on an API 2000 (Applied Biosystems, Darmstadt, Germany) mass spectrometer from the masses of 408.35−276.25 for the nucleoside **11g** and from the masses of 488.25−79.15 for the nucleotide **12g**. Calibration curves were obtained from the pure compounds, and the area under the peak, for each concentration, was quantified.

Acknowledgment. This study was supported by the Deutsche Forschungsgemeinschaft (DFG, GRK804, grant to A.B. and C.E.M.). A.El-T. and J.I. are grateful for a STIBET scholarship by the Deutscher Akademischer Austauschdienst (DAAD). We thank Nicole Florin for performing some of the radioligand binding studies and Sabine Terhart-Krabbe and Annette Reiner for NMR spectra.

Supporting Information Available: Synthetic procedures, ^1H and ^{13}C NMR spectral data, and mass spectra of nucleosides **11a−f,h−j** and nucleotides **12a−f,h−i**, as well as purity determination of synthesized compounds by CE and HPLC and MS spectra from quantitative analyses and HPLC-MS, including MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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