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Design, synthesis, and biological evaluation of novel human 5′-deoxy-5′-methylthioadenosine phosphorylase (MTAP) substrates

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Abstract—The structure-based design, chemical synthesis, and biological evaluation of novel MTAP substrates are described. These compounds incorporate various C5′-moieties and are shown to have different k_{cat}/K_m values compared with the natural MTAP substrate (MTA).
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Cytotoxic chemotherapeutic agents are a proven class of anti-cancer treatments.[1] A subset of these agents function by blocking the activity of key enzymes required for de novo purine biosynthesis including glycinamide ribonucleotide transformylase (GARFT, EC 2.1.2.2) and aminoimidazolecarboxamide ribonucleotide transformylase (AICARFT, EC 2.1.2.3) (Fig. 1).[2] This blockage deprives cancer cells of critical building blocks required for DNA synthesis (AMP and GMP), thereby limiting the potential for tumor growth. Unfortunately, these chemotherapeutic agents often exert cytotoxic effects against non-cancerous (e.g., healthy) cells resulting in a number of toxicities, limiting their utility. We therefore sought to identify a biological mechanism to reduce the toxicity of anti-purine chemotherapeutic agents toward non-cancer cells while preserving their cytotoxic effects against tumors.

Human 5′-deoxy-5′-methylthioadenosine phosphorylase (MTAP) functions in the purine salvage pathway where it catalyzes the reversible phosphorolysis of 5′-deoxy-5′-methylthioadenosine (MTA) and thereby facilitates its conversion to adenine and 5-(methylthio)-ribose-1-phosphate (MTRP) (Fig. 1). Adenine is then converted to AMP via purine salvage pathways, and MTRP is converted via an independent mechanism to methionine.[1] MTAP activity is thus responsible for generation of essentially all the free adenine in human cells,[3] and accounts for the majority of AMP generation when the de novo synthesis pathway is blocked.

In addition, although MTAP is present in all healthy cells, certain cancers are known to have an incidence of MTAP-deficiency.[4] We therefore speculated that co-administration of a MTAP substrate with a de novo purine biosynthesis inhibitor (a chemotherapeutic agent) would reduce the inhibitor's toxicity in MTAP-competent non-tumor cells while retaining its cytotoxic properties against MTAP-deficient cancers. This hypothesis is supported by extensive in vitro studies, which combine MTA with L-alanosine, an inhibitor of AMP synthesis, as well as combined with the de novo purine synthesis inhibitors Lometrexol and AG2037.[5,6a,b] It was our intent to develop MTAP substrates as anti-toxicity agents, which have different biological and physical properties than the enzyme natural substrate in an effort to increase the maximally tolerated dose of AG2037, a cytotoxic inhibitor of GARFT currently in development.[6c] Ideally, these novel MTAP substrates would be converted to adenine by MTAP, which can further enter the de novo AMP synthesis pathway and thereby increase AMP production in MTAP(+) containing (non-tumor) cells, but not in MTAP(−) cancer cells (Fig. 1).[1] The synthesis of such novel MTAP substrates is described below along with an assessment of their k_{cat} and K_m values.[7]

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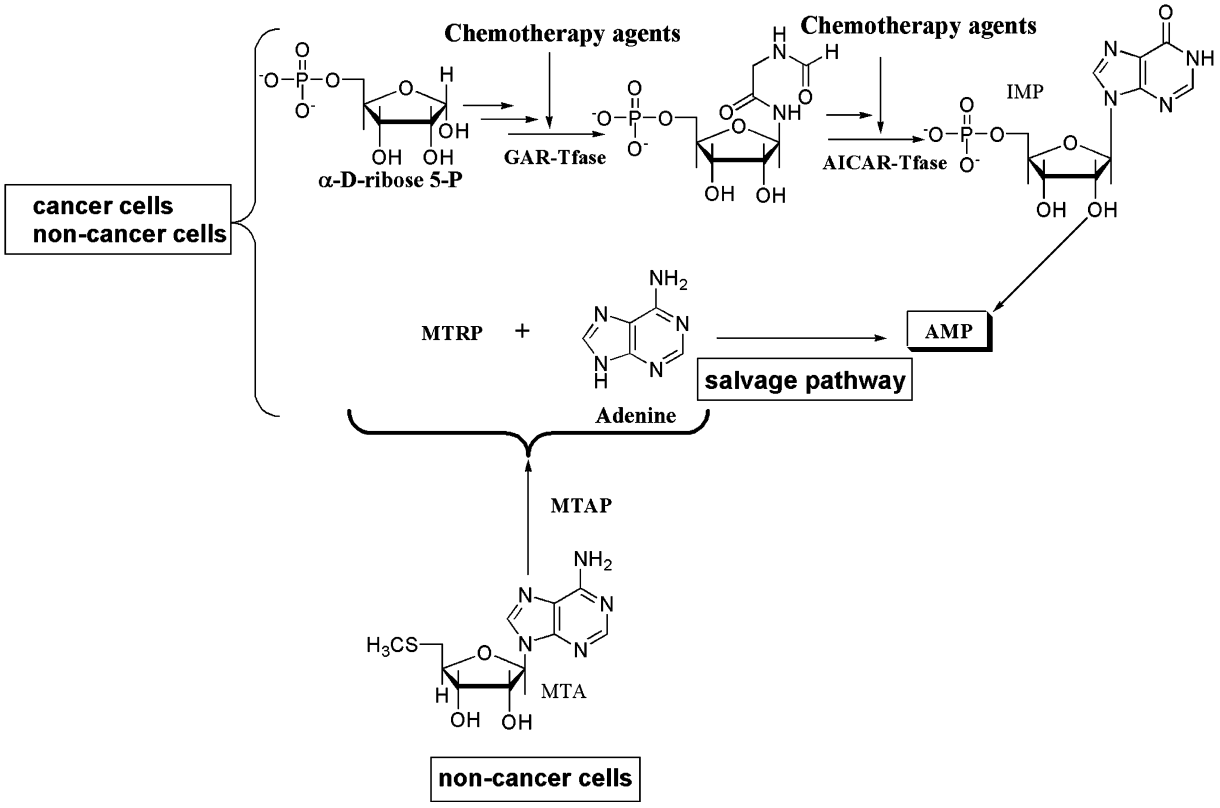
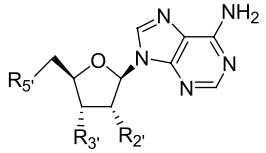


Figure 1.

A previously reported co-crystal structure[8] of MTA (compound **1**) and MTAP was utilized to design the desired substrates although it does not reflect the transition state of the MTA cleavage process. Initial designs explored compounds containing 2′ and 3′ moieties other than hydroxyl groups. Several molecules were synthesized, which replaced either the 2′-OH or 3′-OH substituents with potential hydrogen bonding acceptors and donors such as F, N_3, and NH_2. Unfortunately, these compounds were poorer MTAP substrates as compared with the natural substrate, MTA (compounds **3-8**, Table 1).

In the MTA/MTAP co-crystal structure, there is visible space in the vicinity of the MTA C5′ position between the ligand and the protein. Accordingly, we decided to examine whether modification of the MTA C5′ position would afford novel MTAP substrates. The k_{cat} and K_m values of several such MTA analogues are listed in Table 2. Compounds **2**, **9** (ETA), **10**, and **11** are literature-reported MTAP substrates,[9] and their k_{cat}/K_m values determined in our assays were poorer than that exhibited by MTA (compound **1**). Compounds containing other $C_{5'}$ alkyl chain moieties (compounds **14** and **15**) gave similar catalytic activity compared with ETA

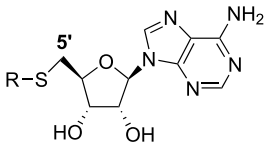
Table 1.

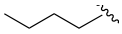
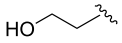
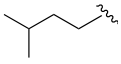
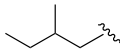
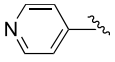
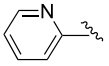
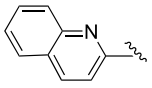
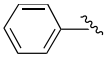
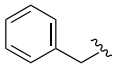
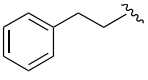
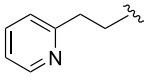


Compound #	$R_{5'}$	$R_{3'}$	$R_{2'}$	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
1 (MTA)	CH_3S	OH	OH	4.6 ± 0.2	1.8 ± 0.3	3.2
2	H	OH	OH	4.8 ± 0.4	6 ± 2	0.8
3	CH_3S	OH	H	0.28 ± 0.005	3.1 ± 0.4	0.09
4	CH_3S	H	OH	0.004 ± 0.0001	3.9 ± 0.5	0.001
5	CH_3S	OH	F	0.0004	ND	ND
6	CH_3S	OH	N_3	0.04 ± 0.01	3600 ± 1500	1.1×10^{-5}
7	CH_3S	OH	OH[a]	0.0065 ± 0.0002	106 ± 9	0.0001
8	CH_3S	OH	NH_2	0.67 ± 0.01	30 ± 3	0.022

[a] Stereochemistry is *R*.

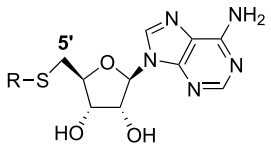
Table 2.

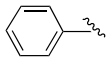
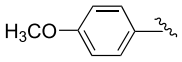
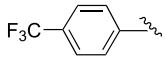
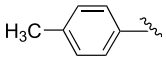
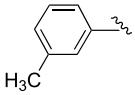
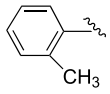


Compound #	R	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
1 (MTA)	CH_3	4.6 ± 0.2	1.8 ± 0.3	3.2
9 (ETA)	CH_3CH_2	2.9 ± 0.1	1.8 ± 0.4	1.6
10		2.3 ± 0.2	3.1 ± 1	0.74
11		5.9 ± 0.3	4 ± 0.8	1.48
12		1.46 ± 0.3	25 ± 2	0.058
13		0.85 ± 0.2	17 ± 2	0.05
14		5.5 ± 0.4	2.8 ± 0.8	1.96
15		4.2 ± 0.2	5 ± 1	0.84
16		0.23 ± 0.004	0.88 ± 0.07	0.26
17		3.44 ± 0.08	5.2 ± 0.7	0.66
18		3.16 ± 0.2	6.9 ± 1	0.46
19		1.5 ± 0.1	3 ± 0.7	0.5
20		3.8 ± 0.2	9 ± 2	0.42
21		2.49 ± 0.09	3.4 ± 0.6	0.73
22		2.5 ± 0.1	4.6 ± 1.1	0.53
23		2.14 ± 0.09	5 ± 1	0.43
24		1.01 ± 0.02	10.2 ± 1	0.09
25		1.57 ± 0.03	3.5 ± 0.2	0.45
26		1.5 ± 0.1	3.2 ± 0.5	0.47

(compound **9**). Compounds containing alkyl chains with a polarized group (compounds **12**) gave reduced catalytic activity. The MTA/MTAP co-crystal structure also showed that the MTA $C_{5'}$ position was surrounded by hydrophobic residues, which created a space larger than that expected to be occupied by a simple alkyl carbon chain. Therefore, some MTA analogues containing phenyl (**19**), pyridyl (**16**, **17**), five-membered heterocycles

Table 3.

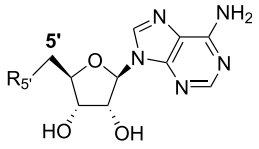


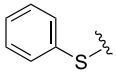
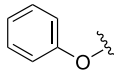
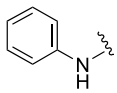
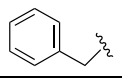
Compound #	*R*	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{1}$)
19		1.5 ± 0.1	3 ± 0.7	0.5
27		0.8 ± 0.07	7 ± 2	0.11
28		0.3 ± 0.02	1.7 ± 0.6	0.17
29		1.4 ± 0.07	1.5 ± 0.4	0.93
30		2.4 ± 0.1	2 ± 0.4	1.2
31		0.18 ± 0.007	5.6 ± 0.7	0.03

(**20**, **21**), quinoline (**18**), benzyl (**22**, **23**, **24**), and saturated carbocyclic rings (**25**, **26**) at the $C_{5'}$ position were also evaluated (Table 2). Several novel MTAP substrates were discovered, which exhibited k_{cat}/K_m values between 0.3 and 0.8. Compounds containing saturated carbocyclic moieties were no better or worse substrates than their aromatic counterparts (compare **25** with **19**, and **26** with **20** and **21**).

Due to the ready synthetic availability of derivatives and analogues, we decided to focus additional SAR studies on improving compound **19**. In Table 3, compounds **27**–**31**, which have different substituents on the phenyl

Table 4.



Compound #	$R_{5'}$	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
19		1.5 ± 0.1	3 ± 0.7	0.5
32		1.11 ± 0.01	12 ± 1	0.09
33		2.02 ± 0.03	8.5 ± 0.6	0.24
34		4.6 ± 0.3	1.3 ± 0.3	3.5

moiety were synthesized and compared. Compounds **29** and **30** displayed similar k_{cat}/K_m values that were approximately 2-fold greater than that exhibited by **19** (note however that all these values are within the experimental error of the assay). Compounds **27** and **28** contain polarized substituents at the *para* position of the phenyl moiety and displayed reduced catalytic activities compared with compound **19**. The 10-fold decrease in k_{cat} displayed by compound **31** may be due to the bulky methyl group present at the *ortho* position of the phenyl moiety.

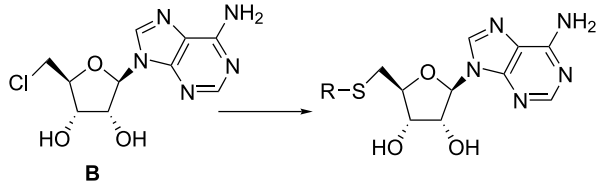
We then chose compound **19** to explore the possibility of replacing the C5′-sulfur atom with oxygen, nitrogen, and carbon atoms. As shown in Table 4, the carbon analogue of compound **19** (compound **34**) gave the greatest k_{cat}/K_m value (3.5 ± 0.3), which closely resembles that exhibited by MTA (k_{cat}/K_m = 3.2).

Compounds **3**, **4**, **5**, and **7** were synthesized by following the reported literature methods.[10] The synthesis of compounds **6** and **8** are described in Scheme 1. Intermediate **A** was prepared via the method described by Gavagnin and Sodano.[11] The $C_{2'}$-hydroxy group was inverted by a S_N2 reaction with sodium acetate then was inverted again with sodium azide. Following deprotection with *tert*-butyl ammonium fluoride (TBAF) to give compound **6**. Compound **6** was then reduced with triphenylphosphine to give compound **8**.

The synthetic approach to the described series of 5′-thioether analogues involves (1) preparation of an alkyl chloride, that is, 5′-deoxy-5′-chloroadenosine; intermediate **B**, which was prepared via the method reported by Robins.[12] (2) reaction of this chloro derivative with appropriate thioalcohols or thiophenols (Scheme 2).

The preparation of compound **32** began with the Mitsunobu reaction of the commercially available 2′,3′-*O*-isopropylideneadenosine and phenol. Subsequent acetic acid deprotection of the isopropylidene group afforded compound **32** in 40% yield (Scheme 3).[13]

Compound **33** was synthesized from intermediate **B** using a modified literature condition.[14] Compound **34** was synthesized through the aldehyde intermediate **C**, which was prepared by following the procedure reported

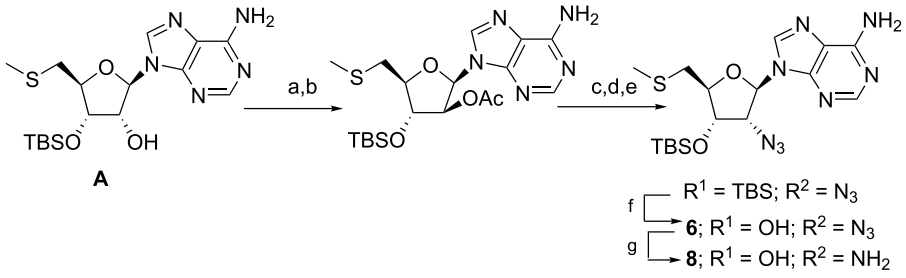


Scheme 2. Reagents and conditions (R see Tables 2 and 3): (a) $K^{+-}Ot$-Bu (1 M in THF), RSH, DMF, 40 °C, 12 h, 40–80%.

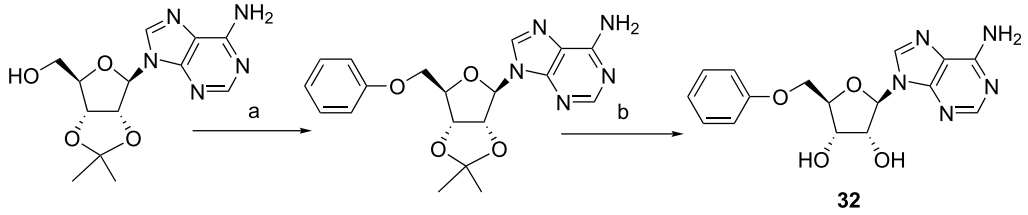
by Montgomery et al.[15] Intermediate **C** was then reacted with a Wittig reagent to give intermediate **D**. Intermediate **D** was reduced by hydrogenation using Pd/C and then fully deprotected to give compound **34** (Scheme 4).

In conclusion, the $C_{5'}$ position of adenosine could tolerate a variety of substituents, however, the $C_{2'}$ and $C_{3'}$ positions are relatively conserved with respect to substrate activity. During the process of probing the C_5 space for MTAP enzyme, we discovered several novel MTAP substrates, especially the carbon analogue (compound **34**), which displays k_{cat}/K_m values similar to the natural substrate (MTA). Those novel MTAP substrates might provide alternative therapeutic strategies for the use of cytotoxic anti-cancer agents.

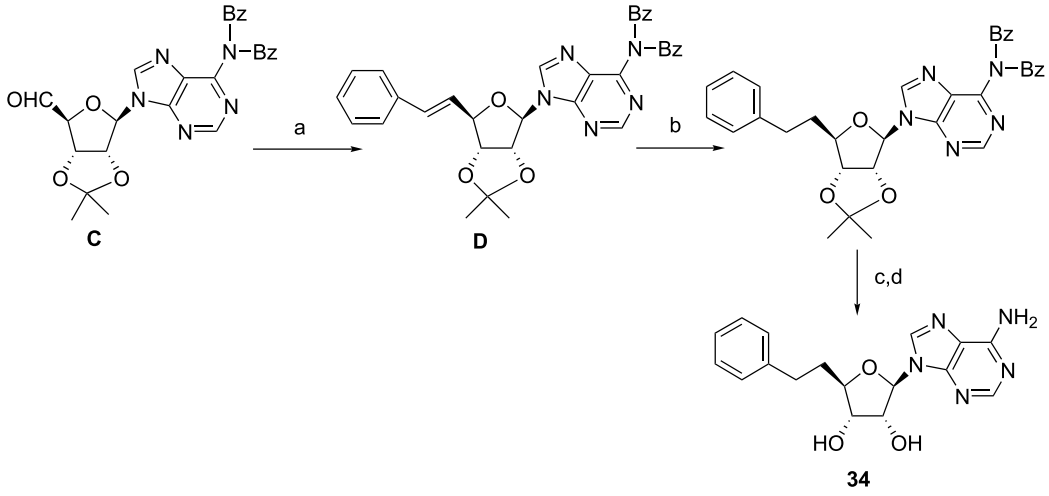
Methylthioadenosine phosphorylase (MTAP) enzymatic assay: Human MTAP containing an N-terminal six-histidine tag was expressed in *E. coli* BL21 DE3 cells. The protein was purified to homogeneity by Ni^{2+} affinity chromatography. Enzymatic activity was measured using a coupled spectrophotometric assay designed to monitor the reaction product adenine.[16] Various concentrations of the indicated 5′-deoxy-5′-methylthioadenosine (MTA) substrate analog were incubated in assay buffer (40 mM potassium phosphate buffer, 1 mM, and DTT 0.8 units/ml xanthine oxidase coupling enzyme) for 5 min at 37 °C. The reaction was initiated by the addition of MTAP. The exact concentration of enzyme used varied for each substrate tested and ranged from 2 to 500 nM. Activity as a function of enzyme concentration was determined for each substrate tested to ensure that the appropriate enzyme concentration was used. Activity was detected by continuous monitoring of absorbance at 305 nm for 10 min (ΔE = 15,500 M^{-1}). Initial velocities were calculated by linear regression. K_m



Scheme 1. Reagents and conditions: (a) triflic anhydride, DMAP, pyridine, DCM, 0 °C, 1 h, 40%; (b) NaOAc, HMPA, 40 °C, 1 h, 22%; (c) NH_3/MeOH, 23 °C, 20 min; (d) triflic anhydride, DMAP, pyridine, DCM, 0 °C, 3 h; (e) NaN_3, DMF, 23 °C, 16 h; (f) TBAF, THF, 23 °C, 30 min, 18% over three steps; (g) PPh_3, pyridine, 23 °C, 18 h, 40%.



Scheme 3. Reagents and conditions: (a) PPh_3, PhOH, diisopropyl azodicarboxylate (DIAD), THF, 23 °C, 18 h, 40%; (b) 80% acetic acid, 100 °C, 6 h, 40%.



Scheme 4. Reagents and conditions: (a) benzyltriphenyl phosphonium chloride, sodium bis(trimethylsilyl)amide (1 M in THF), THF, 55 °C, 3 h, 46%; (b) ethyl acetate, Pd/C, H_2, 4 h; (c) ethanol, $CH_3NH–NH_2$, 55 °C, 12 h, 88%; (d) 80% acetic acid, 100 °C, 6 h, 42.6%.

and k_{cat} values were determined by fitting initial velocity data to the Henri–Michaelis–Menton equation.

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