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# 5′-DEOXY-5′-METHYLTHIOADENOSINE PHOSPHORYLASE—V

## ACYCLOADENOSINE DERIVATIVES AS INHIBITORS OF THE ENZYME

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**Abstract**—Various adenosine acyclonucleoside derivatives were tested as inhibitors of 5′-deoxy-5′-methylthioadenosine (MeSAdo) phosphorylase, an enzyme involved in the salvage of adenine and methionine from MeSAdo. The 2-halogenated derivatives of acyloadenosine [9-(2-hydroxyethoxymethyl)adenine], including the chloro-, bromo- and iodo-congeners, all inhibited murine Sarcoma 180 (S180) MeSAdo phosphorylase, with $K_i$ values in the range of $10^{-6}$ to $10^{-5}$ M. Halogenated derivatives of 9-(1,3-dihydroxy-2-propoxymethyl)adenine, which more closely resemble the natural substrate, were substantially more potent inhibitors of the enzyme, with $K_i$ values in the range of $2–7 \times 10^{-7}$ M. 5′-Methylthio and 5′-halogenated analogs of 2′-deoxy-1′,2′-*seco*-adenosine were weak inhibitors, with $K_i$ values of $10^{-4}$ M or greater. 9-[(1-Hydroxy-3-iodo-2-propoxy)methyl]adenine. (HIPA), the derivative with the lowest $K_i$ value among these analogs, was a competitive inhibitor of S180 MeSAdo phosphorylase. In preliminary studies, HIPA inhibited MeSAdo phosphorylase in intact HL-60 human promyelocytic leukemia cells, as it limited the incorporation of [8-$^{14}$C]MeSAdo into cellular adenine nucleotide pools. In addition, 9-(phosphonoalkyl)adenines, representing potential multisubstrate inhibitors of MeSAdo phosphorylase, were synthesized. Of these the heptyl derivative was the most potent inhibitor, with a $K_i$ of $1.5 \times 10^{-5}$ M at low (3.5 mM) phosphate concentrations. The inhibitory effects of these analogs could be ablated at high phosphate concentrations (50 mM), suggesting that they interact with the phosphate binding site on the enzyme. Some of these novel MeSAdo phosphorylase inhibitors may have a role in cancer chemotherapy as potentiators of agents that block purine *de novo* synthesis, e.g. antifolates and 6-methylmercaptopurine ribonucleoside.

5′-Deoxy-5′-methylthioadenosine(MeSAdo‡),which is produced from decarboxylated *S*-adenosylmethionine during the synthesis of the polyamines spermidine and spermine, is phosphorolytically cleaved by the enzyme MeSAdo phosphorylase in the following reaction [1]: MeSAdo + $P_i$ ⟷ Adenine + 5 - methylthioribose - 1 - phosphate MeSAdo phosphorylase, which is found in virtually all normal mammalian tissues, but is absent from a number of malignant tissues [2–5], has at least two functions: (1) to enable the salvage of adenine back into adenine nucleotide pools via adenine phosphoribosyltransferase (EC 2.4.2.7) [6, 7], and the conversion of 5-methylthioribose-1-phosphate to methionine [8–12], and (2) to limit the intracellular accumulation of MeSAdo, which is growth inhibitory at high concentrations [13].

One rationale for developing inhibitors of MeSAdo phosphorylase is that such inhibitors would limit the recycling of adenine from MeSAdo into purine pools, and thereby might potentiate the antiproliferative effects of agents that act by inhibiting purine *de novo* synthesis, e.g. antifolates, 6-methylmercaptopurine ribonucleoside, and azaserine [14]. This is based on the observation that exogenous MeSAdo relieves the antipurine actions of methotrexate in MeSAdo phosphorylase-containing cell lines, but not in cell lines that lack this enzyme [15]. Previous efforts have led to the identification of several potent inhibitors of mammalian MeSAdo phosphorylase, including the 5′-halogenated formycins [16] and 5′-substituted derivatives of 9-deazaadenosine, e.g. 5′-methylthio-9-deazaadenosine [17]. Unfortunately, the chemical syntheses involved with these classes of C-nucleosides are difficult and employ costly starting materials, making these inhibitors expensive to develop as potential clinical agents. The 7-deaza analog of MeSAdo, 5′-deoxy-5′-methylthiotubercidin, is another known inhibitor of MeSAdo phosphorylase [18]; however, its inhibitory potency is relatively low ($K_m/K_i \simeq 1$).

In the search for potent inhibitors of MeSAdo phosphorylase, we turned out attention to acyclic derivatives of adenosine. This was based on findings that nucleoside-like analogs containing open-chain or "acyclo" structures in the place of the ribose moiety inhibit other mammalian nucleoside phosphorylases. Niedzwicki *et al.* [19, 20] identified acyclouridine [1-(2-hydroxyethoxymethyl)uracil] and its

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‡ Abbreviations: ADA, adenosine deaminase; DHPA, 9-(1,3-dihydroxy-2-propoxymethyl)adenine; HIPA, 9-[(1-hydroxy-3-iodo-2-propoxy)methyl]adenine; MeSAdo, 5′-deoxy-5′-methylthioadenosine; and PNP, purine nucleoside phosphorylase.

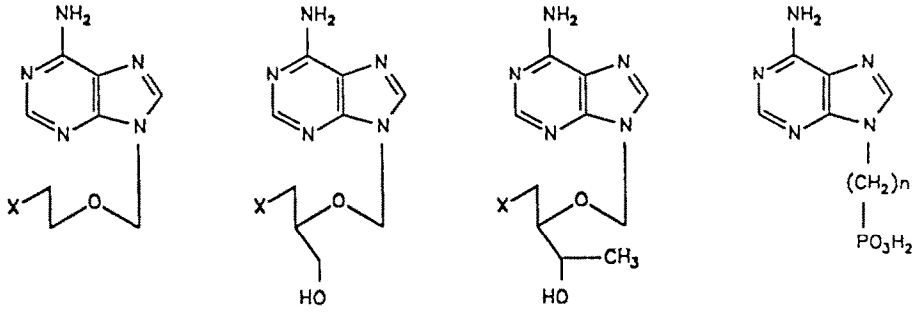


Fig. 1. Structures of acyclic nucleosides studied as inhibitors of MeSAdo phosphorylase. Series I, derivatives of 9-(2-hydroxyethoxymethyl)adenine: X = -OH, -H, Cl, -Br, and -I; Series II, derivatives of (±)9-(1,3-dihydroxy-2-propoxymethyl)adenine; X = -OH, -Cl, -Br, and -I; Series III, derivatives of 2′-deoxy-1′,2′-*seco*-adenosine: X = -OH, -Cl, Br, and -$SCH_3$; and Series IV, 9-(phosphonoalkyl)adenines: $n$ = 3, 4, 5, 6, and 7.

derivatives as specific competitive inhibitors of murine and human uridine phosphorylase (EC 2.4.2.3). Also, Tuttle and Krenitsky [21] reported that the antiherpetic acyclonucleoside, acyclovir [9-(2-hydroxyethoxymethyl)guanine], and its corresponding mono-, di-, and triphosphate esters inhibit human purine nucleoside phosphorylase (PNP; EC 2.4.2.1). This observation was extended by Stein *et al.* [22], who demonstrated that other acyclovir-related compounds such as 9-(1,3-dihydroxy-2-propoxymethyl)guanine inhibit human erythrocytic PNP. In the present study, we examined a number of analogs of acycloadenosine as inhibitors of MeSAdo phosphorylase.

## MATERIALS AND METHODS

*Materials.* MeSAdo, adenine, and xanthine oxidase (Grade III) were obtained from Sigma (St. Louis, MO). [8-$^{14}$C]-5′-Deoxy-5′-methylthioadenosine (56 μCi/μmol) was purchased from Moravek Biochemicals (Brea, CA). 9-(2-Hydroxyethoxymethyl)adenine and 9-(1,3-dihydroxy-2-propoxymethyl)adenine were obtained from Dr. Richard L. Tolman of Merck, Sharp & Dohme Research Laboratories, Rahway, NJ. Syntheses of the other acycloadenosine derivatives are described elsewhere in the form of an extended abstract [23]. While compounds of Series II (see Fig. 1) are racemic, those of Series III, the 1′,2′-*seco*-adenosines, are chiral and have the *S*- and *R*-absolute configurations at carbons 3′- and 4′- respectively. The acyclic moiety of these nucleosides was prepared from suitably protected 2*R*,3*S*-1,2,3-butanetriol, a chiron derived from D-isoascorbic acid according to published procedures [24]. The optical rotations ($[\alpha]_D^{25}$ in EtOH) and melting points of the chiral compounds of Series III were as follows: 2′-deoxy-, −11.73, $c$ = 1.54, m.p. = 145–146°; 2′,5′-dideoxy-5′-methylthio-, +26.08, $c$ = 0.93, m.p. = 88–89°; 5′-chloro-2′,5′-dideoxy-, −0.695, $c$ = 1.495, m.p. = 125–126°; and 5′-bromo-2′,5′-dideoxy-, −0.92, $c$ = 1.27, hygroscopic. Additional details concerning the syntheses of each series of these acyclic nucleosides can be obtained from the authors.

*Preparation of partially purified Sarcoma* 180 *MeSAdo phosphorylase.* Murine Sarcoma 180 ascites cells were obtained from female CD1 mice 6–7 days after i.p. inoculation with approximately 2.5 × $10^6$ cells. Cells were washed in physiological saline and centrifuged, and the cell pellet was resuspended in 1 vol. of 25 mM imidazole, 1 mM dithiothreitol, pH 7.4 (Buffer A). The cell suspension was sonicated, and centrifuged at 105,000 *g* for 1 hr at 4°. The supernatant fluid was then loaded onto a 1.6 × 72 cm chromatofocusing column containing approximately 45 mL of Polybuffer exchanger 94 resin (Pharmacia) equilibrated with Buffer A. The column was pretreated with 30 mL of 8-fold-diluted Polybuffer 74 (Pharmacia), 1 mM dithiothreitol, pH 4.0, before the enzyme preparation was added. MeSAdo phosphorylase was eluted using the latter buffer at a column flow rate of 0.75 mL/min (8.0-mL fractions were collected). The enzyme activity, monitored by a previously described spectrophotometric assay [6], elutes in a single peak at pH 6.0–5.5. MeSAdo phosphorylase-containing fractions were pooled and adjusted to pH 7.4 with potassium hydroxide, and the enzyme was concentrated by negative pressure dialysis against Buffer A using a 75,000 dalton cut-off membrane. The preparation was then dialyzed against Buffer A to remove traces of Polybuffer 74. The resulting samples were placed in aliquots and stored at −80°.

*Enzyme inhibition assays.* Unless noted otherwise, inhibition studies were carried out in reaction mixtures (200 μL) containing 50 mM potassium phosphate, pH 7.4, 5 μM [8-$^{14}$C]-5′-deoxy-5′-methylthioadenosine (0.15 μCi/nmol), 0.075 nmol units of partially purified Sarcoma 180 MeSAdo phosphorylase (1 nmol unit is defined as the amount of enzyme that phosphorolyzes 1 nmol of MeSAdo/min), and various amounts of acycloadenosine-like analog. These reaction mixtures were incubated for periods of up to 5 min at 37° in a shaking water bath. The reaction was stopped with 40 μL of ice-cold 20%

perchloric acid. After neutralization with potassium hydroxide, 20-μL samples were spotted onto Baker microcrystalline cellulose thin-layer chromatography plates that had been prespotted with authentic adenine and MeSAdo, which serve as carriers. The plates were developed at 4° in distilled water. The $R_f$ values of adenine and MeSAdo in this system are 0.21 and 0.41 respectively. Spots were visualized with a 254 nm wavelength lamp, cut out, and quantitated by liquid scintillation counting using Betafluor (National Diagnostics, Manville, NJ) as a scintillant. Apparent $K_i$ values were estimated from Dixon plots (1/reaction velocity vs inhibitor concentration) [25] using least squares fitting. $K_i$ values were determined from the equation $K_{i(app)} = K_{is}(1 + [S]/K_m)$; the $K_m$ value for MeSAdo was 2 μM.

*Incorporation of [8-$^{14}$C]MeSAdo into adenine nucleotide pools of HL-60 human promyelocytic leukemia cells.* HL-60 human promyelocytic leukemia cells [26] were cultured as described previously [27]. HL-60 cells cultured in RPMI 1640 medium supplemented with 10% fetal calf serum were harvested by centrifugation, washed twice in physiological saline, then resuspended in a minimal salt medium consisting of 50 mM potassium phosphate, pH 7.4, 75 mM sodium chloride, 2 mM magnesium chloride, 10 mM dextrose, 5 μM [8-$^{14}$C]MeSAdo (0.5 μCi/mL), and either 45 μM 9-[(1-hydroxy-3-iodo-2-propoxy)methyl]adenine (HIPA) or no addition. Final cell concentration was $1 \times 10^6$ cells/mL (10 mL total volume). After a 2-hr incubation in a shaking water bath, the cells were centrifuged, the supernatant fraction was discarded, and the cell pellet was resuspended in 0.75 mL of 6% perchloric acid on ice. After the addition of 0.1 mL of 500 mM potassium phosphate, pH 7.4, the samples were neutralized with 10 N potassium hydroxide. The nucleotide pools of the entire sample were analyzed by anion-exchange high performance liquid chromatography as previously described [28], at a flow rate of 1.6 mL/min. The eluent was collected in a fraction collector, 1 fraction/min; to each fraction, 8 mL of Aquasol (New England Nuclear, Boston, MA) was added, and the samples were counted by liquid scintillation spectrometry. Various nucleotide peaks were identified based on their retention time, as compared to authentic nucleotide standards.

## RESULTS

*Acyclic adenosine nucleosides as inhibitors of MeSAdo phosphorylase.* The structures of the adenosine-like acyclic derivatives that were synthesized and tested here are shown in Fig. 1. The $K_i$ values of three classes of these analogs for murine Sarcoma 180 MeSAdo phosphorylase are presented in Table 1. The first group includes acycloadenosine [9-(2-hydroxyethoxymethyl)adenine] and various 2-substituted congeners. Although acycloadenosine itself was inactive, its 2-deoxy derivative, i.e. 9-(2-ethoxymethyl)adenine, inhibited the enzyme, albeit weakly ($K_i = 74$ μM). The 2-deoxy-2-halogenated derivatives, including the -chloro, -bromo, and -iodo analogs, were inhibitors of MeSAdo phosphorylase, with $K_i$ values in the range of 3.6 to $9.0 \times 10^{-6}$ M. Of these, the iodo derivative was the most potent. These findings resemble the relative affinities of the enzyme for the corresponding 5′-substituted ribonucleosides, i.e. adenosine binds only very weakly to the enzyme, whereas the 5′-halogenated adenosines interact more strongly, with $K_m$ values in the following order: 5′-deoxy-5′-iodoadenosine < 5′-bromo-5′-deoxyadenosine < 5′-chloro-5′-deoxyadenosine [29, 30].

The second series includes 9-(1,3-dihydroxy-2-propoxymethyl)adenine (DHPA) and related compounds (see Fig. 1 for structures), which are analogous to the antiviral drug ganciclovir. These compounds more closely approximate the structure of the natural substrate than do the acycloadenosine derivatives in that they possess the equivalent of a 3′-carbon, along with its hydroxy group. Once again the parent compound DHPA was inactive, but each of the derivatives in which one of the hydroxyl groups was replaced by a halogen atom inhibited S180 MeSAdo phosphorylase: in fact, the $K_i$ values of the enzyme for these monohalogenated derivatives of DHPA were about one order of magnitude lower than those for the corresponding 2-deoxy-2-halogenated derivatives of acycloadenosine. The importance of the equivalent of the C(3′) hydroxyl group in determining enzyme binding is emphasized by the finding that replacement of both hydroxyl groups of DHPA with halogen atoms, e.g. 9-(1,3-dichloro-2-propoxymethyl)adenine, greatly decreased enzyme affinity. The $K_i$ of the S180 MeSAdo phosphorylase for the most potent inhibitor of this series, 9-[(1-hydroxy-3-iodo-2-propoxy)-methyl]adenine (HIPA) approximates those of some of the most active inhibitors of MeSAdo phosphorylase described to date, including 5′-halogenated formycins ($K_i$ values = $2–5 \times 10^{-7}$ M) [16] and 5′-deoxy-5′-methylthio-9-deazaadenosine ($K_i = 2 \times 10^{-7}$ M) [17].

The third series includes 2′-deoxy-1′,2′-*seco*-adenosine and its analogs (see Fig. 1 for structures). These compounds represent the next logical sequence of acyclonucleosides in that they are DHPA analogs which contain the equivalent of the C(2′) of ribose (in the form of a methyl group); they are closer in structure to MeSAdo than the DHPA analogs, in that they have the equivalent of the five carbons that make up the ribose moiety of MeSAdo. However, none of the 2′-deoxy-1′,2′-*seco*-adenosines, including the 5′-methylthio, 5′-chloro, and 5′-bromo analogs, were potent inhibitors of S180 MeSAdo phosphorylase; the $K_i$ values of these compounds were some three orders of magnitude higher than their corresponding DHPA derivatives (Table 1). None of the acyclic adenosines tested here were substrates for S180 MeSAdo phosphorylase.

One of the more active inhibitors identified in this screening study, HIPA, was studied in more detail. As shown in Fig. 2, HIPA inhibited S180 MeSAdo phosphorylase competitively with respect to MeSAdo. The specificity of HIPA for MeSAdo phosphorylase was tested by examining its reactivity with other adenine nucleoside-metabolizing enzymes. *S*-Adenosylhomocysteine hydrolase (EC 3.3.1.1), which catalyzes the hydrolysis of *S*-adenosylhomocysteine to homocysteine and adenosine, has been shown to be irreversibly inactivated by MeSAdo [31–

Table 1. Inhibition constants of some acyclic adenosine analogs with Sarcoma 180 MeSAdo phosphorylase

| Compound | $K_i$ ($\mu$M) |
|---|---|
| 9-(2-Hydroxyethoxymethyl)adenine analogs | |
| 9-(2-Hydroxyethoxymethyl)adenine | >170 |
| 9-(2-Ethoxymethyl)adenine | 73.5 ± 0.7 |
| 9-(2-Chloroethoxymethyl)adenine | 9.0 ± 0.0 |
| 9-(2-Bromoethoxymethyl)adenine | 4.9 ± 0.3 |
| 9-(2-Iodoethoxymethyl)adenine | 3.6 ± 1.8 |
| 9-(1,3-Dihydroxy-2-propoxymethyl)adenine analogs | |
| 9-(1,3-Dihydroxy-2-propoxymethyl)adenine | >100 |
| 9-(1,3-Dichloro-2-propoxymethyl)adenine | >14 |
| 9-(1-Chloro-3-hydroxy-2-propoxymethyl)adenine | 0.7 ± 0.0 |
| 9-(1-Bromo-3-hydroxy-2-propoxymethyl)adenine | 0.6 ± 0.5 |
| 9-(1-Hydroxy-3-iodo-2-propoxymethyl)adenine | 0.3 ± 0.0 |
| 2′-Deoxy-1′,2′-*seco*-adenosine analogs | |
| 2′-Deoxy-1′,2′-*seco*-adenosine | >300 |
| 2′,5′-Dideoxy-5′-methylthio-1′,2′-*seco*-adenosine | 285 ± 3 |
| 5′-Chloro-2′,5′-dideoxy-1′,2′-*seco*-adenosine | 191 ± 16 |
| 5′-Bromo-2′,5′-dideoxy-1′,2′-*seco*-adenosine | 129 ± 11 |

Values are the averages ± the range of two determinations.

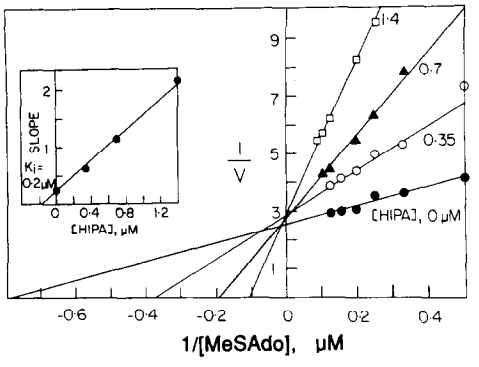


Fig. 2. Double-reciprocal plot for the inhibition of murine Sarcoma 180 MeSAdo phosphorylase by 9-[(1-hydroxy-3-iodo-2-propoxy)methyl]adenine (HIPA). Key: (●) 0 $\mu$M HIPA; (○) 0.35 $\mu$M HIPA; (▲) 0.7 $\mu$M HIPA; and (□) 1.4 $\mu$M HIPA. $V$ = nmol MeSAdo converted/5 min.

33] and MeSAdo-like compounds [34, 35]. Nevertheless, *S*-adenosylhomocysteine activity in extracts of CCRF-CEM human T-cell leukemias was not inhibited by 100 $\mu$M HIPA, even after a 30-min incubation period. However, all the analogs of acycloadenosine and DHPA examined were weak alternative substrates for adenosine deaminase (ADA; EC 3.5.4.4): the reaction rates of ADA from calf intestinal mucosa for the DHPA analogs (at 100 $\mu$M) were only 0.16 to 0.23% that of the natural substrate, adenosine. The $K_m$ and $V_{max}$ values (relative to adenosine) of calf intestinal ADA for HIPA were determined to be 199 ± 20 $\mu$M and 0.48% respectively. The 2-deoxy-2-halogenated acycloadenosine compounds were still less active as substrates of ADA: the relative velocities at 100 $\mu$M substrate levels were in the range of 0.006 to 0.022% that of adenosine. The derivatives of 2′-deoxy-1′,2′-*seco*-adenosine were also poor alternative substrates of ADA (relative velocities at 100 $\mu$M were 0.047 to 0.13% that of adenosine).

If acyclic nucleosides such as HIPA inhibit MeSAdo phosphorylase in intact cells, then these inhibitors should block the incorporation of exogenous MeSAdo into adenine nucleotide pools. Indeed, in preliminary experiments, 45 $\mu$M HIPA inhibited the incorporation of 5 $\mu$M [8-$^{14}$C]MeSAdo (0.5 $\mu$Ci/mL) into the adenine nucleotide pools of MeSAdo phosphorylase-containing HL-60 human promyelocytic leukemia cells. Approximately 13,950 total cpm were incorporated into adenosine 5′-monophosphate pools after 2 hr in untreated HL-60 cells versus approximately 5,350 cpm in HIPA-treated cells (62% inhibition); approximately 78,650 total cpm versus approximately 29,670 cpm were incorporated into adenosine 5′-diphosphate pools in untreated and HIPA-treated cells respectively (63% inhibition), and approximately $1.303 \times 10^6$ total cpm versus approximately 418,600 cpm were incorporated into adenosine 5′-trisphosphate pools in untreated and HIPA-treated cells respectively (68% inhibition). It is unlikely that HIPA produced these effects by blocking the uptake of MeSAdo, since MeSAdo is a relatively lipophilic nucleoside that enters cells via diffusion as well as the nonspecific nucleoside transport system [36]; indeed, the nucleoside transport inhibitor, dipyridamole, is unable to block the conversion of exogenous MeSAdo to methionine in HL-60 cells, presumably owing to the high rate of passive diffusion of MeSAdo into these cells [36]. Furthermore, Mahony *et al.* [37] have demonstrated that ganciclovir, a guanine acyclic nucleoside analogous to HIPA, is only a very weak competitive inhibitor of the nucleoside transport system of human erythrocytes, with a $K_i$ value of 10 mM. These data therefore suggest that this acyclic nucleoside derivative

Table 2. Inhibition constants of 9-(phosphonoalkyl)-adenines with murine Sarcoma 180 MeSAdo phosphorylase

| Compound | $K_i$(μM) |
|---|---|
| 9-(3-Phosphonopropyl)adenine | >250 (2) |
| 9-(4-Phosphonobutyl)adenine | >250 (2) |
| 9-(5-Phosphonopentyl)adenine | 69.6 ± 46.5 (6) |
| 9-(6-Phosphonohexyl)adenine | 43.0 ± 0.2 (2) |
| 9-(7-Phosphonoheptyl)adenine | 15.6 ± 8.0 (3) |

$K_i$ values were determined by using a modification of a previously described coupled spectrophotometric assay [6]. Reaction mixtures (1 mL final volume) consisting of 3.5 mM potassium phosphate, pH 7.4, 21 μM 5′-deoxyadenosine (an alternative substrate of MeSAdo phosphorylase [6]), 1 nmol unit of partially-purified Sarcoma 180 MeSAdo phosphorylase, 1 μM unit buttermilk xanthine oxidase (Sigma, Type III) and various amounts of 9-(phosphonoalkyl)adenines were monitored at 305 nm at 37°. $K_i$ values were determined using Dixon plots as described under Materials and Methods. Numbers in parentheses are the number of independent determinations; values represent the mean ± SD where N > 2, and the mean ± range where N = 2.

can enter cells and inhibit the expected target enzyme.

*9-(Phosphonoalkyl)adenines as inhibitors of MeSAdo phosphorylase.* One approach to develop a potent MeSAdo phosphorylase inhibitor is to construct compounds that incorporate the elements of the two substrates of the enzyme, MeSAdo and orthophosphate, into one molecule, i.e. a "multisubstrate" inhibitor. The strategies involved in designing multisubstrate inhibitors have been outlined recently by Broom [38]. With this idea in mind, a series of compounds were synthesized containing an adenine base with an acyclic tail consisting of an alkyl chain terminated by a phosphonic acid moiety (see Fig. 1). The design of these 9-(phosphonoalkyl)adenines, which perhaps represent the simplest multisubstrate inhibitors of this enzyme, is based on the findings that (1) acyclic adenosine derivatives are capable of binding to MeSAdo phosphorylase (see above), and (2) 9-(phosphonoalkyl)hypoxanthines inhibit purine nucleoside phosphorylase, the only other mammalian enzyme that phosphorolyzes purine nucleosides [39]. As shown in Table 2, at relatively low potassium phosphate concentrations (3.5 mM), the higher alkyl derivatives (pentyl, hexyl and heptyl) inhibited Sarcoma 180 MeSAdo phosphorylase with $K_i$ values in the range of $10^{-5}$–$10^{-4}$ M; 9-(7-phosphonoheptyl)adenine was the most potent inhibitor of this series. On the other hand, the shorter alkyl chain derivatives, i.e. the propyl and butyl derivatives, were relatively inactive. The inhibitory effects of pentyl, hexyl and heptyl phosphonates on MeSAdo phosphorylase were abolished in the presence of 50 mM potassium phosphate (data not shown), suggesting that these compounds interact with the phosphate binding site on the enzyme.

## DISCUSSION

We have found that various adenosine acyclic nucleosides, including hydroxy-substituted derivatives of 9-(2-hydroxyethoxymethyl)adenine (acycloadenosine) and 9-(1,3-dihydroxy-2-propoxymethyl)adenine (DHPA), as well as 9-(phosphonoalkyl)adenines, inhibit mammalian MeSAdo phosphorylase. Since all the previously reported inhibitors of this enzyme, i.e. 5′-substituted derivatives of 7-deazaadenosine [18], formycin [16], and 9-deazaadenosine [17], represent analogs of MeSAdo possessing modifications in the purine ring, these acyclonucleosides, which are ribose-modified derivatives of MeSAdo, constitute novel classes of MeSAdo phosphorylase inhibitors. Thus, MeSAdo phosphorylase becomes the third mammalian nucleoside phosphorylase reported to date that is inhibited by acyclonucleoside analogs of its natural substrate: uridine phosphorylase is inhibited by acyclouridine and its congeners [19, 20, 40], and purine nucleoside phosphorylase is inhibited by the guanosine-like acyclovir and its derivatives [21, 22].

These findings offer several insights into the nature of the pentose binding site of MeSAdo phosphorylase. Perhaps the most significant observation is that some of these acyclic nucleosides bind as well or better than their ribonucleoside counterparts. For example, 9-(2-iodoethyoxymethyl)adenine, which lacks the equivalent of both C(3′) and C(2′) and their attendant hydroxyls, binds more tightly to Sarcoma 180 MeSAdo phosphorylase ($K_i = 3.6$ μM) than its corresponding pentose-containing derivative, 5′-deoxy-5′-iodadenosine ($K_m = 8$ μM) [29], and is comparable in affinity to the natural substrate, MeSAdo ($K_m = 2$ μM). The $K_i$ value of HIPA, 0.2 μM, is about 40-fold lower than the $K_m$ value of 5′-deoxy-5′-iodadenosine, and is comparable to the most potent MeSAdo phosphorylase inhibitors identified to date, e.g. the pentose-containing 5′-deoxy-5′-iodoformycin ($K_i = 0.17$ μM) [16]. These data suggest that the flexibility of the acyclo tail permits a more favorable orientation for enzyme binding than a rigid pentose moiety.

Another striking finding was the approximately 10-fold increase in enzyme binding that was achieved when a hydroxymethyl group was added to the 2-deoxy-2-halogenated acycloadenosines to form compounds, i.e. the monohalogenated derivatives of DHPA, that possess the equivalent of the C(3′)-OH of methylthioribose (see Fig. 1). This increase in binding affinity was lost when this hydroxyl group was replaced by a halogen atom [i.e. 9-(1,3-dichloro-2-propoxymethyl)adenine], or when an additional methyl group was added to the equivalent of the 3′-carbon (i.e. the 2′-deoxy-1′,2′-*seco*-adenosine analogs). Based on this, one can presume that the C(3′)-OH plays an important role in the binding of MeSAdo and its analogs to MeSAdo phosphorylase. In contrast, the fact that acycloadenosine derivatives lacking the equivalent of the C(2′)-OH can bind to the enzyme with high affinity suggests that this portion of the pentose moiety of MeSAdo is relatively unimportant to substrate binding.

Previous structure–activity relationship studies [29] using pentose-containing MeSAdo analogs established that replacement of the 5′-methylthio group of MeSAdo with a simple hydrogen atom (i.e. 5′-deoxyadenosine) results in a modest decrease in

enzyme binding ($K_m \simeq 20\ \mu M$), whereas replacement with an hydroxyl group (i.e. adenosine) greatly diminishes enzyme binding ($K_m \simeq 1500\ \mu M$). Substituting the methylthio moiety by halogen atoms produced compounds with relatively high affinity for the enzyme ($K_m$ values $< 20\ \mu M$), with $K_m$ values decreasing as the size of the halogen atom increases (fluorine to iodine). Considering the 2-carbon of the acyclo tail of acycloadenosine to be analogous to the 5'-carbon of the methylthioribose portion of MeSAdo (see Fig. 1, series I for structure), the same general pattern of binding affinity to Sarcoma 180 MeSAdo phosphorylase was observed with acycloadenosine and its 2-deoxy derivatives, i.e. the $K_i$ of acycloadenosine, the congener of adenosine $\gg$ 9-(2-ethoxymethyl)adenine, the congener of 5'-deoxyadenosine > 9-(2-chloroethoxymethyl)adenine > 9-(2-bromoethoxymethyl)adenine > 9-(2-iodoethoxymethyl)adenine. These parallels strongly suggest that the 2-carbon and its substituent are interacting with the site on MeSAdo phosphorylase that is responsible for binding the 5'-portion of substrates.

While other investigators have tried to design multisubstrate inhibitors of purine nucleoside phosphorylase [41, 42], the studies presented here on the 9-(phosphonoalkyl)adenines represent the first attempt to develop multisubstrate inhibitors of MeSAdo phosphorylase. The pentyl, hexyl and heptyl derivatives inhibited the enzyme with $K_i$ values in the range of 70–16 $\mu M$, indicating that this approach is a potentially fruitful one. It is noteworthy that the analogs with shorter-length alkyl chains, i.e. the propyl and butyl derivatives, were inactive. Although the reasons for this are not yet understood, it is possible that the adenine and the phosphate binding sites of MeSAdo phosphorylase are separated by a distance that is greater than the length of an *n*-butane and/or that the greater degree of rotational freedom of the methylenes of the higher alkyl chains (n = 5,6,7) permits these compounds to attain the conformation needed to have both the adenine and phosphate binding sites occupied. Remarkably similar results have been obtained when 9-(phosphonoalkyl)hypoxanthines and guanines were tested as inhibitors of the related enzyme, purine nucleoside phosphorylase: 9-(2-phosphonoethyl)guanine [22] and 9-(3-phosphonopropyl)hypoxanthine [39] have $K_i$ values for human erythrocytic PNP greater than $10^{-3}$ M, whereas the hypoxanthine-containing pentyl, hexyl, and heptyl derivatives have $K_i$ values in the order of $10^{-6}$ M [39]. Further studies with additional multisubstrate analogs of this type should help in defining the relative orientations of the purine and phosphate binding sites of these phosphorylases.

By limiting the salvage of adenine from MeSAdo, inhibitors of MeSAdo phosphorylase may make cells more dependent upon purine *de novo* synthesis, and thereby may potentiate antineoplastic agents that act in whole or in part by blocking *de novo* purine synthesis. This concept is based on the observation of Kamatani *et al.* [15], who demonstrated that the antipurine effects of methotrexate (MTX) on MeSAdo phosphorylase-containing leukemic cell lines could be ameliorated by coadministration of MeSAdo, but not in leukemic lines that lack MeSAdo phosphorylase activity. Evidence has been obtained that 5'-deoxy-5'-chloroformycin, a competitive inhibitor of MeSAdo phosphorylase ($K_i$ = 0.5 $\mu M$) [16], potentiates the growth inhibitory actions of MTX in the MeSAdo phosphorylase-containing HL-60 human promyelocytic leukemia line [43]. Because of their potency and relative ease of synthesis, acyclic adenosine analogs have potential for development as potentiators of *de novo* purine synthesis inhibitors.

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