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METABOLIC FORMATION OF NUCLEOSIDE-MODIFIED ANALOGUES OF S-ADENOSYLMETHIONINE

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Summary: Evidence is presented for the metabolism of six different adenosine analogues (formycin, 7-deazaadenosine, 8-azaadenosine, 2-fluoroadenosine, purine ribonucleoside and 3'-deoxyadenosine) to their corresponding S-nucleosidylmethionine derivatives in human erythrocytes and mouse lymphocytes. The identification of these novel metabolites was based upon chromatographic and enzymatic characterization of the unique radioactive substance found in cells incubated with each of these adenosine analogues plus L-[methyl-^{3}H]methionine. The metabolic formation of analogues of S-adenosylmethionine may contribute to the inhibition of cellular methylation reactions caused by various purine analogues.

Purine analogues are capable of inhibiting S-adenosylmethionine (AdoMet)-dependent methylations of cellular constituents in a variety of ways, the simplest mechanism being direct inhibition of a methyltransferase by a purine antimetabolite (1,2). Alternatively, compounds such as N^{6}-(Δ^{2}-isopentenyl)-adenosine (3) and xylosyladenine (4) may inhibit the biosynthesis of AdoMet from ATP and L-methionine and thereby repress enzymatic reactions which utilize this methyl donor. Due to the fact that S-adenosylhomocysteine (AdoHcy) is a potent inhibitor of many different methyltransferases (5), the inhibition of AdoHcy hydrolase (EC 3.3.1.1) by agents such as 3-deazaadenosine (5-8), 2'-deoxyadenosine (9-11) and adenine arabinoside (9,10) results in both an elevation of intracellular AdoHcy (5,6,8,12,13) and a consequent inhibition of cellular methylation reactions (5,8,13-17). By a similar yet distinct mechanism,

Abbreviations: AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; c^{7}Ado, 7-deazaadenosine; c^{7}AdoMet, S-7-deazaadenosylmethionine; COMT, catechol-O-methyltransferase (EC 2.1.1.6); 3'-dAdo, 3'-deoxyadenosine; 3'-dAdoMet, S-3'-deoxyadenosylmethionine; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; FAdo, 2-fluoroadenosine; FAdoMet, S-2-fluoroadenosylmethionine; For, formycin A; ForMet, S-formycinylmethionine; HPLC, high-performance liquid chromatography; Neb, nebularine; NebMet, S-nebularylmethionine; z^{8}Ado, 8-azaadenosine; z^{8}AdoMet, S-8-azaadenosylmethionine.

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adenosine analogues such as 3-deazaadenosine (5-8,12,13) and N^6-methyladenosine (18) can be condensed intracellularly with L-homocysteine via AdoHcy hydrolase to yield analogues of AdoHcy which are themselves inhibitory to various methyltransferases. We have begun to examine the possibility that purine analogues might also interfere with cellular methylation reactions as a result of their metabolic conversion to analogues of AdoMet. This communication presents evidence that a number of adenosine analogues can indeed be metabolized to their corresponding S-nucleosidylmethionine derivatives in human and mouse cells.

Methods: 3'-Deoxyadenosine and 3,4-dihydroxybenzoic acid were obtained from Calbiochem. L-[Methyl-^{3}H]methionine (12 Ci/mmol) was from Amersham. S-[Methyl-^{14}C]adenosylmethionine was purchased from New England Nuclear and was mixed with unlabeled S-adenosylmethionine from P-L Biochemicals to give a specific activity of 0.3 mCi/mmol. Ribavirin was a product of ICN Life Sciences Group. Pig liver catechol-O-methyltransferase (2250 units/mg protein) was from Sigma. S-Tubercidinylmethionine and S-8-azaadenosylmethionine were generously provided by Dr. Ronald T. Borchardt of the University of Kansas. Other materials were obtained from sources identified previously (12,19).

Human blood was withdrawn from healthy volunteers into heparinized Vacutainers (Becton, Dickinson). The blood cells were washed several times with (Dulbecco's) phosphate-buffered saline and the buffy coat was discarded. Erythrocyte incubations were carried out with 5.5% cell suspensions in 10-ml volumes of phosphate-buffered saline supplemented with 5.0 mM D-glucose and 50 μM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA). Cells were incubated for 2 hr at 37° with saline or 200 μM adenosine analogue and were then supplemented with 40 μCi of L-[methyl-^{3}H]methionine for an additional 1 hr of incubation. The cells were then washed and extracted with 5.0 ml of cold 1.0 M perchloric acid. The resultant extracts were clarified by centrifugation, neutralized with KOH, filtered through glass wool to remove the insoluble potassium perchlorate, evaporated to dryness under reduced pressure (in a Buchler Evapo-Mix apparatus) and reconstituted in 500 μl of deionized water. These samples were stored at -20° until their analysis.

Cytolytic lymphocytes were obtained from CD-1 mice as described (20). Lymphocytes (8.6 x 10^6 cells per 5.0 ml of phosphate-buffered saline supplemented with 10% heat-inactivated fetal calf serum and 7.9 μM EHNA) were incubated for 1 hr with saline or 150 μM adenosine analogue and were then supplemented with 10 μCi of L-[methyl-^{3}H]methionine for an additional 1 hr of incubation. Acid-soluble extracts of the lymphocytes were prepared as described above for the erythrocytes.

Acid-soluble extracts of cells were analyzed for ribonucleoside 5'-triphosphates by anion-exchange high-performance liquid chromatography (HPLC) (19,20). Radioactive metabolites of L-[methyl-^{3}H]-methionine were analyzed both by reversed-phase HPLC (12) and by cation exchange HPLC. Cation-exchange HPLC was carried out with a Reeve Angel Partisil-10 SCX column (0.46 x 25 cm) mounted in a Varian model LCS-1000 liquid chromatograph. This column was eluted with a convex gradient from 5 to 500 mM potassium phosphate, pH 3.5. The starting volume of low concentration eluent in the mixing chamber was 30 ml. The column flow rate was 30 ml/hr and the gradient flow rate was 20 ml/hr. An auxilliary ultraviolet (280 nm) flow monitor (Schoeffel model SF770) was connected in series with the standard 254 nm monitor to allow simultaneous recording at

these two wavelengths. Fifty- or 100-µl samples of each cell extract were injected into the chromatograph. Full-scale absorbance ranges of 0.04 A unit were employed. The effluent of the liquid chromatograph was collected at 1.0-min intervals and these fractions were either monitored in a liquid scintillation spectrometer or pooled and concentrated for enzymatic analysis.

For enzymatic analysis of presumptive AdoMet analogues, cell extracts were fractionated by reversed-phase or cation-exchange HPLC and 1.0-min fractions of the column effluent were collected. A small portion of each such fraction was monitored by liquid scintillation counting in order to locate precisely the peak fractions of putative [^{3}H]AdoMet analogue and to assure that each such peak was well separated from [^{3}H]AdoMet. These peak fractions of radioactive metabolite (2-5 ml) were pooled, acidified and desalted on small columns (0.55 x 5.0 cm) of SP-Sephadex by the method of Glazer and Peale (21). After sample application, each column was washed with 20 ml of 150 mM HCl. Each radioactive metabolite was then eluted with 8.0 ml of 500 mM HCl and was evaporated to dryness under reduced pressure and reconstituted in 350 µl of deionized water. Overall recovery of [^{14}C]AdoMet by this procedure was 70%.

A portion (150 µl) of each desalted [^{3}H]metabolite was tested for reactivity with catechol-O-methyltransferase (COMT) under conditions similar to the assay procedure of Nikodejevic et al. (22). Each complete incubation mixture contained in 400 µl: 20 µmol of Tris/HCl buffer, pH 9.1; 0.4 µmol of magnesium chloride; 0.2 µmol of dithiothreitol; 0.2 µmol of 3,4-dihydroxybenzoic acid; 1150-10,220 dpm of [^{3}H]AdoMet analogue; 200 units of COMT. Each [^{3}H]AdoMet analogue was assayed in the presence and absence of 3,4-dihydroxybenzoic acid. After 2 hr the reactions were terminated by the addition of 200 µl of 1.0 M HCl and the methylated product (vanillic acid) was extracted with 10 ml of toluene/isoamyl alcohol (7/3). After centrifugation, 4.0 ml of the organic phase was mixed with 10 ml of Aquasol 2 (New England Nuclear) and monitored in a liquid scintillation spectrometer with a counting efficiency of 38% for tritium.

Results and Discussion: AdoMet is formed biosynthetically from ATP and L-methionine via the action of AdoMet synthetase (EC 2.5.1.6) (23):

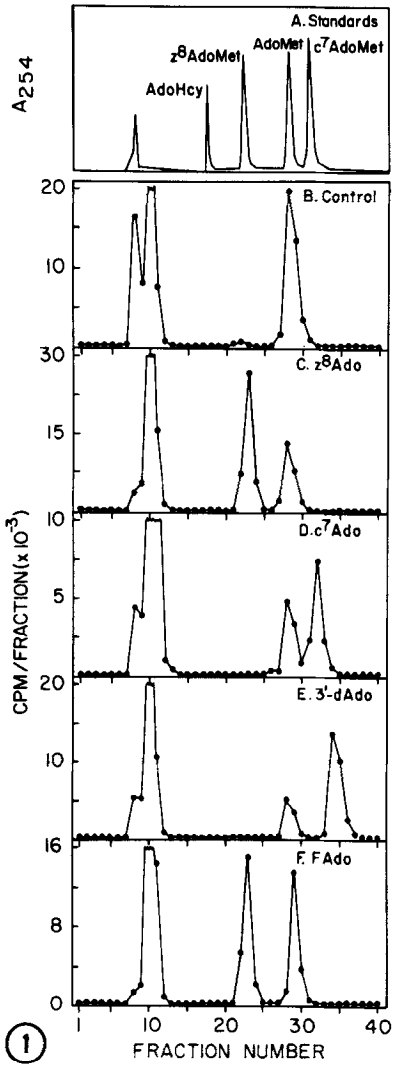
$$\text{ATP} + \text{L-methionine} \rightarrow \text{AdoMet} + \text{PP}_i + \text{P}_i$$

Since a nucleoside 5'-triphosphate is required as the immediate metabolic precursor for AdoMet biosynthesis, adenosine analogues known to undergo metabolism to 5'-triphosphates were selected for investigation of possible AdoMet analogue formation. Compounds investigated on this basis with human erythrocytes included 2-fluoroadenosine (FAdo), 7-deazaadenosine (c^7Ado), formycin (For), 2-aminoadenosine, purine ribonucleoside (nebularine, Neb), 8-azaadenosine (z^8Ado), 3'-deoxyadenosine (3'-dAdo), 2'-deoxyadenosine and ribavirin. The experimental design was pretreatment of cells with each adenosine analogue, in order to allow metabolic formation of the respective ATP analogue, and then supplementation of the cellular suspension with L-[methyl-^{3}H]methionine for an additional period of incubation. Subsequently, extracts of these cells were prepared for chromatographic analyses of radioactive metabolites. EHNA, an

inhibitor of adenosine deaminase (24), was included in all of the cellular incubations even though some of the adenosine analogues studied are not substrates for the deaminase.

Anion-exchange HPLC was employed in order to confirm the extensive metabolism of all of the above-mentioned adenosine analogues to their corresponding 5'-triphosphates in human erythrocytes. At the end of the 3-hr erythrocyte incubations with these different analogues, intracellular levels of 2'-dATP and ribavirin 5'-triphosphate were approximately 50% those of ATP; all of the other ATP analogues examined were present at levels greater than those of ATP.

Extracts of adenosine analogue-treated/L-[methyl-^{3}H]methionine-labeled cells were analyzed both by cation exchange HPLC (Fig. 1) and by reversed-phase HPLC (Fig. 2). Fig. 1A shows the separation obtained by cation-exchange HPLC of authentic samples of AdoHcy, S-8-azaadenosylmethionine (z^{8}AdoMet), AdoMet and S-7-deazaadenosylmethionine (c^{7}AdoMet); radioactive methionine was eluted in the breakthrough region (fractions no. 6-13) of the chromatogram. Extracts of L-[methyl-^{3}H]methionine-labeled control cells yielded a relatively simple elution profile of radioactivity from the cation-exchange column (Fig. 1B): a split peak in the breakthrough region of the chromatogram and a later prominent peak having the same retention time as authentic AdoMet. In addition to these peaks of radioactivity, extracts of cells treated with z^{8}Ado (Fig. 1C), c^{7}Ado (Fig. 1D), 3'-dAdo (Fig. 1E) or FAdo (Fig. 1F) each contained a unique species of radioactive material which was eluted in the region of the chromatogram near AdoMet. In the two cases where authentic AdoMet analogues were available as chromatographic standards, the novel radioactive metabolite peaks found in cells treated with z^{8}Ado (Fig. 1C) and c^{7}Ado (Fig. 1D) were eluted with the same retention times as z^{8}AdoMet and c^{7}AdoMet, respectively (Fig. 1A). In none of these cases was sufficient material present to allow ultraviolet detection or characterization of the putative S-nucleosidylmethionine metabolites. None of the other five adenosine analogues examined in this erythrocyte system yielded unique, analogue-related peaks of radioactivity under these conditions of cation-exchange HPLC.



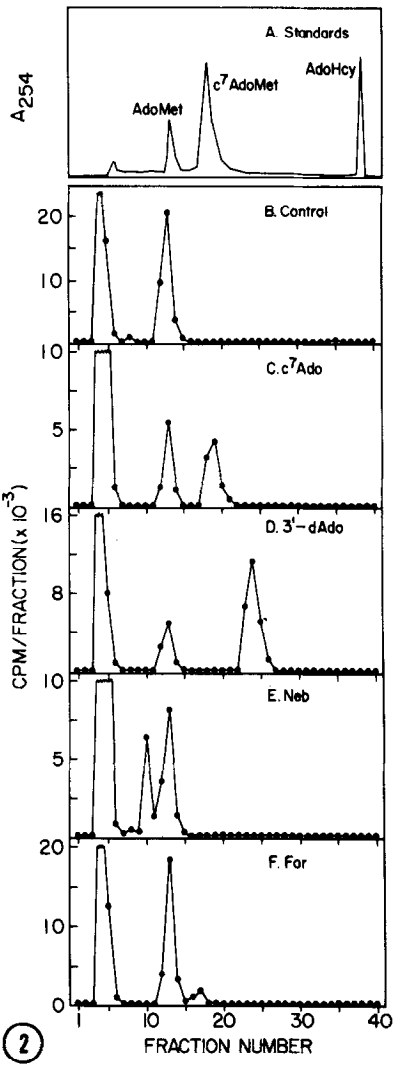


Fig. 1. Cation-exchange HPLC of standards and cell extracts. Panel A shows the ultraviolet (254 nm) elution profile of several standard compounds. Panels B-F show the radioactive elution profiles of extracts from human erythrocytes incubated with (B) saline, (C) z^8Ado, (D) c^7Ado, (E) 3'-dAdo or (F) FAdo and then labeled with L-[methyl-^{3}H]methionine.

Fig. 2. Reversed-phase HPLC of standards and cell extracts. Panel A shows the ultraviolet (254 nm) elution profile of several standard compounds. Panels B-F show the radioactive elution profiles of extracts from human erythrocytes incubated with (B) saline, (C) c^7Ado, (D) 3'-dAdo, (E) Neb or (F) For and then labeled with L-[methyl-^{3}H]methionine.

Reversed-phase HPLC has provided additional evidence for the metabolic formation of AdoMet analogues. Fig. 2A illustrates the resolution obtained by this technique with a mixture of AdoMet, c^7AdoMet and AdoHcy standards. Radio-

active methionine was eluted in the breakthrough region (fractions no. 4-6) of the chromatogram, and z^8AdoMet co-chromatographed with AdoMet in this system. Extracts of L-[methyl-^{3}H]methionine-labeled control cells gave peaks of radioactivity in the breakthrough region of the chromatogram and at the retention time corresponding to AdoMet (Fig. 2B). Extracts from cells incubated with radioactive methionine plus c^7Ado (Fig. 2C), 3'-dAdo (Fig. 2D), Neb (Fig. 2E) or For (Fig. 2F) were each found to contain a unique, additional peak of radioactivity which was eluted near AdoMet. None of the other adenosine analogues examined under these conditions, including z^8Ado and FAdo, led to the appearance of novel peaks of radioactivity by reversed-phase HPLC; this failure to resolve a peak of putative [^{3}H]z^8AdoMet is explicable by the demonstrated co-elution of authentic z^8AdoMet and AdoMet in this chromatographic system.

Similar experiments with mouse cytolytic lymphocytes have provided chromatographic evidence for the metabolic formation of these same six AdoMet analogues (results not shown).

As a means of characterizing these novel radioactive metabolites further, each of the putative AdoMet analogues was tested for its ability to donate its radioactive moiety to 3,4-dihydroxybenzoic acid in the presence of COMT. Under the conditions of the assay, approximately 50% of the radioactivity of commercial S-[methyl-^{14}C]AdoMet was transferred into extractable product; this reaction was shown to be completely dependent upon the presence of both the non-radioactive substrate and the enzyme. The [^{3}H]AdoMet formed in L-[methyl-^{3}H]methionine-labeled control erythrocytes donated 83% of its radioactivity in this assay. In a similar manner, the presumptive S-nucleosidylmethionine metabolites of For, Neb, c^7Ado, FAdo, z^8Ado and 3'-dAdo were found to donate 46-91% of their radioactivity to 3,4-dihydroxybenzoic acid in the presence of COMT. The reactivity of these L-[methyl-^{3}H]methionine-labeled metabolites of adenosine analogues with an AdoMet-dependent methyltransferase such as COMT constitutes strong evidence that these metabolites are indeed AdoMet analogues. Borchardt and his co-workers have synthesized a number of AdoMet analogues, including c^7AdoMet,

z^8AdoMet and S-3'-deoxyadenosylmethionine (3'-dAdoMet), and have shown that these compounds are utilized with varying degrees of efficiency by several different methyltransferases (25).

While several methionine analogues have been shown to undergo metabolism to the respective amino acid-modified analogues of AdoMet (26 and references contained therein), the present communication provides the first known examples of the metabolic formation of nucleoside-modified analogues of AdoMet. 2'-dATP (27) and UTP (28) have been reported to be alternate substrates for AdoMet synthetase, although chemical purity and kinetic parameters were not determined for these two nucleotides. The results of the present study indicate that a variety of ATP analogues can serve as alternate substrates for this enzyme.

Several nucleoside-modified analogues of AdoMet have been synthesized chemically and shown to be competitive inhibitors of different AdoMet-utilizing methyltransferases (25). It is, therefore, plausible that AdoMet analogues formed metabolically from purine (nucleoside) antimetabolites may interfere with cellular methylation reactions. This possibility is under investigation.

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