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5′-DEOXY-5′-METHYLTHIOADENOSINE PHOSPHORYLASE—IV

BIOLOGICAL ACTIVITY OF 2-FLUOROADENINE-SUBSTITUTED 5′-DEOXY-5′-METHYLTHIOADENOSINE ANALOGS*

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Abstract—5′-Deoxy-5′-methylthioadenosine phosphorylase (MTAPase) phosphorolyzes 5′-deoxy-5′-methylthioadenosine (MTA) generated during polyamine biosynthesis to adenine and 5-methylthioribose-1-phosphate. Two doubly-substituted, 2-fluoroadenine-containing analogs of MTA, 5′-deoxy-2-fluoroadenosine (5′-dFAdo) and 5′-deoxy-5′-iodo-2-fluoroadenosine (5′-IFAdo), were synthesized and studied as substrates of MTAPase: their reaction with this enzyme resulted in the liberation of the cytotoxic base, 2-fluoroadenine, as well as potentially cytotoxic analogs of 5-methylribose-1-phosphate. The activities of these MTA analogs were compared to that of the singly-substituted analog, 5′-deoxy-5′-methylthio-2-fluoroadenosine (5′-MTFAdo). The cytotoxic action of these MTA analogs depended primarily on their conversion to 2-fluoroadenine-containing nucleotides, as a cell line that contains both MTAPase and adenine phosphoribosyltransferase (APRT) activity (HL-60 human promyelocytic leukemia) readily converted these MTA analogs to 2-fluoroadenine-containing nucleotides (especially 2-fluoroadenosine triphosphate) and was highly sensitive to the growth-inhibitory effects of all three compounds (IC_{50} values in the 10^{-8} M range), whereas cell lines lacking MTAPase (CCRF-CEM human T-cell leukemia) or APRT (HL-60/*aprt*$_1$ cells) did not form analog nucleotides and were relatively insensitive to these compounds (IC_{50} values in the 10^{-5} M range). The doubly-substituted analogs were not more growth inhibitory than 5′-MTFAdo in wild type HL-60 cells as the potent effects of 2-fluoroadenine may mask the activity of the 5-methylthioribose-1-phosphate analogs generated in the reaction of these compounds with MTAPase. 5′-dFAdo and 5′-IFAdo also were irreversible inhibitors of *S*-adenosylhomocysteine hydrolase, which may explain in part the weak but observable growth inhibitory action of these compounds against MTAPase-deficient cell lines.

5′-Deoxy-5′-methylthioadenosine (MTA‡) is generated from *S*-adenosylmethionine (AdoMet) during the synthesis of the polyamines spermidine [1] and spermine [2]. In mammalian cells, MTA is phosphorolyzed by the enzyme 5′-deoxy-5′-methylthioadenosine phosphorylase (MTAPase) to yield adenine and 5-methylthioribose-1-phosphate [3–10]; these products are then salvaged to adenine nucleotides [11, 12] and methionine [13–19], respectively. Thus, MTAPase acts as a salvage enzyme in both purine and methionine metabolism (for a review, see Ref. 20).

Over the past several years we have made a systematic search for analogs of MTA that are capable of reacting with MTAPase. It was anticipated that MTA-like compounds could be found whose reaction with MTAPase would produce growth-inhibitory metabolites; such MTA analogs might have potential for development as chemotherapeutic agents. Two classes of MTA analogs that show promise as antimetabolites have been identified to date. The first class includes MTA analogs whose adenine moiety has been replaced with an adenine analog, such as 2-fluoroadenine or 2,6-diaminopurine. These compounds react with MTAPase to generate free adenine analog bases, which are then converted to cytotoxic nucleotides [21]. The second class includes compounds in which the 5′-methylthio group of MTA has been replaced by a halogen atom, e.g. 5′-bromo-5′-deoxyadenosine or 5′-deoxy-5′-iodoadenosine. Such compounds have been shown to behave as substrates of MTAPase and to exert growth inhibitory effects, especially against MTAPase-containing cell lines [22]; the metabolites responsible for the

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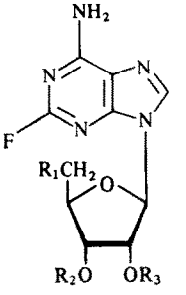
‡ Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoHcy hydrolase, *S*-adenosyl-L-homocysteine hydrolase; APRT, adenine phosphoribosyltransferase; 5′-dFAdo, 5′-deoxy-2-fluoroadenosine; 2-FATP, 2-fluoroadenosine triphosphate; 5′-IFAdo, 5′-deoxy-5′-iodo-2-fluoroadenosine; MTA, 5′-deoxy-5′-methylthioadenosine; MTAPase, 5′-deoxy-5′-methylthioadenosine phosphorylase; 5′-MTFAdo, 5′-deoxy-5′-methylthio-2-fluoroadenosine; and PNP, purine nucleoside phosphorylase.

growth inhibition are believed to be the 5-halogenated ribose-1-phosphates produced by the reaction of these derivatives with MTAPase [22].

Based on these structure–activity studies, we attempted to design "second generation" MTA analogs that might possess high antiproliferative activity. Two approaches were taken. The first involved the formulation of doubly-substituted MTA analogs that would have the potential of liberating both a cytotoxic adenine analog and a pentose-1-phosphate analog. Since MTA analogs containing 2-fluoroadenine are by far the most potent inhibitors of cell growth among the adenine-substituted compounds tested to date [21], whereas 5′-deoxy-5′-iodoadenosine is the most active among the 5′-substituted compounds yet studied [22], the hybrid compound, 5′-deoxy-5′-iodo-2-fluoroadenosine (5′-IFAdo), was conceived. The second approach was to design MTA analogs that are rapidly cleaved by MTAPase and, thereby, capable of delivering antimetabolites to cells at high rates. It was postulated that, since 5′-deoxyadenosine is a substrate for MTAPase with a V_{max} value 1.8-fold greater than that of MTA [11], the corresponding 2-fluoroadenine-containing nucleoside, 5′-deoxy-2-fluoroadenosine (5′-dFAdo), might also be cleaved rapidly. The biochemical pharmacology of these two rationally-designed MTA analogs including their activation by MTAPase and APRT is compared and evaluated in this report. Portions of this work have been published in preliminary form [23–25].



1 R_1 = OH, R_2 = R_3 = H

2 R_1 = OH, R_2, R_3 = $HCOCH_2CH_3$

3 R_1 = OTs, R_2 = R_3 = $HCOCH_2CH_3$

Fig. 1. Structures of intermediates in the synthesis of 2-fluoroadenine-substituted MTA analogs.

MATERIALS AND METHODS

Materials

5′-Deoxy-5′-methylthioadenosine, xanthine oxidase (Grade III), hexokinase, and snake venom (*Crotalus adamanteus*) were obtained from Sigma (St. Louis, MO). 5′-Deoxy-5′-iodoadenosine was purchased from the Aldrich Chemical Co. (Milwaukee, WI). 5′-Deoxyadenosine was obtained from P-L Biochemicals (Milwaukee, WI).

Chemical synthesis of the 2-fluoradenine-substituted MTA analogs was carried out as described below. Melting points were determined on a Mel-Temp apparatus and are uncorrected. NMR spectra were recorded with a Varian XL-100-15 spectrometer operating at 25.16 MHz for ^{13}C and 100.6 MHz for ^{1}H or a Nicolet NMC 300NB spectrometer operating at 75.16 MHz for ^{13}C and 300.635 MHz for ^{1}H. Chemical shifts are expressed in parts per million downfield from tetramethylsilane. Ultraviolet absorption spectra were determined on a Cary 17 spectrophotometer by dissolving the compound in methanol and diluting 10-fold with 0.1 N HCl, pH 7 buffer, and 0.1 N NaOH. Microanalyses were performed by Atlantic Microlab, Inc., Atlanta, GA, and the Molecular Spectroscopy Section, Southern Research Institute. Mass spectra were recorded on a Varian MAT 311A mass spectrometer in the fast atom bombardment (FAB) mode.

5′-Deoxy-2-fluoro-5′-iodoadenosine (5′-IFAdo). A solution of Compound **3** (2′,3′-*O*-ethoxymethylidene-2-fluoro-5′-*O*-[*p*-methylbenzenesulfonyl]adenosine) (for structure, see Fig. 1) 2.8 g, 5.6 mmol) and dry NaI (2.5 g, 16.8 mmol) in dry acetone (40 ml) was heated in a 75° bath for 16 hr. Insoluble solid was removed by filtration, and the filtrate was evaporated to dryness. This crude residue (2 g) was diluted with 0.06 N HCl (95 ml) and stirred at room temperature for 2 days. After some insoluble solid was removed, the reaction was evaporated to dryness, triturated with dilute ethanolic ammonia, evaporated, and crystallized after charcoal treatment from hot MeOH to give 679 mg of crude product. Pure material was obtained after four recrystallizations from MeOH, yield 368 mg (17%); m.p. 197° (dec). TLC: 5:1 $CHCl_3$–MeOH, R_f 0.65; MS (FAB) m/z 396 $(M + 1)^+$; UV λ_{max}, nm ($\varepsilon \times 10^{-3}$) [pH 1] 262 (14.5), 268 (sh), [pH 7] 261 (15.1), 268 (sh), [pH 13] 262 (15.3), 268 (sh); ^{1}H NMR (100 MHz, Me_2SO-d_6) δ 3.36–3.72 (m, 2, H-5′a,5′b), 3.94–4.27 (n, 2, H-3′,4′), 4.75 (m, 1, H-2, $J_{2',3'}$ = 4.4 Hz), 5.46 (d, 1, 3′-OH, J = 4.5 Hz), 5.58 (d, 1, 2′-OH, J = 6 Hz), 5.86 (d, 1, H-1′, $J_{1',2'}$ = 6 Hz), 7.84 (br s, 2, NH_2), 8.35 (s, 1, H-8); ^{13}C NMR (25 MHz, Me_2SO-d_6) δ 7.56 (C-5′), 72.80, 73.09 (C-2′,3′), 83.84, 87.56 (C-1′,4′), 117.54 (C-5), 140.10 (C-8), 150.70 (C-4), 157.67 (C-6), 158.60 (C-2), J_{C_2F} = 204.2 Hz, J_{C_4F} = 20.3 Hz, J_{C_5F} = 4.2 Hz, J_{C_6F} = 21.3 Hz, J_{C_8F} = 1.7 Hz. Anal. Calc. for $C_{10}H_{11}FIN_5O_3$*CH_3OH: C, 30.93; H, 3.54; N, 16.39. Found: C, 30.87; H, 3.28; N, 16.63.

5′-Deoxy-2-fluoroadenosine (5′-dFAdo). The synthesis of 5′-deoxy-2-fluoroadenosine has been reported previously [26]. This procedure involved the coupling of 1,2,3-tri-*O*-acetyl-5-deoxy-D-ribofuranose and 2,6-dichloropurine by a fusion procedure to produce 9-(2,3-di-*O*-acetyl-5-deoxy-β-D-ribofuranosyl)-2,6-dichloropurine and its anomer. The β anomer was then carried on by a standard series of transformations to the target compound. We have modified the procedure in order to avoid obtaining significant quantities of the α anomer by carrying out the coupling to form the nucleoside through a mercuric cyanide catalyzed condensation

between 2,6-dichloropurine and 1-chloro-2,3-di-*O*-acetyl-D-ribofuranose under standard conditions. The procedure for this step is provided below.

9-(2,3-Di-O-acetyl-5-deoxy-β-D-ribofuranosyl)-2,6-dichloropurine. To a mixture of mercuric cyanide (10.28 g, 40.7 mmol) and 2,6-dichloropurine (6.75 g, 35.7 mmol) in 615 ml of molecular sieve-dried nitromethane was added a solution of 1-chloro-2,3-di-*O*-acetyl-5-deoxy-D-ribofuranose (35.35 mmol, prepared from 1,2,3-tri-*O*-acetyl-5-deoxy-D-ribofuranose with hydrogen chloride gas in ether for 3 days) in nitromethane. The mixture was stirred at reflux until thin-layer chromatographic analysis showed that the chlorosugar was no longer present (*ca.* 3 hr). The mixture was filtered through Celite and evaporated to dryness. The residue was dissolved in chloroform (500 ml) and washed with 30% potassium iodide (2 × 100 ml) and water. The dried ($MgSO_4$) organic layer was evaporated to dryness and crystallized from ethanol, affording 4.53 g (31.4%) of the β anomer, m.p. 88–90°. The filtrate, which did not crystallize, weighed 9.8 g and was also suitable for carrying on to the next step. ^1H NMR (300 MHz, $CDCl_3$) δ 1.55 (d, 3, 5'-CH_3, J = 7 Hz), 2.10, 2.17 (2 s, 6, 2 $COCH_3$), 4.38 (m, 1, H-4'), 5.35 (m, 1, H-3'), 5.83 (m, 1, H-2'), 6.13 (d, 1, H-1', J = 5.5 Hz), 8.22 (s, 1, H-8).

5'-Deoxy-2-fluoro-5'-methylthioadenosine (5'-MTFAdo). A solution of Compound **3** (2.4 g, 4.8 mmol) in dry dimethylformamide (DMF) (60 ml) at room temperature was treated in one portion with $NaSCH_3$ (390 mg, 5.6 mmol). The reaction in a tightly sealed flask was held in a preheated 80° bath for 12 hr before it was evaporated to dryness, dissolved in $CHCl_3$, washed with H_2O, and dried over $MgSO_4$. After the solvent was evaporated, the residue was triturated with 0.06 N HCl (95 ml) for 3 days. The black tar present solidified after addition of EtOH and was collected after a 24-hr trituration. The filtrate containing the product was evaporated to dryness, triturated with dilute ethanolic ammonia, evaporated, and crystallized from boiling MeOH to give 260 mg of crude product. Pure material was obtained as crystals from EtOH after chromatographic purification on successive thick-layer plates (Analtech GF, 2000-μm layer), eluting with 5:1 $CHCl_3$–MeOH, yield 76.3 mg (5%), m.p. 213°. TLC: 5:1 $CHCl_3$–MeOH, R_f 0.70; MS (FAB) m/z 316 $(M + 1)^+$; UV λ_{max}, nm ($\varepsilon \times 10^{-3}$) [pH 1] 262 (14.0), 268 (sh), [pH 7] [pH 13] 261 (15.1), 268 (sh); ^1H NMR (100 MHz, Me_2SO-d_6) δ 2.07 (s, 3, SCH_3), 2.76–2.91 (m, 2, H-5'a,5'b), 3.95–4.24 (m, 2, H-3',4'), 4.68 (m, 1, H-2', $J_{2',3'}$ = 4.6 Hz), 5.32 (d, 1, 3'-OH, J = 5 Hz), 5.50 (d, 1, 2'-OH, J = 6 Hz), 5.82 (d, 1, H-1', $J_{1',2'}$ = 5.5 Hz), 7.84 (br s, 2, NH_2), 8.34 (s, 1, H-8); ^{13}C NMR (25 MHz, Me_2SO-d_6) δ 15.53 (SCH_3), 36.02 (C-5'), 72.49, 72.64 (C-2',3'), 83.81, 87.40 (C-1',4'), 117.53 (C-5), 139.99 (C-8), 150.69 (C-4), 157.63 (C-6), 158.56 (C-2), J_{C_2F} = 204.1 Hz, J_{C4F} = 20.2 Hz, J_{C_5F} = 4.2 Hz, J_{C_6F} = 21.3 Hz, J_{C8F} = <1 Hz. Anal. Calc. for $C_{11}H_{14}FN_5O_3S$: C, 41,90; H, 4.48; N, 22.21. Found: C, 41.70; H, 4.48; N, 22.07.

2',3'-O-Ethoxymethylidene-2-fluoroadenosine (Compound 2). Crude 2-fluoroadenosine (Compound **1**) [27, 28] (4.3 g, 12 mmol) in dry DMF (50 ml) at room temperature was treated in one portion with triethyl orthoformate (3.77 g, 25.4 mmol) followed by 5.3 N HCl in DMF (2.9 ml, 15 mmol). After stirring at room temperature in a tightly stoppered flask for 19 hr, the reaction was complete as indicated by TLC (9:1 $CHCl_3$:MeOH, R_f 0.60). Triethylamine (2.1 ml, 15 mmol) was added, and the resulting mixture was stirred for 1 hr, after which time the solid was removed by filtration, washed with DMF, and discarded. The combined filtrate and washings were evaporated and dried *in vacuo* to give a dark gum that solidified during a 3-hr trituration with water (128 ml) containing 1% Et_3N. The solid was collected after chilling and dried *in vacuo* to give 3.5 g of crude Compound **2**. Extraction of the filtrate with $CHCl_3$ (4 × 100 ml), drying ($MgSO_4$), and evaporation of the extract gave more crude Compound **2** (1.0 g). These solids were combined and used directly in the next step.

2',3'-O-Ethoxymethylidene-2-fluoro-5'-O-(p-methylbenzenesulfonyl)adenosine (Compound 3). Blocked 2-fluoroadenosine derivative Compound **2** (~12 mmol), in dry pyridine (100 ml) at −20° was treated in one portion with *p*-toluenesulfonyl chloride (3.9 g, 20 mmol) and stored at −20° for 4 days. TLC analysis (9:1 $CHCl_3$:MeOH) showed one major product, R_f 0.70. Aliquots of this freezer-stored reaction were worked up as needed. Typically, a 10-ml aliquot was treated with H_2O (0.1 ml), stirred for 0.5 hr, and poured into cold saturated $NaHCO_3$ (20 ml). After 15 min, $CHCl_3$ (25 ml) was added and the layers were separated. The aqueous layer was extracted with $CHCl_3$ (2 × 25 ml). The combined organic layer and extracts were washed with H_2O (3 × 25 ml), dried with $MgSO_4$, and evaporated to give 490 mg of crude Compound **1c** that was used directly in displacement reactions.

A small sample was purified chromatographically on thick-layer plates (Analtech GF, 2000-μm layer), eluting with EtOAc, and was solidified by ether trituration. MS m/e 495; MS (FAB m/z 496 $(M + 1)^+$; ^1H NMR (300 MHz, Me_2SO-d_6) δ 1.12 (t, 3, OCH_2CH_3), 2.38 (s, 3 $ArCH_3$), 3.54 (q, 2, OCH_2CH_3), 4.18–4.27 (m, 1, H-5'a, $J_{4',5'a}$ = 8.6 Hz, $J_{5'a,5'b}$ = 11.6 Hz), 4.32–4.42 (m, 2, H-4', H-5'b, $J_{3',4'}$ = 3.1 Hz), 5.04 (dd, 1, H-3', $J_{2',3'}$ = 6.3 Hz), 5.35 (dd, 1, H-2'), 6.13 (d, 1, H-1', $J_{1',2'}$ = 2.0 Hz), 6.22 (s, 1, $HCOCH_2CH_3$), 7.25, 7.57 (2 d, 4, ArH), 7.95 (br s, 2, NH_2), 8.22 (s, 1, H-8); ^{13}C NMR (75 MHz, Me_2SO-d_6) δ 14.79 (CH_2CH_3), 21.06 ($ArCH_3$), 59.00 (CH_2CH_3), 69.50 (C-5'), 79.69, 81.89 (C-2',3',4'), 88.40 (C-1'), 116.41 (H-C), 117.46 (C-5), 127.20, 129.56, 131.45, 144.89 (Ar), 140.06 (C-8), 149.46 (C-4), 157.54 (C-6), 158.20 (C-2), J_{C_4F} = 20.4 Hz, J_{C_5F} = 3.0 Hz, J_{C_6F} = 21.4 Hz, J_{C_8F} = 1 Hz. Anal., Calc. for $C_{20}H_{22}FN_5O_7S \cdot 1/2H_2O$: C, 47.62; H, 4.60; N, 13.88. Found: C, 47.63; H, 4.78; N, 14.16.

Cell culture

CCRF-CEM human T-cell leukemia cells [29] and HL-60 human promyelocytic leukemia cells [30] were cultured as described previously [18].

Isolation of an HL-60 subline deficient in adenine phosphoribosyltransferase activity

HL-60 cells lacking adenine phosphoribo-

syltransferase (APRT) were selected in the following manner. Wild type HL-60 cells (1.75×10^5 cells/ml) in RPMI 1640 medium supplemented with 10% fetal calf serum were treated with the mutagen *N*-methyl-*N*-nitro-*N*-nitroguanidine (1 μg/ml) for 1 hr at 37°. The cells were then washed, replated in fresh medium, and allowed to replicate for 3–5 generations. The cells were then harvested and cloned in soft agar [31] in medium containing 0.5 μM 2-fluoroadenine. Each 35 mm dish (Falcon Plastics) contained 3×10^5 cells. After 14 days of incubation, isolated colonies were plucked and replated in serum-supplemented RPMI 1640 medium. Each of these clones were passaged over several weeks in the presence of 2-fluoroadenine, starting at 0.5 μM, then increasing to 1.5 μM, 5 μM, and finally 10 μM. Cells that grew at normal rates in 10 μM 2-fluoroadenine (i.e. approximately 100 times the IC_{50} for this compound against wild type HL-60 cells [21]) were then examined for APRT activity using the assay of Kelley *et al.* [32] as modified by Crabtree *et al.* [33]; no detectable activity was observed in each of the 2-fluoroadenine-resistant clones studied. One clone that was studied in detail was designated HL-60/*aprt*$_1$. This cell line contained MTAPase activity (1.07 nmol units/mg protein) comparable to wild type HL-60 cells but lacked APRT activity (<0.01 nmol units/mg protein).

Partial purification of MTAPase from HL-60 cells

The partial purification of MTAPase from HL-60 cells by chromatofocusing and gel filtration was performed using previously reported methods [22].

Reversed-phase high performance liquid chromatographic assay of nucleoside cleavage activity

The liberation of 2-fluoroadenine from 2-fluoroadenine-containing MTA analogs was monitored directly by reversed-phase HPLC using a Varian 4200 high performance liquid chromatograph equipped with a Waters μBondapak C_{18} analytical column (0.39 × 30 cm). In these studies, which employed a modification of a previously described method [21], the low concentrate eluent was 10 mM potassium phosphate (pH 5.5), and the high concentrate eluent was 50% methanol, 5 mM potassium phosphate (pH 5.5). The gradient program consisted of a linear increase in a high concentrate eluent from 0 to 100% at a rate of 9%/min for 15 min, followed by an additional 6 min of isocratic elution at 100% high concentrate eluent. The flow rate was 1.0 ml/min. 2-Fluoroadenine, 2-fluoroadenosine, adenine and adenosine were monitored at 254 nm and were identified by comparing their retention times with those of authentic standards. Reversed-phase HPLC was used to perform kinetic studies on MTAPase with 2-fluoroadenine-containing MTA analogs. Cleavage activity was found to be linear with respect to protein (in the range of 1–10 μg protein) and time (0–10 min) using this assay.

Determination of 2-fluoroadenine nucleotide formation by anion-exchange high performance liquid chromatography

The intracellular formation of 2-fluoroadenine nucleotides from 2-fluoroadenine-containing MTA analogs was measured using a modification of a previously described anion-exchange HPLC technique [21]. A Varian model 5000 liquid chromatograph equipped with a Whatman Partisil PXS 10/25 SAX column (0.26 × 25 cm) was used, and various natural and 2-fluoroadenine-containing nucleotides were separated by the use of a programmed gradient of 1 mM potassium phosphate (pH 3.9) as the low concentrate eluent and 500 mM potassium phosphate (pH 3.9) as the high concentrate eluent. The gradient profile consisted of a linear increase of high concentrate eluent from 0 to 100% over a 40-min period, followed by a 20-min isocratic period at 100% high concentrate. Absorbance was monitored at 254 nm; the flow rate was 1.2 ml/min.

A 2-fluoroadenosine triphosphate (2-FATP) standard was prepared and identified by the following method. 2-FATP was isolated by anion-exchange HPLC from extracts of human erythrocytes (20% suspension in a phosphate-buffered minimal salt medium) that had been incubated with 1 mM 2-fluoroadenosine as previously described [34]. To identify this putative 2-FATP peak as a triphosphate derivative, a hexokinase peak shift assay was performed, using the method of Parks and Brown [34]. The putative 2-FATP peak was converted to a diphosphate compound (presumably 2-fluoroadenosine diphosphate) in hexokinase-treated samples as determined by HPLC, but not in samples that were not treated with hexokinase, thus establishing the compound as a triphosphate derivative. To confirm that the putative 2-FATP sample was a derivative of 2-fluoroadenosine, an aliquot of 2-FATP (28 nmol) was incubated for 30 min at 37° with 0.2 ml of *C. adamanteus* snake venum (1000 μg/ml) containing 5′-nucleotidase activity. The reaction was stopped by boiling the samples for 2 min. The samples were then centrifuged, and aliquots were analyzed for the presence of 2-fluoroadenosine using the reversed-phase HPLC method described above. A peak appeared with a retention time corresponding to that of authentic 2-fluoroadenosine but not to that of adenosine. Thus, the putative 2-FATP sample was derived from 2-fluoroadenosine. The 2-FATP sample, whose purity was confirmed by anion-exchange HPLC, was quantitated by spectrophotometry [35] and used as standard to identify and quantitate 2-FATP concentrations in analog nucleotide incorporation studies.

Enzyme assays

MTAPase was assayed as described elsewhere [11]; purine nucleoside phosphorylase was assayed by the method of Kim *et al.* [36]. *S*-Adenosylhomocysteine hydrolase activity was measured as described by Hershfield [37].

RESULTS

2-Fluoroadenine-substituted MTA analogs as substrates of human MTAPase

5′-Deoxy-5′-iodo-2-fluoroadenosine (5′-IFAdo) and 5′-deoxy-2-fluoroadenosine (5′-dFAdo) represent 2-fluoroadenine-containing MTA analogs in which the methylthioribose moiety has been replaced. The biological activities of these doubly-

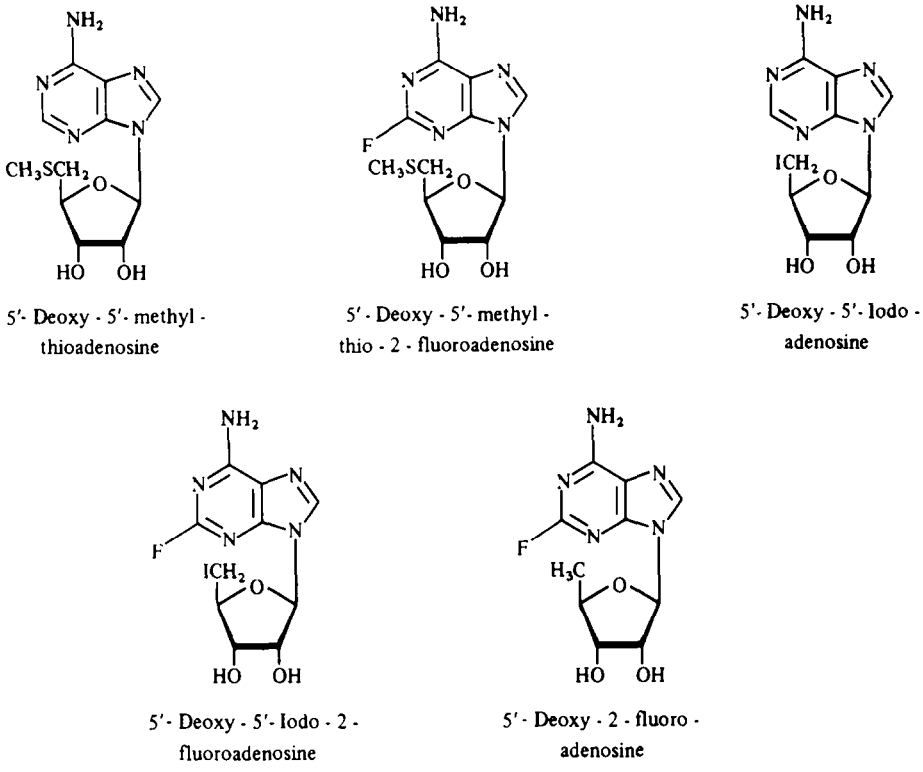


Fig. 2. Structures of MTA, 5'-deoxy-5'-iodoadenosine, and various 2-fluoroadenine-substituted derivatives.

substituted compounds were compared to that of 5'-deoxy-5'-methylthio-2-fluoroadenosine (5'-MTFAdo), in which the 5'-methylthio group has been left unaltered (see Fig. 2 for the structures of these compounds). Three lines of evidence indicate that each of these analogs is a substrate of MTAPase. First, when crude extracts of MTAPase-containing HL-60 promyelocytic leukemia cells (0.8 nmol units/mg protein) were incubated with 5'-MTFAdo, 5'-IFAdo, or 5'-dFAdo, the formation of 2-fluoroadenine was observed by reversed-phase HPLC. The results obtained with 5'-dFAdo are shown in Fig. 3, A and B. In contrast, crude extracts of the CCRF-CEM human T-cell leukemia line, which lacks MTAPase activity [18, 38], were unable to produce 2-fluoroadenine at detectable levels from 5'-dFAdo or from any of these MTA analogs (Fig. 3, C and D). Second, when MTAPase from HL-60 cells was partially purified by chromatofocusing, 5'-IFAdo cleaving activity remained associated with the MTAPase activity but not with the purine nucleoside phosphorylase (PNP) activity, the only other purine-specific phosphorylase known in mammalian tissues (Fig. 4). Similarly, 5'-dFAdo and 5'-MTFAdo cleaving activities were found to coelute with MTAPase activity (data not shown). Finally, these analogs were converted to 2-fluoroadenine-containing nucleotides in intact MTAPase-containing HL-60 cells, but not in MTAPase-deficient CCRF-CEM cells (see below).

The kinetic constants of MTAPase derived from HL-60 cells for these 2-fluoroadenine-substituted MTA analogs and the corresponding adenine-containing derivatives are presented in Table 1. Substitution of a fluorine atom in the 2-position of the adenine ring (e.g. 5'-MTFAdo) did not alter substrate binding. Also, the replacement of the 5'-methylthio group by either an iodine (e.g. 5'-deoxy-5'-iodoadenosine) or a hydrogen atom (e.g. 5'-deoxyadenosine) had only a small effect on the K_m value, decreasing binding by a factor of less than 2-fold relative to the natural substrate, MTA. Even the doubly-substituted analogs, 5'-IFAdo and 5'-dFAdo, had binding constants that were not markedly different from MTA. In regard to relative V_{max} values, 5'-MTFAdo > 5'-dFAdo > 5'-IFAdo. It is not surprising that the 5'-iodo derivative had the lowest relative V_{max} value, since 5'-iodinated derivatives are poor substrates of human MTAPase [22]. It was unexpected, however, that the relative V_{max} of 5'-MTFAdo exceeded that of 5'-dFAdo, since the corresponding adenine compound, 5'-deoxyadenosine, had a greater V_{max} value than MTA itself with both murine Sarcoma 180 [11] and human HL-60 leukemia MTAPase (Table 1).

Role of MTAPase and APRT in the activation and cytotoxicity of 2-fluoroadenine-substituted MTA analogs

The role of MTAPase, along with APRT, in the biological activation of 5'-MTFAdo, 5'-IFAdo, and 5'-dFAdo was examined by employing several mutant cell lines. The growth inhibitions affected by these analogs were compared in a cell line containing both MTAPase and APRT (HL-60 wild type cells), a cell line that has APRT activity but lacks MTAPase

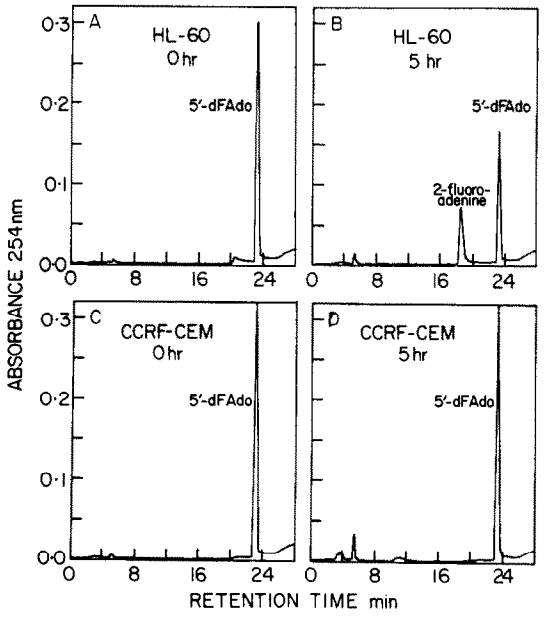


Fig. 3. (A and B) Reversed-phase HPLC profiles demonstrating the liberation of adenine from 5'-dFAdo in the presence of MTAPase-containing HL-60 cell extracts. (C and D) Reversed-phase HPLC profiles demonstrating the inability of MTAPase-deficient CCRF-CEM extracts to liberate adenine from 5'-dFAdo. Exponentially-growing HL-60 or CCRF-CEM cells (approx. 10^7 cells) were harvested by centrifugation and washed twice in 0.9% saline. Cells were suspended in 25 mM imidazole (pH 7.4), 1 mM dithiothreitol, and then sonicated and centrifuged at 40,000 *g* for 1.5 hr. The supernatant fluids were dialyzed against the above buffer for several hours at 4°. Reaction mixtures (1 ml) containing 100 μM 5'-dFAdo, 50 mM potassium phosphate (pH 7.4) and cell extract (500 μg protein) were incubated in a shaking water bath at 37°. At the indicated times, samples were prepared for reversed-phase HPLC analysis as described in Ref. 21; HPLC analysis was carried out as described under Materials and Methods. 2-Fluoroadenine was identified based on the retention time of authentic standards.

(CCRF-CEM cells), and in a line that has MTAPase activity, but lacks APRT (HL-60/$aprt_1$).

As shown in Table 2, 5'-MTFAdo, 5'-IFAdo, and 5'-dFAdo were potent growth inhibitors of the wild type HL-60 cell line, with IC_{50} values in the 10^{-8} M range. In fact, these three compounds represent the most cytotoxic MTA analogs identified to date [21, 22]. In accord with this growth inhibition data, these cells readily incorporated 5'-MTFAdo, 5'-IFAdo, and 5'-dFAdo into 2-fluoroadenine-containing nucleotides, especially 2-fluoroadenosine triphosphate (2-FATP); such a process requires both MTAPase and APRT activity. The nucleotide profile of wild type HL-60 cells treated with 100 μM 5'-dFAdo for 2 hr is shown in Fig. 5B. The free base, 2-fluoroadenine, also was rapidly converted to analog nucleotides by these cells (Fig. 5C). Significantly, the doubly-substituted analogs, 5'-IFAdo and 5'-dFAdo, were not more active than the singly-substituted derivative, 5'-MTFAdo, in inhibiting the growth of wild type HL-60 cells. In fact, the three analogs were equipotent. This suggests that the antiproliferative activity of all of these compounds is primarily associated with the liberation and activation of their 2-fluoroadenine moieties.

In contrast, MTAPase-deficient CCRF-CEM cells were much less sensitive to these agents, with IDC_{50} values in the order of 250- to 1000-fold greater than the corresponding values with the MTAPase-containing HL-60 cell line (Table 2). As expected, 5'-dFAdo was not converted to detectable levels of 2-FATP in this MTAPase-deficient line (Fig. 5E), nor were 5'-MTFAdo or 5'-IFAdo (data not shown); however, when the MTAPase step was circumvented by administration of 2-fluoroadenine, 2-FATP accumulated in this APRT-containing cell line (Fig. 5F). The APRT-deficient line, HL-60/$aprt_1$, was also relatively insensitive to the antiproliferative effects of 5'-MTFAdo, 5'-IFAdo, and 5'-dFAdo (IC_{50} values in the range of 125- to 2000-fold greater than those found with the wild type cells), in spite of the fact that these cells contain MTAPase activity (Table

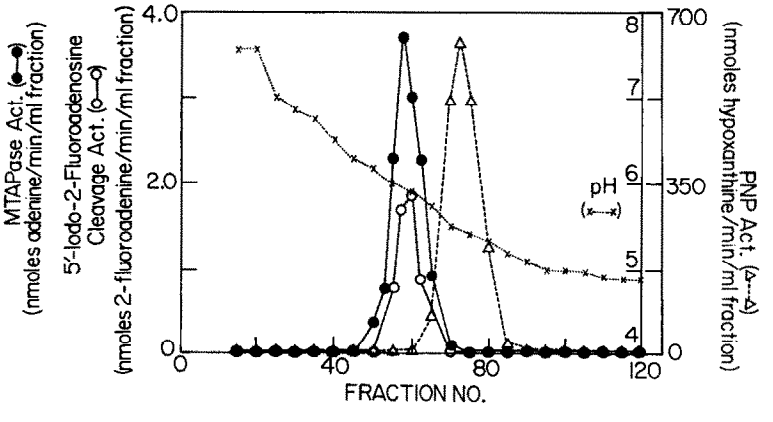


Fig. 4. Association of MTAPase and 5'-IFAdo cleaving activities during partial purification of HL-60 human promyelocytic leukemia cell MTAPase by chromatofocusing. The chromatofocusing procedure employed is described in Ref. 22. 5'-IFAdo cleaving activity was determined by reversed-phase HPLC. Reaction mixtures (0.4 ml) containing 200 μM 5'-IFAdo, 50 mM potassium phosphate and 150 μl of individual fractions were incubated in a shaking water bath at 37° for 30 min. Reactions were stopped with perchloric acid (final concn 4%); samples were then adjusted to pH 6.5–7.5 with 5 N KOH. The reversed-phase HPLC method used is described under Materials and Methods. MTAPase [11] and PNP [36] activities were determined by previously described spectrophotometric assays.

Table 1. Kinetic constants of MTAPase from HL-60 human promyelocytic leukemia cells for 2-fluoroadenine-containing MTA analogs and related compounds

	K_m (μM)	V_{max} (% rel. to MTA)
5′-Deoxy-5′-methylthioadenosine	5.5 ± 1.1*	100*
5′-Deoxy-5′-methylthio-2-fluoroadenosine	5.3 ± 0.8	144 ± 55
5′-Deoxy-5′-iodoadenosine	8.1 ± 0.4*	25 ± 5*
5′-Deoxy-5′-iodo-2-fluroadenosine	6.9 ± 0.5	48 ± 14
5′-Deoxyadenosine	10.7 ± 3.7	129 ± 5
5′-Deoxy-2-fluoroadenosine	6.8 ± 0.8	59 ± 7

Kinetic parameters for 5′-MTFAdo, 5′-IFAdo and 5′-dFAdo were determined using the reversed-phase HPLC technique described in Materials and Methods. Reaction mixtures contained 50 mM potassium phosphate (pH 7.4), partially-purified HL-60 MTAPase (approx. 70 μg protein), and various concentrations of nucleoside in a total volume of 400 μl. Mixtures were incubated for periods up to 7 min at 37° in a shaking water bath. Reactions were stopped with cold perchloric acid (final conc 4%), and the samples were then adjusted to pH 7.4 with 5 N KOH. Samples were kept frozen at −20° prior to HPLC analysis. The kinetic parameters of 5′-deoxyadenosine were determined using a previously described spectrophotometric assay [11]. Values represent the mean and standard deviation of two to four independent determinations.

* Data from Ref. 22.

2). Again, in agreement with the IC_{50} values, neither the MTA analogs (see Fig. 5H for 5′-dFAdo) nor 2-fluoroadenine (Fig. 5I) was converted to analog nucleotides in this subline. These results indicate that both MTAPase and APRT activities are required for the formation of 2-fluoroadenine-containing nucleotides from these three analogs, and that the presence of both enzymes is required for the major cytotoxic actions of these analogs to be expressed. It should be noted that these MTA analogs exerted relatively low but detectable growth inhibition against the MTAPase-deficient and APRT-deficient lines, where no metabolism of these compounds to analog nucleotides was observed, suggesting that the nucleosides themselves have weak growth inhibitory properties.

Once the pathway by which these 2-fluoradenine-substituted MTA analogs are converted to 2-FATP was identified, the question of which step in this process is rate-limiting was explored. The answer was found by comparing the rate of conversion of these analog nucleosides to 2-FATP relative to the free base, 2-fluoroadenine. As shown in Fig. 6, the rate of 2-FATP formation from 2-fluoroadenine was at least 8-fold greater than that of any of the MTA derivatives after 1 hr of incubation. This indicates that the phosphorolysis of these compounds, catalyzed by MTAPase, is the rate-limiting step in the synthesis of 2-FATP from these nucleosides. In agreement with this concept, the analog with the highest relative V_{max} value for MTAPase, 5′-MTFAdo (see Table 1), was converted to 2-FATP at the fastest rate, whereas the analog with the lowest relative V_{max} value, 5′-IFAdo, was converted at the slowest rate. Again this indicates that the rate at which a given analog is cleaved by MTAPase determines the rate of conversion to nucleotide metabolites.

MTAPase-independent actions of 2-fluoroadenine-substituted MTA analogs

These analogs exert modest but measurable growth inhibitory actions on the MTAPase-deficient CCRF-CEM line, despite the fact that these compounds are not metabolized to 2-fluoroadenine containing nucleotides by these cells (see Table 2 and

Table 2. Comparison of the growth-inhibitory effects of 2-fluoroadenine-substituted MTA analogs and related compounds on HL-60 promyelocytic leukemia cells, APRT-deficient HL-60 cells, and MTAPase-deficient CCRF-CEM T lymphoblastic leukemia cells

	IC_{50} (μM)		
	HL-60	CCRF-CEM	HL-60/*aprt*$_1$
5′-Methylthioadenosine	>200	101.0	>200
5′-Methylthio-2-fluoroadenosine	0.07	34.0	9.0
5′-Iodoadenosine	2.6	66.0	31.0
5′-Iodo-2-fluoroadenosine	0.05	13.0	7.0
5′-Deoxyadenosine	>100	>100	ND*
5′-Deoxy-2-fluoroadenosine	0.05	52.0	100.0
2-Fluoroadenine	0.15	0.1	>100

The IC_{50} values were determined as previously described [21]. Values represent the mean of two to four independent determinations.

* ND = no data.

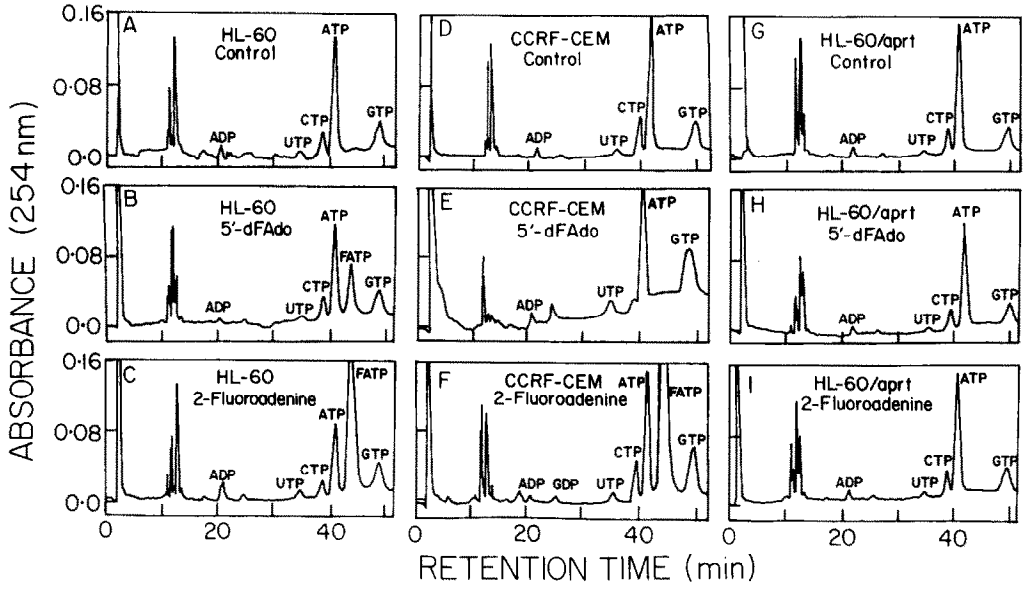


Fig. 5. (A, B, and C): Anion-exchange HPLC profiles of HL-60 promyelocytic leukemia cells treated for 2 hr with medium alone (A), medium containing 100 μM 5'-dFAdo (B), or medium containing 100 μM 2-fluoroadenine (C). (D, E and F): Anion-exchange HPLC profiles of MTAPase-deficient CCRF-CEM leukemia cells treated for 2 hr with medium alone (D), medium containing 100 μM 5'-dFAdo (E), or medium containing 100 μM 2-fluoroadenine (F). (G, H and I) Anion-exchange HPLC profiles of HL-60/*aprt*$_1$ cells treated for 2 hr with medium alone (G), medium containing 100 μM 5'-dFAdo (H), or medium containing 100 μM 2-fluoroadenine (I). All cell types were grown in RPMI 1640 medium containing 15% fetal calf serum; cells were harvested by centrifugation and washed twice in 0.9% saline. The cells were then resuspended at a concentration of 1×10^6 cells/ml in a minimal salt medium containing 50 mM potassium phosphate (pH 7.4), 2 mM $MgSO_4$, 75 mM NaCl, 10 mM D-glucose, and with or without a 100 μM concentration of the indicated compounds. After incubation in a shaking water bath for 2 hr at 37°, aliquots were prepared for anion-exchange HPLC analysis as described previously [21].

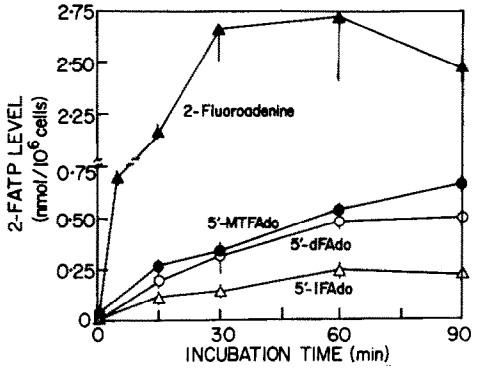


Fig. 6. Relative rates of incorporation of 2-fluoroadenine, 5'-MTFAdo, 5'-dFAdo, and 5'-IFAdo into 2-FATP pools in wild type HL-60 cells. HL-60 cells (1×10^6 cells/ml) were incubated (50 ml final volume) in the minimal salt medium described in Fig. 4 in the presence of a 20 μM concentration of the respective compounds at 37° in a shaking water bath. At the indicated times aliquots of 8×10^6 cells were prepared for HPLC analysis as described previously [21]. The conditions used to separate the natural and analog nucleotides by anion-exchange HPLC are described under Materials and Methods. An authentic sample of 2-FATP (see Materials and Methods) was used to identify and quantitate 2-FATP formation. Data points and error bars represent the average value and the range, respectively, of duplicate samples.

Fig. 5). This activity indicated that the nucleosides themselves may have biological effects. To examine the nature of this nucleoside-mediated inhibition, cell growth recovery experiments were performed. MTAPase-containing HL-60 cells and MTAPase-deficient CCRF-CEM cells were treated with 5 and 60 μM 5'-IFAdo, respectively, for a period of 24 hr. At this time the cells were washed free of drug and replated in fresh drug-free medium. Cell growth was monitored for 4 days following the replating. As shown in Fig. 7, 5'-IFAdo-treated HL-60 cells did not recover their ability to grow following removal of the drug, whereas CCRF-CEM cells exhibited a reversal of growth inhibition once 5'-IFAdo was removed. These results suggest that 5'-IFAdo exerts a cytotoxic effect on the HL-60 cells where analog nucleotides are formed, but a cytostatic action against the CCRF-CEM line where nucleoside remains unmetabolized.

A possible target site for these MTA analog nucleosides is *S*-adenosylhomocysteine hydrolase (AdoHcy hydrolase; EC 3.3.1.1). This enzyme, which converts *S*-adenosylhomocysteine (AdoHcy), generated from *S*-adenosylmethionine by methylation reactions, to adenosine and homocysteine, is apparently crucial for (1) preventing the accumulation of AdoHcy, which can inhibit *S*-adenosylmethionine-dependent methylation reactions by product inhibition [39], (2) allowing the recycling of the adenosyl moiety of AdoHcy to purine pools, and

Table 3. Protection of CCRF-CEM AdoHcy hydrolase activity from inactivation by 2-fluoroadenine-substituted MTA analogs

Inhibitor	Substrate	Percent of control activity remaining after 60-min incubation
	None	63 ± 9
5′-dFAdo (25 μM)	Adenosine (10 μM)	128 ± 8
	AdoHcy (10 μM)	84 ± 8
	Homocysteine (100 μM)	63 ± 7
5′-IFAdo (25 μM)	None	33 ± 3
	Adenosine (10 μM)	112 ± 5
	AdoHcy (10 μM)	151 ± 10
	Homocysteine (100 μM)	32 ± 3

CCRF-CEM cells were sonicated in 200 mM potassium phosphate, pH 7.4, 4 mM dithiothreitol and 2 μM 2′-deoxycoformycin and centrifuged at 105,000 *g*. Supernatant fluids (180–835 μg protein) were incubated at 37° in 100 mM potassium phosphate, pH 7.4, 2 mM dithiothreitol, 1 μM 2′-deoxycoformycin, and with or without the indicated concentrations of substrates or 2-fluoroadenine-substituted MTA analogs in a shaking water bath (final concentration, 200 μl). After 60 min, 20-μl aliquots were transferred to AdoHcy hydrolase assay mixtures described in Fig. 8. Control values (no inhibitors present during preincubation) were 3.78 and 1.44 nmol/hr-mg protein for experiments with 5′-dFAdo and 5′-IFAdo respectively. Values represent the mean ± SD of four independent samples.

(3) providing the source of homocysteine for the B_{12}-dependent methionine synthase (5-methyltetrahydropteroyl-1-glutamate:1-homocysteine *S*-methyltransferase, EC 2.1.1.13) reaction, which regenerates tetrahydrofolate from 5-methyltetrahydrofolate as homocysteine is salvaged to methionine. A number of adenine-containing nucleosides, including MTA [40, 41] and several other 5′-substituted adenosines [42], have been shown to inhibit AdoHcy hydrolase. Therefore, the 2-fluoroadenine-substituted MTA analogs were examined for their ability to inhibit AdoHcy hydrolase activity in extracts of CCRF-CEM cells. As shown in Fig. 8, incubation of CCRF-CEM extracts with either 5′-dFAdo or 5′-IFAdo led to an inactivation of AdoHcy activity. This inactivation occurred via pseudo first-order kinetics, suggesting that an enzyme–MTA analog complex is formed. From a plot of the reciprocal of the pseudo first-order rate constants versus nucleoside analog concentration, an estimate of the equilibrium constant (K_i) for this complex can be obtained [43]. For both 5′-dFAdo and 5′-IFAdo, K_i values of 8 μM were calculated. 5′-MTFAdo also inactivated AdoHcy hydrolase from CCRF-CEM cells, although detailed kinetic analysis was not performed. This pattern of pseudo first-order inactivation kinetics suggested that these nucleosides may be acting as k_{cat} or "suicide" inactivators of AdoHcy hydrolase, i.e. these substrate analogs undergo catalysis to reactive intermediates which then become irreversibly bound to the enzyme. In support of this, (1) extensive dialysis did not reac-

Table 4. Effect of 5′-dFAdo on AdoHcy hydrolase activity in cultured CCRF-CEM cells

Time after initiation of treatment (hr)	AdoHcy hydrolase activity (nmol adenosine converted/min/mg protein)	
	Control	Treated with 50 μM 5′-dFAdo
0	0.268 ± 0.073	0.253 ± 0.093
6	0.354 ± 0.042	0.154 ± 0.023
24	0.322 ± 0.037	0.129 ± 0.010
48	0.392 ± 0.011	0.166 ± 0.031

CCRF-CEM cells were grown in RPMI 1640 medium supplemented with 10% horse serum, antibiotics, and buffers. cells were placed in Falcon 25 cm^2 cultured flasks at a density of 3×10^5 cells/ml, 50 ml/flask. At 0 time, 5′-dFAdo was added to appropriate flasks to a concentration of 50 μM. At all time points, 10 ml of the cell suspensions were withdrawn and centrifuged for 10 min at 1300 *g* at 4°, and the supernatant fluid was removed. The pellet was resuspended in 200 μl of 200 mM potassium phosphate, pH 7.4, 4 mM dithiothreitol 2 μM 2′-deoxycoformycin, and sonicated. The sonicate was centrifuged for 15 min at 4° in a microfuge, and 20 μl of the resulting supernatant fluid was added to AdoHcy hydrolase assay mixtures (see legend of Fig. 8) and incubated for 30 min. The protein content was determined by the method of Bradford [44]. Reaction mixtures were processed as described in the legend of Fig. 8. Values represent the mean ± SD of three independent samples.

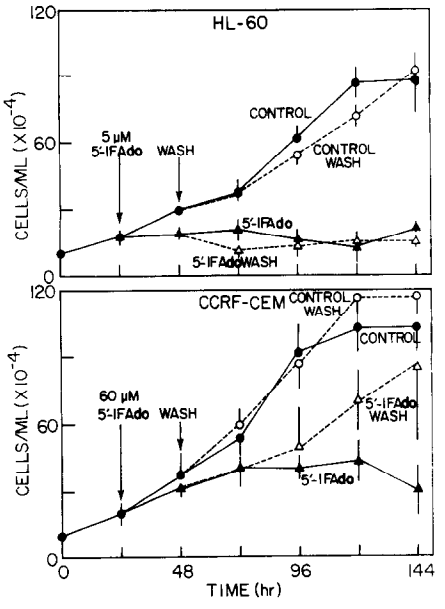


Fig. 7. Ability of MTAPase-containing and deficient cell lines to recover growth following treatment with 5'-IFAdo. Top panel: MTAPase-containing HL-60 cells. HL-60 cells were plated in RPMI 1640 containing 15% horse serum at 1×10^5 (3 ml final volume). After 24 hr, the cells were replated in 5 μM 5'-IFAdo or drug-free medium and incubated for 24 hr. At this time, the samples treated with 5'-IFAdo were divided into two groups. One set of samples, designated "5'-IFAdo Wash" (△), was centrifuged, the medium was removed, and the cells were washed twice in normal saline and replated in fresh drug-free medium. The other samples, designated "5'-IFAdo" (▲), were not washed; 5'-IFAdo remained in these samples for the duration of the experiment. At the same time, samples not treated with 5'-IFAdo were divided into two groups; those designated "Control Wash" (○) were centrifuged, washed in saline, and replated in drug-free medium as described above. The other group of samples, designated "Control" (●) were left as originally plated. At the indicated times, cell counts were taken using a hemacytometer. Data points represent the mean ± SD of four samples run in two separate experiments. Bottom panel: MTAPase-deficient CCRF-CEM cells. The experimental procedure used was exactly as described above for HL-60 cells, except that the CCRF-CEM cells were treated with 60 μM 5'-IFAdo.

tivate CCRF-CEM AdoHcy hydrolase that had been inactivated by incubation with 5'-dFAdo or 5'-IFAdo, and (2) AdoHcy and adenosine, i.e. the nucleoside substrates of the enzyme, protected the enzyme from inactivation by either of these MTA analogs (Table 3).

These MTA analogs were then tested as inhibitors of AdoHcy hydrolase in intact CCRF-CEM cells in culture at concentrations close to their IC_{50} values against this cell line. When CCRF-CEM cells were incubated with 50 μM 5'-dFAdo, AdoHcy hydrolase activity decreased to approximately 40% relative to untreated controls after 6 hr of exposure (see Table 4), and remained at this depressed level through the duration of the study (48-hr exposure). Qualitatively similar results were obtained when CCRF-CEM cells were incubated with 60 μM 5'-IFAdo (data not shown). This inhibition of AdoHcy hydrolase apparently does not result in a folate deficiency (due to the trapping of folates in the 5-methyltetrahydrofolate form owing to a lack of availability of homocysteine for the B_{12}-dependent methionine synthase reaction [45]), as neither homocysteine nor folinic acid nor the combination of the two compounds abrogated the growth inhibitory action of 5'-dFAdo or 5'-IFAdo. Nevertheless, this key enzyme may be inhibited to the extent that AdoHcy accumulation occurs and critical methylation reactions are unfavorably affected. Thus, inhibition of AdoHcy hydrolase is one possible mechanism by which these MTA analogs exert their action on MTAPase-deficient (or APRT-deficient) cells. It should be noted, however, that Zimmerman *et al.* [46] could not correlate an MTA-mediated inhibition of lymphocyte cytolytic activity with a perturbation of lymphocyte AdoHcy levels. Since MTA has been demonstrated to exert inhibitory actions on cyclic AMP phosphodiesterase [47, 48], this mechanism may also be involved in the action of 2-fluoroadenine-substituted analogs in MTAPase-deficient cells.

DISCUSSION

Based on previous structure–activity relationship studies [21, 22], we have attempted to develop a "second generation" of MTA analogs with improved biological activity. 5'-IFAdo was conceived as an analog whose reaction with MTAPase would generate two growth inhibitory products, 2-fluoroadenine and 5-iodoribose-1-phosphate. 2-Fluoroadenine has long been known as a cytotoxic adenine analog, which is converted via APRT and adenine nucleotide kinases into its active metabolites, namely 2-fluoroadenosine-containing nucleotides [49]. 5-Iodoribose-1-phosphate recently has been implicated as the growth inhibitory metabolite of 5'-deoxy-5'-iodoadenosine in MTAPase-containing cells, and of 5'-deoxy-5'-iodoinosine in purine nucleoside phosphorylase-containing cells [22]. This pentose phosphate analog has been shown to inhibit a number of 5-phosphoribosyl-1-pyrophosphate-utilizing enzymes, such as PRPP amidotransferase and orotate phosphoribosyltransferase in cell-free systems [50], but the precise site of action of this metabolite has not yet been established. However, in these studies, 5'-IFAdo was not a more potent growth inhibitor of wild type HL-60 cells than the singly substituted analog, 5'-MTFAdo. This is because the potent growth inhibitory actions of 2-fluoroadenine mask the weaker actions of the 5-iodoribose-1-phosphate, as evidenced by the finding that wild type HL-60 cells were about 17-fold more responsive to 2-fluoroadenine than they were to 5'-deoxy-5'-iodoadenosine (Table 2). Furthermore, the advantages of having a doubly-substituted MTA analog should have become apparent in the MTAPase-containing, APRT-deficient HL-60/*aprt*$_1$ cells, which can produce but cannot activate 2-fluoroadenine from either 5'-IFAdo or 5'-MTFAdo. Here, an antimetabolite is generated only in the case of 5'-IFAdo, namely 5-iodoribose-1-phosphate. 5'-IFAdo, however, was no more active with this cell line than 5'-MTAFdo (Table 2). It is possible that, in the absence of APRT, 2-fluoroadenine liberated from 5'-IFAdo may

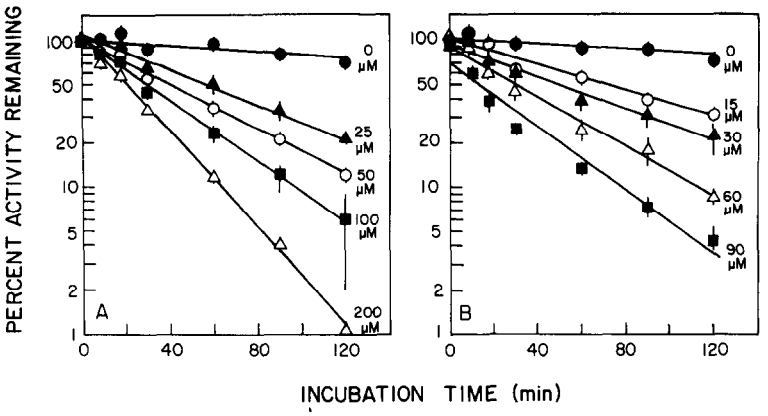


Fig. 8. Kinetics of inactivation of CCRF-CEM AdoHcy hydrolase by 5′-dFAdo (A) and 5′-IFAdo (B). CCRF-CEM cells grown in culture were harvested by centrifugation, adjusted to 2×10^7 cells/ml in 20 mM potassium phosphate, pH 7.3, and preincubated with 1 μM 2′-deoxycoformycin for 30 min at 4° to inactivate adenosine deaminase. The cells were then sonicated and spun at 38,000 *g* for 50 min, and the resulting supernatant fluid was used as the source of AdoHcy hydrolase activity. Enzyme extracts (0.6 to 1.7 mg protein) were preincubated in 100 mM potassium phosphate, pH 7.4, 1 μM 2′-deoxycoformycin, and the indicated concentrations of MTA analog (1 ml final volume) at 37° in a shaking water bath. At the times described in the figure, aliquots of 20 μl were withdrawn and added to a AdoHcy hydrolase reaction mixture consisting of 100 mM potassium phosphate, pH 7.4, 1 μM 2′-deoxycoformycin, 10 μM [8-^{14}C]adenosine (0.1 μCi/nmol) and 5 mM homocysteine (200 μl final volume). The reaction was allowed to proceed at 37° for 20 min and then stopped by the addition of perchloric acid (4% final concentration). The samples were neutralized with 5 N KOH, and 10-μl aliquots were spotted onto cellulose thin-layer plates (Baker) which were prespotted with unlabeled AdoHcy and adenosine. Chromatograms were developed in *n*-butanol/H_2O/methanol/NH_4OH (60:20:20:1, by vol.) at 4°. Spots were identified under ultraviolet light, cut out, and counted in 8 ml of toluene-based scintillation fluid. Control (0 min) AdoHcy hydrolase activity = 1.24 ± 0.20 nmol/hr/mg protein (N = 4). Results represent the average and range of two determinations run in two separate experiments.

accumulate, leading to a "pseudo-product" inhibition of the MTAPase reaction [12]. This, in turn may interfere with the continued production of 5-iodoribose-1-phosphate from 5′-IFAdo. Product inhibition of MTAPase by accumulated adenine may also explain why 5′-deoxy-5′-iodoadenosine was about 15-fold less active against the HL-60/*aprt*$_1$ line than the wild type line. Thus, based on this *in vitro* data, there is no evidence to encourage the further development of 5′-IFAdo as a chemotherapeutic agent over 5′-MTFAdo.

The other doubly-substituted MTA analog studied here was 5′-dFAdo. This compound was examined since it was known that the parent compound, 5′-deoxyadenosine, is an unusually good substrate of MTAPase, with a higher relative V_{max} value than the natural substrate, MTA (Ref. 11 and Table 1). It was hoped that 5′-dFAdo might also be rapidly phosphorolyzed, thereby serving as an efficient prodrug of 2-fluoroadenine. However, 5′-dFAdo was found to have a V_{max} value relative to MTA of only 59%. The reasons for this low activity are not apparent, but this result demonstrates the difficulty of predicting the activity of doubly-substituted compounds on the basis of kinetic values obtained with singly substituted agents (in this case, 5′-MTFAdo and 5′-deoxyadenosine). Like 5′-IFAdo, 5′-dFAdo was no more cytotoxic to wild type HL-60 cells than 5′-MTFAdo. This may be because (1) 5′-dFAdo is not cleaved at a faster rate than 5′-MTFAdo, and (2) the 5-deoxyribose-1-phosphate generated by the MTAPase reaction with 5′-dFAdo apparently does not affect growth, as evidenced by the fact that 100 μM 5′-deoxyadenosine did not inhibit the growth of the wild type HL-60 line (Table 2). Based on these findings, 5′-dFAdo also would not be expected to have a definite pharmacological advantage over 5′-MTFAdo.

Preliminary *in vivo* antitumor experiments have been performed with 2-fluoroadenine-containing MTA analogs. One of the original compounds of this type, 5′-deoxy-5′-ethylthio-2-fluoroadenosine, was found to have neither antitumor activity nor toxicity in mice bearing the L1210 lymphoblastic leukemia [51]. However, it was later appreciated that the L1210 leukemia cell line lacks MTAPase [5, 52] and therefore is resistant to the cytotoxic effects of these compounds [52]. More recent preliminary *in vivo* antitumor studies using the MTA compounds discussed here were performed in CD_1 mice bearing the MTAPase-containing [11] murine Sarcoma 180 ascites cell line. At its LD_{10}, 17.5 mg/kg (qd × 5), 5′-dFAdo displayed only weak antitumor activity, increasing the survival time of only two of ten Sarcoma 180-bearing mice approximately 76% over that of saline-treated controls. Preliminary pharmacokinetic data, however, indicate that the biological $T_{1/2}$ of 5′-dFAdo in the plasma of mice may be as low as 3 min (Dr. Donald Hill, personal communication, cited with permission). This finding might explain the low antitumor activity of 5′-dFAdo by this treatment schedule. The reason for this remarkably short $T_{1/2}$ value has not been established, but it seems possible that vascular endothelium may be the responsible

tissue. Other studies indicate that the activities of purine nucleoside phosphorylase and a number of enzymes of purine metabolism are very high in vascular endothelial cells isolated from coronary blood vessels [53]. It is conceivable that MTAPase is similarly elevated. In any case because of this low $T_{1/2}$ value, analogs such as 5'-dFAdo will have to be administered by continuous infusion in future antitumor studies, or in the form of a prodrug. Another approach may be to coadminister a nucleoside transport inhibitor, such as *p*-nitrobenzyl-6-thioinosine, to block drug uptake in the vasculature, thus prolonging the $T_{1/2}$ values of the MTA analogs. Paterson and others [54] have demonstrated that nucleoside transport inhibitors can be coadministered with cytotoxic purine nucleoside analogs without adversely altering the antineoplastic action of these agents.

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