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METABOLIC CONVERSION OF NEPLANOCIN A TO S-NEPLANOCYLMETHIONINE BY MOUSE L 929 CELLS

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Summary: Treatment of mouse L cells with 1 μM Neplanocin A, results in the metabolic formation of the corresponding S-nucleosidylmethionine derivative. Identification of S-neplanocyl-L-methionine is based upon chromatographic analysis of the radiolabelled compound isolated from Neplanocin A-treated cells incubated with either [methyl-^{3}H] or [carboxyl-^{14}C]methionine and upon the results of in vivo studies using specific metabolic inhibitors. The intracellular level of this AdoMet analogue increases to approximately 500 pmoles/10^{6} cells within 12 hours of Neplanocin A administration, after which it slowly decreases. The apparent metabolic utilization of this unique derivative is supported by our observation that it is capable of serving as a substrate for catechol-O-methyltransferase in vitro. These results provide evidence for a second mechanism by which Neplanocin A can affect cellular S-adenosylmethionine-dependent methylation reactions.

S-Adenosyl-L-methionine (AdoMet)-dependent transmethylation reactions are involved in the biosynthesis and/or metabolism of a wide variety of small molecules and in modulating the activity of macromolecules (1,2). To elucidate the physiological and pharmacological significance of these reactions, investigators have sought to perturb the cellular metabolic balance with specific inhibitors to one or more of the enzymes involved in these biochemical pathways. Much work in this area has focused on the design and evaluation of purine analogues. As noted by Zimmerman and his colleagues (3), such analogues are capable of inhibiting AdoMet-dependent transmethylations by one or more distinct mechanisms. These include: (a) inhibition of AdoMet synthesis from ATP and L-methionine, thereby reducing the intracellular AdoMet concentration (4,5); (b) inhibition of AdoMet-dependent methyltransferases by purine antimetabolites (6,7); and (c) indirect inhibition of these

Abbreviations: AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; NpcA, Neplanocin A; Ado, adenosine; HPLC, high-performance liquid chromatography; COMT, catechol-O-methyltransferase (EC 2.1.1.6); DHB, dihydroxybenzoic acid; UV, ultraviolet; NpcAMet, S-neplanocylmethionine.

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methyltransferases by direct inhibition of S-adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1) and subsequent elevation of intracellular AdoHcy (a product inhibitor of many methyltransferases) (8-11), or via AdoHcy hydrolase-catalyzed condensation with L-homocysteine to yield the corresponding AdoHcy derivatives as methyltransferase inhibitors (8,9,12-14), or by metabolic conversion to the corresponding AdoMet analogue similarly yielding a potential inhibitor of AdoMet-dependent methyltransferases (3,15).

Currently our laboratory has been investigating the effects of Neplanocin A (NpcA), on cellular transmethylation reactions. NpcA, an adenosine (Ado) analogue in which the ribose moiety is replaced by a cyclopentene ring, is a novel antibiotic first reported by Yaginuma and coworkers to have potent antitumor activity (16,17). We have shown that this compound is also a potent inhibitor of AdoHcy hydrolase and of vaccinia virus multiplication in mouse L 929 cells (18). In the present communication we demonstrate the metabolic conversion of this novel nucleoside to its corresponding AdoMet derivative, which appears to be active as a substrate for, at least, some AdoMet-dependent methyltransferases.

Materials and Methods: Neplanocin A [(-)-9-[trans-2,trans-3-dihydroxy-4(hydroxymethyl)-cyclopent-4-enyl]adenine] (NpcA) was generously provided by the Toyo Jozo Co., Ltd., Japan. Adenosine dialdehyde was prepared by periodate oxidation of adenosine as previously described (19). Cycloleucine was obtained from Sigma Chemical Co. (St. Louis, MO).

Stock cultures of mouse L 929 cells were grown in suspension (37° C) in Waymouth's modified 752/1 spinner medium (KC Biological, Inc., Lenexa, KS) supplemented with 5% bovine calf serum. Experimental cultures were plated in 60 mm polystyrene tissue culture dishes at a density of 2.5 X 10^6 cells/dish and allowed to attach for 4 hours. The medium was then replaced by chemically-defined KU-1 medium (20) and the cells allowed to acclimate in serum-free conditions for 12 hours.

To initiate experiments the monolayer cultures of L cells were refed with 5 mL of fresh KU-1 medium containing 1 μM NpcA and, where indicated, 15 μCi L-[methyl-^{3}H]methionine (15 Ci/mmole) or 5 μCi L-[carboxyl-^{14}C]methionine (51 mCi/mmole) (Amersham Corp., Arlington Heights, IL). After incubation at 37° C for the designated time period, the culture medium was removed and the cells were rinsed with ice cold phosphate-buffered saline (PBSA), detached by trypsinization and pelleted by centrifugation (2 minutes) in an Eppendorf microfuge. The cell pellet was resuspended in 125 μL 0.4 N $HClO_4$, rapidly frozen on dry-ice and stored at -70° C prior to HPLC analysis.

Acid-soluble cell extracts were analyzed by reverse-phase HPLC using a Perkin-Elmer Series 3 instrument equipped with a 25 cm x 4.6 mm Zorbax C-8 column (DuPont Co., Wilmington, DE). Separation was based on a 2-step gradient program at a flow rate of 1.0 mL/min. - Solvent A: acetonitrile; Solvent B: 50 mM sodium phosphate (pH 3.2), 10 mM heptane sulfonic acid; Program: 5-20% A, 15 min.; 20-40% A, 20 min. Eluted material was continuous-

ly monitored for absorbance at 254 nm (0.1 A unit full-scale) and quantitated using a Perkin-Elmer Sigma Series 10-B Console Data Station, after which fractions were collected at 1.0 min intervals. Radioactivity in each fraction was determined by diluting a small sample (50-100 µL) in 10 mL of 3a70B scintillation cocktail (Research Products International, Elk Grove Village, IL) followed by liquid scintillation spectrometry.

Preparation of [methyl-^{3}H]AdoMet and the corresponding radiolabelled NpcA analogue for enzymatic analysis were carried out as described by Zimmerman et al. (3). Following isolation of the [^{3}H]metabolites, they were examined for enzymatic activity with catechol-O-methyltransferase (COMT) according to the procedure of Borchardt (21). Each final reaction volume of 250 µL contained: 20 µmol N-tris[hydroxymethyl-2-aminoethane] sulfonic acid (TES), pH 7.6; 0.3 µmol magnesium chloride; 1 µmol dithiothreitol; 0.25 µmol 3,4-dihydroxybenzoic acid; 15,000 dpm [^{3}H]NpcAMet; 129 units of COMT. Blanks were prepared in identical fashion with the exception that 3,4 dihydroxybenzoic acid was omitted. After 60 minutes at 37° C, the reactions were terminated by the addition of 100 µL 0.1 N HCl and the methylated product (vanillic acid) was extracted with 3 mL of toluene/isoamyl alcohol (7:3). Following centrifugation duplicate 1.0 mL samples of the organic phase were counted for radioactivity in 10 mL 3a70B scintillation cocktail.

Results and Discussion: Our laboratory has demonstrated that NpcA is a potent inhibitor of AdoHcy hydrolase and of vaccinia virus multiplication in mouse L 929 cells (18). Moreover, HPLC analyses of the NpcA-treated, virus-infected cells indicated that the antiviral activity of this nucleoside analogue appears to be related to an elevation of intracellular AdoHcy and presumably to a perturbation of cellular methylation reactions. During the course of these analyses, we inadvertently observed the appearance of a unique, UV-absorbing peak (254 nm) in the acid-soluble extracts from NpcA-treated cells (Figure 1A, 17.96 minutes). Initial attempts to identify this material revealed that it was not NpcA itself, which elutes at 10.9 minutes, nor deaminated NpcA, which elutes at 4.8 minutes (Tsujino and coworkers previously reported NpcA to be a substrate for adenosine deaminase, EC 3.5.4.4, both *in vitro* and *in vivo*; reference 22). In addition, our previous indications that NpcA is not a substrate for AdoHcy hydrolase (16), thus eliminating the corresponding S-nucleosidylhomocysteine derivative as a possibility, were confirmed by the observation that a 2 hour pretreatment of L cells with 5 µM adenosine dialdehyde (another potent inhibitor of L cell AdoHcy hydrolase; reference 23) had no effect on the appearance of this HPLC peak (data not shown).

Considering the close structural resemblance of NpcA to Ado, the similarity of retention times for the NpcA-induced peak and AdoMet on reverse

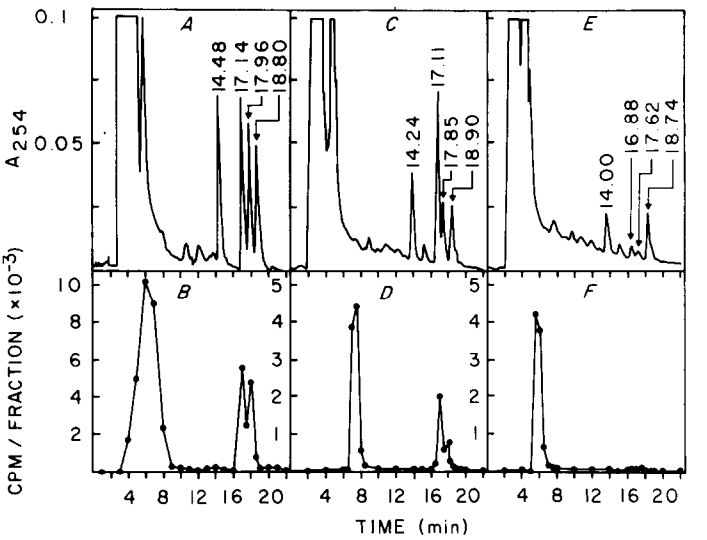

Figure 1. Reverse-phase HPLC profiles of L cell extracts. Panels A,C,E - absorbance 254 nm; Panels B,D,F - [^{3}H]radioactivity. Panels A,B - 10 hour treatment, 1 μM NpcA and L-[methyl-^{3}H]methionine; Panels C,D - 4 hour treatment, 1 μM NpcA and L-[methyl-^{3}H]methionine; Panels E,F - 30 minute pretreatment, 20 μM cycloleucine, 4 hour treatment, 1 μM NpcA and L-[methyl-^{3}H]methionine. On Panels A, C and D, respectively: AdoHcy = 14.48, 14.24 and 14.00 min.; AdoMet = 17.14, 17.11 and 16.88 min.; NpcAMet = 17.96, 17.85 and 17.62 min.; and Tryptophan = 18.80, 18.90 and 18.74 min.

phase HPLC (Figure 1A, 17.96 vs. 17.14, respectively) prompted us to investigate whether this material might be the S-nucleosidylmethionine derivative of NpcA. Consequently, L cells were treated with 1 μM NpcA in the presence of L-[methyl-^{3}H]methionine for 10 hours and cell extracts prepared for HPLC analysis. As shown in Figure 1B, two peaks of radioactivity were detected in the 16-18 minute region of the chromatogram; one corresponding to the AdoMet UV-absorbing peak and the other to the NpcA-induced peak. A similar experiment in which the cells were pretreated for 30 minutes with 20 μM cycloleucine, a known inhibitor of AdoMet synthetase (24), and then exposed to 1 μM NpcA and radioactive methionine for 4 hours indicated that the appearance of this second peak (like AdoMet) is related to cellular AdoMet synthetase activity. Figures 1C-F illustrate that, under these conditions, significant decreases were apparent in the UV-absorbance peaks (93.4 and 93.4 percent inhibition) and the associated radioactivity peaks (92.2 and 88.5 percent inhibition) for both AdoMet and the NpcA-induced peak, respectively. Virtually identical results were obtained with L-[carboxyl-^{14}C]methionine

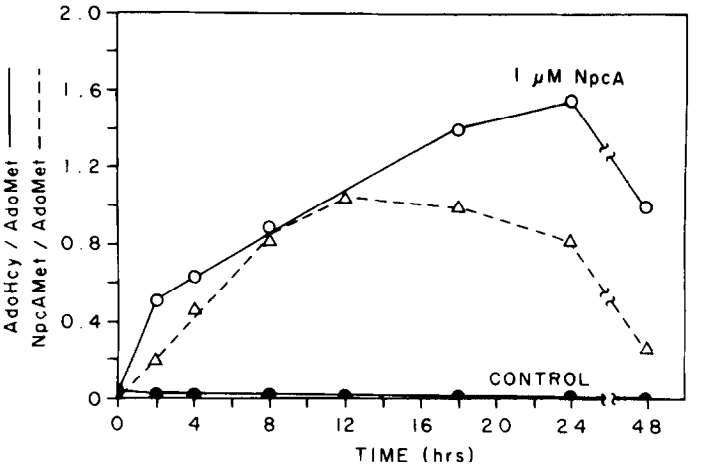

Figure 2. NpcAMet/AdoMet and AdoHcy/AdoMet ratios of L cell extracts. L cell cultures were refed with KU-1 medium containing (○, △) or lacking (●) 1 μM NpcA and incubated (37° C) for the indicated times. The cells were harvested and perchloric acid extracts were prepared and analyzed by reverse-phase HPLC for UV-absorbing material (254 nm).

(data not shown), confirming that the material in this peak is S-Neplanocylmethionine (NpcAMet).

Our ability to detect a UV-absorbing peak for NpcAMet in NpcA-treated L cells permitted us to evaluate the intracellular concentrations of this derivative at different times after treatment and to compare these values to the corresponding AdoMet levels in the same cells. As shown in Figure 2, the intracellular ratio of NpcAMet/AdoMet begins to increase within 2 hours of NpcA administration and reaches a maximum value of 1.0 after 12 hours. This represents an intracellular level of NpcAMet which is approximately 500 pmoles/10^6 cells. In contrast to the AdoHcy/AdoMet ratio of the NpcA-treated cells which continues to rise throughout 24 hours (Figure 2), the NpcAMet/AdoMet ratio exhibits a plateau between 12 and 24 hours and then rapidly declines. The NpcAMet is no longer detectable 72 hours after NpcA administration.

The disappearance of intracellular NpcAMet suggests that it is metabolized by the cells. To examine this possibility, [methyl-^{3}H]NpcAMet was isolated from NpcA-treated cells and tested for its substrate activity *in vitro* with purified COMT. Employing 3,4-dihydroxybenzoic acid (DHB) as a

methyl acceptor, we found that at least 68% of the radioactivity was transferred from the labeled NpcAMet to DHB. This finding further confirms that the unique derivative is NpcAMet and provides an explanation for its disappearance in L cells, i.e., metabolic utilization.

The fact that the intracellular level of NpcAMet begins to decline before the AdoHcy/AdoMet ratio reaches its maximum value in the treated cells raises important questions about the methyl donating activity of this analogue and the ability of AdoHcy to inhibit methylations which can utilize NpcAMet as a methyl donor. In light of our recent findings that elevation of intracellular AdoHcy does not produce complete (100%) inhibition of methyltransferase reactions in cells (23), it will be interesting to compare the activity of this analogue to AdoMet as a methyl donor for various methyltransferases.

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