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Purification and Characterization of Some Metabolic Effects of S-Neplanocylmethionine

BRADLEY T. KELLER, RICHARD S. CLARK, ANTHONY E. PEGG, AND RONALD T. BORCHARDT

Departments of Biochemistry and Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas 66045 and Department of Physiology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

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SUMMARY

Our laboratory has previously demonstrated that treatment of mouse L929 cells with 1 μM neplanocin A results in the metabolic formation of *S*-neplanocylmethionine (Keller, B. T., and R. T. Borchardt, *Biochem. Biophys. Res. Commun.* **120**:131–137 (1984)). The present study describes an efficient procedure for the purification of this analog from L cells based on its inherent chemical stability in alkaline conditions. Several metabolic effects of *S*-neplanocylmethionine are also reported. In L cells, *S*-neplanocylmethionine was determined to have an apparent half-life of 13 hr compared to 1 hr for *S*-adenosylmethionine during the initial 2 hr of a cycloleucine block. Analysis of polyamine levels in neplanocin A-treated cells showed a 3.8-fold decrease in putrescine and a 1.7-fold decrease in spermidine by 24 hr, reflecting a decrease in the cell growth rate in response to neplanocin A rather than a direct effect of *S*-neplanocylmethionine on the cellular *S*-adenosylmethionine decarboxylase. Consistent with these results are our findings that *S*-neplanocylmethionine does not significantly inhibit purified rat prostate or *Escherichia coli* *S*-adenosylmethionine decarboxylase and that [*carboxy*-^{14}C]*S*-neplanocylmethionine exhibits no substrate activity with either enzyme. Purified *S*-neplanocylmethionine was observed to be a weak inhibitor of both *S*-adenosylmethionine-dependent protein carboxymethyltransferase and lipid methyltransferase in L cell extracts, having an IC_{50} value of 205 μM (*S*-adenosylmethionine = 10 μM). Similar studies with [*methyl*-^{3}H]*S*-neplanocylmethionine indicate that the analog has little substrate activity in these two L cell methylation reactions and thus appears to act as a poor competitive inhibitor.

INTRODUCTION

Neplanocin A is a naturally occurring carbocyclic adenosine analog in which the ribose moiety is replaced by a cyclopentene ring (1). Interest in the biological activity of this novel antibiotic was stimulated by the initial observation that it possessed significant antitumor activity despite only minimal antimicrobial and antifungal properties (2). Subsequent studies in our laboratory demonstrated that neplanocin A is also a potent inhibitor of AdoHcy[1] hydrolase and of vaccinia virus multiplication in mouse L929 cells (3). The inhibition of cellular AdoHcy hydrolase by neplanocin A results in the intracellular accumulation of AdoHcy and consequential inhibition of AdoMet-dependent macromolecular methylation reactions (*e.g.* protein, lipid, and nucleic acid) (3, 4). The latter effect, and in particular the inhibition of methylation of the 5′-terminal cap structure on viral mRNAs, is presumed to be a major factor in the mechanism of antiviral action of this compound.

In conjunction with these findings, we also reported the metabolic conversion of neplanocin A to its corresponding AdoMet analog, NpcMet (Fig. 1), by the L cells (5). NpcMet was observed to accumulate to intracellular levels equal to or slightly greater than AdoMet itself (*i.e.*, 500 pmol/10^6 cells) within 12–18 hr of administration of 1 μM neplanocin A to the culture medium. Implied in these results is that neplanocin A is phosphorylated to the corresponding nucleotide triphosphate prior to being utilized by AdoMet synthethase. This has been independently confirmed by two other research groups using different cell culture lines. Saunders and co-workers demonstrated the appearance of neplanocin triphosphate in neplanocin A-treated Chinese hamster ovary cells, although they were unable to make a direct correlation between this nucleotide and the high cytotoxicity of the drug (6). Using HT-29 carcinoma cells, Glazer and Knode (7) not only reported the appearance of neplanocin A nucleotides but also found the major metabolite to be

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[1] The abbreviations used are: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; HPLC, high pressure liquid chromatography; IC_{50}, concentration of drug eliciting 50% inhibition of control value; NpcHcy, *S*-neplanocylhomocysteine; NpcMet, *S*-neplanocylmethionine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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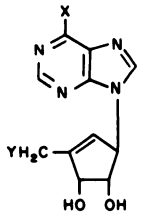
NpcMet (7). Moreover, the observed cytocidal activity of neplanocin A to these cells was determined to be specifically related to a decrease in RNA methylation apparently mediated by this AdoMet analog.

Although the effects of inhibitors of AdoHcy hydrolase (*e.g.*, adenosine dialdehyde, 3-deazaadenosine, and 3-deazaaristeromycin) on cellular methylation reactions have been demonstrated in numerous systems (8–10), the metabolic consequences due to the concomitant formation of NpcMet following neplanocin A treatment have not been thoroughly examined. The potential for combined effects on AdoMet-dependent methylation reactions either as an alternate substrate and/or inhibitor, as well as on AdoMet decarboxylase and the polyamine biosynthetic pathway, certainly exist. In the studies presented here we describe an efficient procedure for the purification of NpcMet from L cells and report on some of its metabolic effects using L cell lysates or purified enzyme systems.

EXPERIMENTAL PROCEDURES

Materials. Neplanocin A was kindly donated by the Toyo Jozo Co., Ltd., Shizuoka, Japan. Radiochemicals were obtained as follows: [*methyl*-^{3}H]AdoMet (18 Ci/mmol) from ICN Radiochemicals, Irvine, CA and [*carboxy*-^{14}C]AdoMet (60 mCi/mmol) and [*methyl*-^{3}H]Met (80 Ci/mmol) from New England Nuclear. The remaining chemicals and supplies were purchased from the following commercial suppliers: Waymouth's 752/1 low calcium spinner medium (KC Biologicals, Inc., Lenexa, KS); bovine calf serum (Hazelton Dutchland Laboratories, Denver, PA); Zorbax C-8 reverse phase HPLC column (DuPont Co., Wilmington, DE); Partisil-10 SCX cation exchange HPLC column (Whatman); and gentamycin, cycloleucine, 1-heptanesulfonic acid, and SP-Sephadex C-25 (Sigma).

Cell culture. Stock cultures of clone 929 mouse L cells, strain L (Earle) were grown in suspension at 37° in Waymouth's modified 752/1 spinner medium supplemented with 5% bovine calf serum and 70 μg/ml gentamycin. Experimental cultures for the analysis of polyamines were inoculated at 1.5 × 10^6 cells/60-mm dish and allowed to attach for 4 h. The medium was then replaced by a chemically defined modified version of Waymouth's MD 705/1 medium (11). Neplanocin A was prepared as a sterile concentrated stock solution (1.5–10 mM) in glass-distilled water and added directly to the culture medium at the indicated concentrations.



	X	Y
Neplanocin A	$-NH_2$	$-OH$
Neplanocin D	$-OH$	$-OH$
Neplanocin Triphosphate	$-NH_2$	$-O(PO_3)_3^{-4}$
S-Neplanocylmethionine	$-NH_2$	$-\overset{\oplus}{S}(CH_2)_2CHCOOH$ (CH$_3$; NH$_2$)
decarboxylated S-Neplanocylmethionine	$-NH_2$	$-\overset{\oplus}{S}(CH_2)_2CH_2NH_2$ (CH_3)
S-Neplanocylhomocysteine	$-NH_2$	$-S(CH_2)_2CHCOOH$ (NH_2)

FIG. 1. *Structures of potential neplanocin A metabolites*

Purification of NpcMet. Neplanocin A-treated cells were collected by centrifugation, washed with ice-cold phosphate-buffered saline, and lysed in 1 volume of 0.4 N perchloric acid. The acid supernatant was adjusted to approximately pH 10 with 5 N potassium hydroxide, incubated at 37° for 20 min, and then reacidified to pH 3 with 70% perchloric acid. The potassium perchlorate precipitate was removed by centrifugation, and 800-μl aliquots of the supernatant injected onto a Zorbax C-8 reverse-phase HPLC column (25 cm × 4.6 mm). Separation of NpcMet was accomplished using a two-step gradient of acetonitrile (A) in 50 mM sodium phosphate, pH 3.3, 10 mM heptane sulfonic acid (B) at a flow rate of 1 ml/min: 5–20% A, 15 min; 20–25% A, 10 min.

The material corresponding to NpcMet was collected and adjusted to 10 mM HCl, absorbed to SP-Sephadex C-25, washed with 150 mM HCl, and eluted in 500 mM HCl (12). The eluant was evaporated to dryness under reduced pressure and resolubilized in glass-distilled water.

Lipid methylation assay. Cells were lysed in 10 mM potassium phosphate buffer, pH 7.5 (3 × 10^6 cells/50 μl) by swelling on ice for 10 min followed by rapid freezing on dry ice. Lipid methylation was measured using a modification of the procedure of Hirata *et al.* (13). A total reaction volume of 350 μl included 50 μl of cell lysate in 300 mM phosphate buffer (pH 7.5), 15 mM magnesium chloride, 10 μM [*methyl*-^{3}H]AdoMet (5.5 μCi) and the indicated concentration of NpcMet. In some experiments various concentrations of [*methyl*-^{3}H]NpcMet (10–21.1 Ci/mol) were used in place of AdoMet in order to test the substrate activity of the analog. After 60 min at 37°, the reaction was stopped by the addition of 1 ml of 20% trichloroacetic acid, 40 mM methionine, pH 7.5, and the samples centrifuged at 18,000 × *g* for 20 min at 4°. The pellets were suspended in 3 ml of chloroform/methanol (2:1) and extracted twice with 2.0 ml of 0.1 M potassium chloride in 50% methanol. A 2-ml aliquot of the organic phase was evaporated and the radioactivity determined.

Protein carboxymethylation assay. Cell lysates were prepared as described for the lipid methylation assay. Protein carboxymethylation was measured using a modified procedure of Diliberto *et al.* (14). A total reaction volume of 350 μl included 50 μl of cell lysate, 200 μg of gelatin, 200 mM citric phosphate buffer (pH 6), and 10 μM [*methyl*-^{3}H]AdoMet (5.5 μCi) and the indicated concentration of NpcMet. As before, [*methyl*-^{3}H]NpcMet was used in place of AdoMet in some experiments. After incubation for 60 min at 37°, the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid, and 50 μl of bovine serum albumin (20 mg/ml) was added as carrier protein. The acid precipitate was collected by centrifugation and the protein methyl esters hydrolyzed with 750 μl of 1.0 M sodium borate (pH 11) in 1% methanol at room temperature for 20 min. The [^{3}H]methanol generated upon hydrolysis was extracted with 3 ml of toluene/isoamyl alcohol (3:2), and two 1-ml aliquots were transferred to scintillation vials. One aliquot was counted directly in 10 ml of scintillation cocktail and the other evaporated to dryness in a vacuum oven at 80° prior to counting. The difference in radioactivity between the evaporated and nonevaporated samples was used as the measure of protein carboxymethylation.

Purified protein carboxymethyltransferase, obtained by the method of Kim *et al.* (15) with the modifications of Aswad and Deight (16), was used in place of the cell lysates in the indicated experiments. Each assay tube contained 21 μg of protein having a specific activity of 25.5 units/min/mg where 1 unit = 1 pmol of [^{3}H]CH_3 transferred per min.

AdoMet decarboxylase assay. Rat prostate and *Escherichia coli* AdoMet decarboxylase were purified and assayed as previously described by Pegg and Jacobs (17). The reaction (30 min) was followed by measuring the production of $^{14}CO_2$ in an assay mixture (200 μl) containing 100 μM [*carboxy*-^{14}C]AdoMet (0.075 μCi), 100 mM HEPES, pH 7.5, 2.5 mM 2-mercaptoethanol, and the enzyme. For assays of the prostate enzyme 2.5 mM putrescine was included; this was replaced by

10 mM $MgCl_2$ for the *E. coli* enzyme. The acid supernatants of the reaction mixtures as well as the stock solutions of NpcMet were analyzed by HPLC on a Partisil SCX (10 × 0.46 cm) cation exchange column with isocratic elution (1 ml/min) in 300 mM ammonium formate, pH 4.0, 10% acetonitrile at 40°.

Analysis of polyamine levels in L cells. Cells (1.5×10^6 cells/60-mm dish) were refed with fresh medium containing or lacking 1 μM neplanocin A and incubated for either 12, 24, or 36 hr. The cells were removed by trypsinization, washed with phosphate-buffered saline and collected by centrifugation. The pellets were suspended in 75 μl of 0.4 N perchloric acid and sonicated for 30 min. The acid precipitate was removed by centrifugation and assayed for protein according to the method of Lowry *et al.* (18), and the supernatant was analyzed for polyamines using an amino acid analyzer with fluorescence detection as previously described (19).

RESULTS

Intracellular turnover of NpcMet. Our initial approach to assess the metabolic potential of NpcMet in L cells was to examine the "apparent" intracellular turnover rate of the analog following inhibition of AdoMet synthetase with cycloleucine (20). A comparison of this rate to that of AdoMet would provide a qualitative indication of the utilization of NpcMet in the relevant metabolic processes. Experimentally, cells were treated with 1 μM neplanocin A for 12 hr to promote the intracellular accumulation of NpcMet, then exposed to culture medium containing 20 mM cycloleucine for 1–4 hr. Fig. 2 illustrates that initially the "apparent" turnover rate for NpcMet ($t_{1/2}$ = 13 hr) was much slower than that of AdoMet ($t_{1/2}$ = 1 hr), suggesting that the analog is poorly metabolized in comparison to the natural substrate. However, after 1 hr when the intracellular amount of AdoMet had been reduced by approximately 50%, its turnover decreased significantly to a rate similar to that of NpcMet. These findings suggest that there may be two distinct pools of AdoMet in L cells, one being more easily accessible than the other (see "Discussion"). Fig. 2 also demonstrates that the turnover of AdoMet in cells not exposed to neplanocin A is virtually identical to that in the treated cells, indicating that the presence of NpcMet has little effect on the utilization of AdoMet.

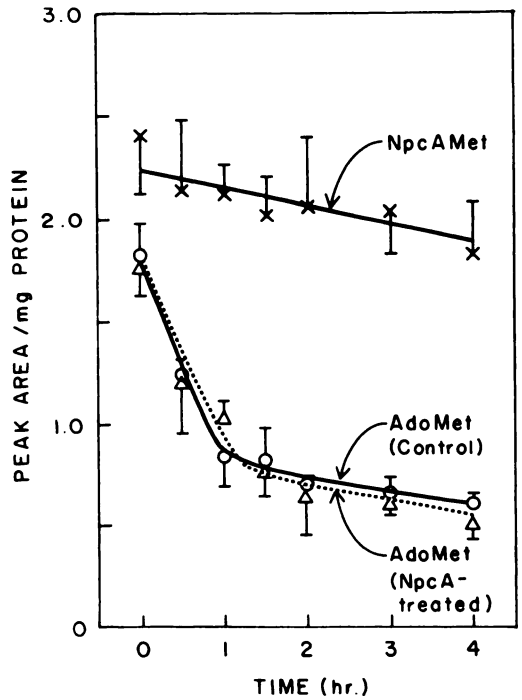


FIG. 2. *Intracellular turnover of NpcMet and AdoMet in mouse L cells*

L cells were treated with or without 1 μM neplanocin A for 12 hr and then refed with fresh culture medium lacking neplanocin A but containing 20 mM cycloleucine. At the indicated times after cycloleucine addition, cultures were harvested, the cells extracted with 0.4 N $HClO_4$, and the acid-soluble material analyzed by reverse-phase HPLC as described under "Experimental Procedures." Each point represents the mean ± SE of 4–6 experiments. ×, NpcMet; Δ, AdoMet, neplanocin A-treated cells; O, AdoMet, untreated cells.

In light of these findings, it should be reported that the 20 mM cycloleucine block was determined to inhibit only 73% of the AdoMet synthetase activity within 4 hr of administration. Consequently, the stated $t_{1/2}$ values are overestimated and represent "apparent" turnover rates under these conditions. Because we are only interested in the relative turnover of NpcMet to AdoMet, the lack of a complete block will have little, if any, effect on the present results.

Purification of NpcMet from mouse L cells. To investigate the direct effects of NpcMet on AdoMet-dependent metabolism the analog was purified from perchloric acid extracts of mouse L cells grown in suspension culture ($1.5–2.0 \times 10^6$ cells/ml) following a 12-hr exposure to 3 μM neplanocin A. The general procedure for separation of NpcMet involved reverse-phase HPLC on a Zorbax C8 column as described under "Experimental Procedures." It was initially observed, however, that AdoMet and NpcMet eluted with very similar retention times in this system and often appeared as partially overlapping peaks. This was particularly true when large amounts of material (i.e., from $1.5–2.0 \times 10^8$ cells) were applied to the column. As a consequence, the preparation of NpcMet was usually contaminated by a detectable amount of AdoMet which posed a significant problem when assaying NpcMet for substrate and/or inhibitory activity in AdoMet-related metabolic processes.

In an attempt to overcome this problem, we investigated the stability of NpcMet under alkaline conditions. As previously reported by Borchardt (21), AdoMet is extremely labile under such conditions (due to cleavage of the glycosidic bond) while its carbocyclic analog, *S*-aristeromycinylmethionine, is stable. Based on the similar carbocyclic structure of the NpcMet analog we reasoned that this compound might also be stable to the alkaline conditions, providing a selective means of removing AdoMet from the extract. As shown in Fig. 3 (*A* and *B*), a 20-min incubation of the cell extract at pH 10 resulted in complete degradation of AdoMet (17.24 min) with less than 3% loss of the material in the NpcMet peak (17.96, 17.79 min). The loss of AdoMet, together with the simultaneous degradation of 5′-methylthioadenosine (22.80 min), totally account for the appearance of adenine at 12.44 min.

NpcMet collected following alkaline treatment was desalted by SP-sephadex chromatography (12) and evaporated to dryness. Rechromatographing this material

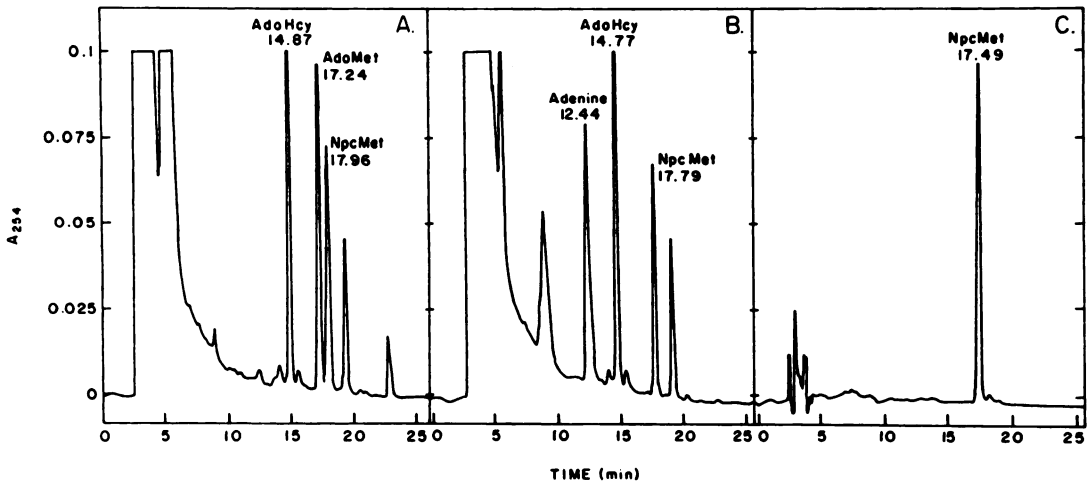


FIG. 3. *Reverse-phase HPLC profiles of untreated and alkaline-treated L cell extracts and purified NpcMet*

L cells were treated with 3 μM neplanocin A for 12 hr and then extracted with 0.4 N $HClO_4$. NpcMet was subsequently purified by reverse-phase HPLC as described in the text. Panel A, untreated cell extract; panel B, same cell extract following 20-min incubation at pH ~10 (sample was reacidified prior to injection onto HPLC); panel C, purified NpcMet.

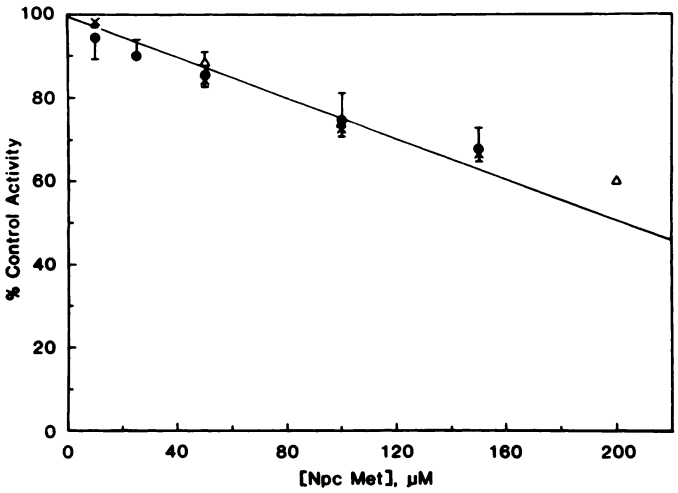


FIG. 4. *Inhibition of lipid and protein methylation in L cell lysates by NpcMet*

L cell lysates were prepared and assayed for either [*methyl*-^{3}H] AdoMet-dependent lipid or protein carboxymethyltransferase activity in the presence of increasing concentrations of unlabeled NpcMet as described under "Experimental Procedures." ×, lipid methyltransferase activity; ●, carboxymethyltransferase activity (mean value ± the SE of 4 experiments); △, purified calf thymus carboxymethyltransferase (average value of 2 experiments).

confirmed the absence of AdoMet, yielding a final product of greater than 96% purity (Fig. 3*C*).

In vitro effects of NpcMet on protein and lipid methylation. To assess the inhibitory effects of NpcMet on AdoMet-dependent transmethylation reactions, we studied the macromolecular processes of lipid and protein carboxyl methylation in lysates of L cells. In addition, we examined the latter reaction using purified calf thymus protein carboxylmethyltransferase prepared in our laboratory. The results presented in Fig. 4 show that, although the neplanocin analog produced similar concentration-dependent inhibition in all three methylation reactions, it was found to be a relatively weak inhibitor.

TABLE 1

Substrate activity of NpcMet in lipid and protein carboxymethylation

L cell lysates were prepared and assayed for lipid methylation (13) or protein carboxymethylation (14) using either [*methyl*-^{3}H]NpcMet or [*methyl*-^{3}H]AdoMet as the substrate at 25, 50, and 100 μM: K_m and V_{max} were determined by linear regression analysis of the best-fitting line through the data points

Substrate	Acceptor	K_m	V_{max}
		μM	*pmol/min*
NpcMet	Protein	122	0.80
NpcMet	Lipid	195	1.17
AdoMet	Protein	4.2	1.24
AdoMet	Lipid	7.5	1.07

TABLE 2

Effect of neplanocin A on L cell polyamine content

Cell cultures were treated with 1 μM neplanocin A for the indicated time and harvested by trypsinization. Perchloric acid cell extracts were prepared and the acid-soluble material analyzed for polyamine content as described by Pegg (19). Each value represents the average of two determinations ± the standard deviation.

Sample	Cell number	Putrescine	Spermidine	Spermine
	×10⁶	*nmol/10⁶ cells*		
Control, 12 hr	1.75	0.89 ± 0.05	4.95 ± 0.48	1.70 ± 0.20
Neplanocin A, 12 hr	1.80	0.30 ± 0.06	4.20 ± 0.39	1.79 ± 0.29
Control, 24 hr	2.85	0.65 ± 0.02	4.43 ± 0.14	1.28 ± 0.05
Neplanocin A, 24 hr	2.50	0.17 ± 0.02	2.67 ± 0.00	1.34 ± 0.02
Control, 36 hr	4.25	0.57 ± 0.06	4.78 ± 0.41	1.32 ± 0.09
Neplanocin A, 36 hr	2.85	0.18 ± 0.00	3.44 ± 0.08	1.68 ± 0.03

The IC_{50} value for these reactions was determined to be 205 μM or 20-fold higher than the concentration of AdoMet used in the assays (*i.e.*, 10 μM).

The substrate activity of NpcMet as a methyl donor was similarly examined for the two L cell methylation reactions. [*methyl*-^{3}H]NpcMet was purified as described above from L cells incubated in the presence of [*methyl*-

^{3}H]methionine and 3 μM neplanocin A for 12 hr. The samples were calculated to have relatively low specific activities between 10–21 μCi/μmol. For comparison, commercially available [*methyl*-^{3}H]AdoMet was diluted to an equivalent specific activity and assayed in parallel with the [^{3}H]NpcMet. Table 1 displays the K_m and V_{max} determined for each system. While the K_m values for the two NpcMet reactions are approximately 30-fold higher than those of AdoMet, there is less than 40% difference observed in the corresponding V_{max} values. These findings, along with the inhibition studies using unlabeled NpcMet, indicate that NpcMet is a weak competitive inhibitor of lipid and protein methylation in L cells.

Effects of NpcMet on cellular polyamine levels and AdoMet decarboxylase activity. Another aspect of NpcMet metabolism which we sought to evaluate was its effect on AdoMet decarboxylase and the biosynthesis of polyamines. Glazer and Knode (7) previously reported that the formation of NpcMet had no effect on polyamine levels in HT-29 cells and that coaddition of methylglyoxal bis(guanylhydrazone), an inhibitor of the decarboxylase, did not potentiate the effects of neplanocin A. Although the slow turnover of NpcMet in L cells suggested that the analog was probably not an efficient substrate for this enzyme, it provided no indication as to whether NpcMet might be an effective inhibitor of the decarboxylase. To investigate this possibility, polyamine levels were measured in L cells which had been treated with 1 μM neplanocin A for 12–36 hr to induce the maximal intracellular accumulation of NpcMet. As shown in Table 2, neplanocin treatment produced a 3.8-fold decrease in putrescine and a 1.7-fold decrease in spermidine by 24 hr while spermine levels were relatively unaffected. In contrast to the elevation of putrescine and depletion of spermidine and spermine that would be expected if AdoMet decarboxylase was directly inhibited by NpcMet, the changes described here appear to be indirectly related to a prior effect of neplanocin treatment on the polyamine biosynthetic pathway. These changes, and in particular the decrease in spermidine, coincide with the onset of L cell growth inhibition observed in the present studies and those previously reported (3).

The lack of an inhibitory effect of NpcMet on the L cell decarboxylase is also supported by two other observations. First, no difference in the intracellular level of 5′-methylthioadenosine was detected by reverse-phase HPLC analysis of extracts from neplanocin-treated and untreated L cells (data not shown). In addition, *in vitro* studies using enzyme purified from either rat prostate or *E. coli* (Table 3) demonstrated that concentrations of the analog as high as 100 μM produced only 7 and 12% inhibition of the mammalian and bacterial enzymes, respectively, whereas a potent inhibitor such as ethylglyoxal bis(guanylhydrazone) at 10 μM produces 98 and 47% inhibition, respectively, under identical conditions (17). The substrate activity of NpcMet was also examined using these two purified AdoMet decarboxylase enzymes. [*carboxy*-^{14}C]NpcMet (21.3 μCi/μmol) was prepared by incubating L cells with [*carboxyl*-^{14}C]methionine for 12 hr as described above for [*methyl*-^{3}H]NpcMet.

TABLE 3

Effect of NpcMet on AdoMet decarboxylase activity

AdoMet decarboxylase from either *E. coli* or rat prostate was assayed in the presence of the indicated amount of unlabeled NpcMet for 30 min according to the method of Pegg and Jacobs (17). Each value is the mean of 2–4 individual determinations. ND, not determined.

Concentration NpcMet	*E. coli*	Rat prostate
μM	% control	
2	98	100
10	92	ND
20	94	98
25	78	ND
50	81	90
75	91	92
100	88	93

During a 45-min assay, little, if any, release of [^{14}C]CO_2 from the labeled analog was detected with either enzyme source, and a parallel assay using [*carboxy*-^{14}C]AdoMet indicated that the neplanocin analog had less than 1/1000th the comparable activity of the authentic substrate.

DISCUSSION

The effects of neplanocin A on AdoMet-dependent metabolism in mouse L929 cells have been attributed to the inhibition of AdoHcy hydrolase and subsequent accumulation of AdoHcy (3). As previously demonstrated for adenosine dialdehyde-treated L cells (8) and neplanocin A-treated neuroblastoma N2a cells (4), the dramatic increase in the intracellular ratio of AdoHcy/AdoMet induced by treatment of L cells with neplanocin A can result in significant suppression of AdoMet-dependent transmethylation such as 50% inhibition of lipid methylation and 30% inhibition of protein carboxylmethylation. Identification of the AdoMet analog, NpcMet, as a major neplanocin metabolite in these cells, however, posed the additional possibility of combined effects on AdoMet-dependent methylations as well as concomitant effects on AdoMet decarboxylase and polyamine biosynthesis. The present studies were designed to evaluate the action of NpcMet on these processes and clarify its role in the response of L cells to neplanocin A.

The turnover studies of NpcMet and AdoMet in intact cells (Fig. 2) yielded several observations regarding the metabolism of the analog which were subsequently supported by *in vitro* studies using the purified compound. Noting the slightly higher initial intracellular level of NpcMet, a comparison of the "apparent" turnover rate of this analog to AdoMet ($t_{1/2}$ = 13 hr *versus* 1 hr, respectively) indicates that the analog is not readily metabolized in L cells. Although our $t_{1/2}$ value for AdoMet is considerably longer than the 4–18 min reported for other cell lines (presumably due to the residual activity of AdoMet synthetase in the presence of 20 mM cycloleucine (22, 23)), it is the relative turnover of the two compounds which we are primarily concerned with in these studies. The present results, therefore, reflect the low substrate activity of NpcMet with either AdoMet-dependent methyltransferases or AdoMet decarboxylase. Consistent with this interpretation was our

limited ability to detect release of either the corresponding radioactive and/or UV-absorbing products *in vitro* (*i.e.*, NpcHcy or decarboxylated NpcMet). Unlike AdoMet decarboxylase which displayed virtually no substrate activity with or inhibition by NpcMet, some activity was observed for lipid and protein carboxyl methylations. Together with the moderate inhibitory effect of the unlabeled compound on [*methyl*-^{3}H]AdoMet-dependent methylation (IC_{50} = 205 μM (Fig. 4)), the increased K_m and relatively constant V_{max} determined for [*methyl*-^{3}H]NpcMet (Table 1) indicate that the neplanocin analog functions as a weak competitive inhibitor of these reactions. Moreover, recognizing that the IC_{50} value represented a 20.5:1 ratio of NpcMet/AdoMet, the intracellular effects of the analog (where the ratio is less than 2:1) would appear to be negligible.

The low inhibitory activity of NpcMet on AdoMet-dependent methyltransferases and AdoMet decarboxylase is also apparent from the nearly identical turnover of AdoMet in neplanocin A-treated and untreated cells. If accumulation of the analog had a significant inhibitory effect on both AdoMet-dependent processes, turnover of AdoMet would be detectably delayed in the treated cells. Moreover, based on the fact that protein and lipid methylation are inhibited by the accumulation of AdoHcy in treated cells, the similar turnover curves for AdoMet imply that a majority of the cellular AdoMet pool is utilized for polyamine biosynthesis. In Friend erythroleukemia cells it has been reported that greater than 50% of the cellular AdoMet pool is utilized for this process (22). An alternative possibility which is also negated by the present findings is that the synthesis of NpcMet by AdoMet synthetase could result in a simultaneous reduction in the synthesis of AdoMet itself, eventually depleting cellular levels of the natural substrate. It is clear from Fig. 3, however, that the initial levels and subsequent turnover of AdoMet are in no way affected by the presence of NpcMet.

A final point of interest regarding the turnover studies is the biphasic shape of the two AdoMet curves. These findings seem to imply that L cells contain two distinct pools of AdoMet having significantly different metabolic accessibilities. Since approximately half of the total cellular material appears to be contained in each, considering the turnover of the pools independently would reduce the $t_{1/2}$ of the readily available pool to 25–30 min. Cellular compartmentalization of AdoMet has also been reported for *Saccharomyces cerevisiae* and isolated rat hepatocytes, with the labile pools existing in the cytosol and the more stable pools being identified as the vacuolar and mitochondrial fractions, respectively (23). Presently, the significance of this characteristic is unclear.

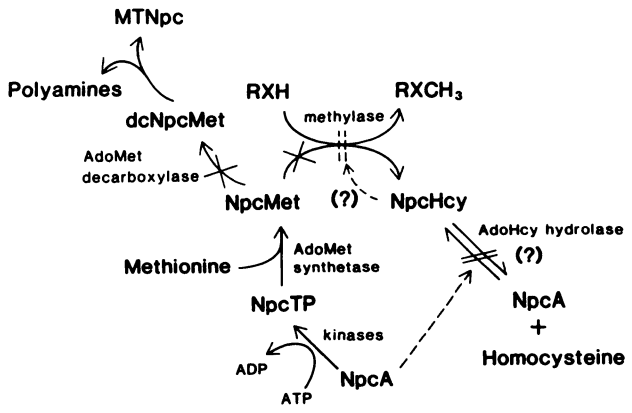


FIG. 5. *Metabolic scheme for neplanocin A in mouse L cells*
dcNpcMet, decarboxylated *S*-neplanocylmethionine; MTNpc, 5′-methylthioneplanocin; NpcA, neplanocin A; NpcTP, neplanocin A triphosphate; NpcMet, *S*-neplanocylmethionine; NpcHcy, *S*-neplanocylhomocysteine; RXH, unmethylated acceptor molecule; $RXCH_3$, methylated acceptor molecule.

Other investigations of structural analogs of AdoMet have demonstrated a broad range of activities with small molecule methyltransferases. Zimmerman and his colleagues (24) have reported the metabolic conversion of six adenosine analogs (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 substrate activities of these derivatives with catechol-*O*-methyltransferase were found to range from 46–91% utilization. Similarly, previous work from this laboratory has demonstrated that a number of synthetically prepared AdoMet analogs are also utilized with varying degrees of efficiency by several small molecule methyltransferases (25). Of particular interest to our present work is the carbocyclic derivative, *S*-aristeromycinylmethionine, which exhibited relatively good substrate activity with all the methyltransferases tested. Although this AdoMet analog differs from NpcMet only by the unsaturation at the 4–5 position of the latter's cyclopentenyl moiety, this change results in the formation of a planar bond to carbon 6 and significantly alters the orientation of the amino acid side chain. It is not unlikely, therefore, that an alteration in this crucial part of the molecule would result in significant changes in its methyl-donating activity.

A general scheme for the metabolism of neplanocin A in mouse L929 cells is presented in Fig. 5. In light of our present results, it would appear that the major effects of this nucleoside analog are, in fact, mediated by its inhibition of AdoHcy hydrolase and subsequent accumulation of AdoHcy. The metabolic conversion to NpcMet in these cells seems to have little, if any, consequence on AdoMet-dependent processes. In contrast, Glazer and Knode (7) have attributed the cytotoxicity of neplanocin A on HT-29 carcinoma cells to the inhibitory effects of the AdoMet analog on RNA methylation. Although we have not yet investigated the direct effects of NpcMet on L cell RNA methylation, we have not observed any cytotoxic effects of neplanocin A on these cells in the concentration range ($\leq$3 μM) which elicits greater than 95% inhibition of AdoHcy hydrolase and substantial intracellular accumulation of both AdoHcy and NpcMet. These findings, together with the minimal effects observed on lipid and protein methylation, suggest that either there is not a dramatic effect on L cell RNA methylation or that inhibition of RNA methylation does not lead to cytotoxicity in L cells.

As indicated in Fig. 5, the lack of methyltransferase activity with NpcMet in L cells has not allowed us to

evaluate the metabolic effects of the demethylated product, NpcHcy. However, considering that AdoHcy hydrolase has become a prime target for the design of pharmacological inhibitors due to the potent inhibitory action of endogenous AdoHcy and that NpcHcy might also directly inhibit AdoMet-dependent methylations as recently shown for the carbocyclic analog *S*-aristeromycinylhomocysteine (26), this AdoHcy analog has the potential to be an important pharmacological agent for further evaluating the physiological role of these methylation reactions. Future studies elucidating the biochemical action of NpcHcy will provide a more complete scheme for the overall metabolic effects of neplanocin A.

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REFERENCES

1. Hayashi, M., S. Yaginuma, H. Yoshioka, and K. Nakatsu. Studies on neplanocin A, new antitumor antibiotic II. Structure determination. *J. Antibiot.* (*Tokyo*) **34**:675–680 (1981).
2. Yaginuma, S., N. Muto, M. Tsujino, Y. Sudate, M. Hayashi, and M. Otani. Studies on neplanocin A, new antitumor antibiotic. I. Producing organism, isolation and characterization. *J. Antibiot.* (*Tokyo*) **34**:359–366 (1981).
3. Borchardt, R. T., B. T. Keller, and U. Patel-Thombre. Neplanocin A: a potent inhibitor of *S*-adenosylhomocysteine hydrolase and of vaccinia virus multiplication in mouse L929 cells. *J. Biol. Chem.* **259**:4353–4358 (1984).
4. Ramakrishnan, V., and R. T. Borchardt. Adenosine dialdehyde and neplanocin A: potent inhibitors of *S*-adenosylhomocysteine hydrolase and *S*-adenosylmethionine-dependent transmethylations in mouse neuroblastoma N2a cells. *Fed. Proc.* **43**:1556, abstr. 814 (1984).
5. Keller, B. T., and R. T. Borchardt. Metabolic conversion of neplanocin A to *S*-neplanocylmethionine by mouse L929 cells. *Biochem. Biophys. Res. Commun.* **120**:131–137 (1984).
6. Saunders, P. P., M.-T. Tan, and R. K. Robins. Metabolism and action of neplanocin A in Chinese hamster ovary cells. *Biochem. Pharmacol.* **34**:2749–2754, (1985).
7. Glazer, R. I., and M. C. Knode. Neplanocin A: a cyclopentenyl analog of adenosine with specificity for inhibiting RNA. *J. Biol. Chem.* **259**:12964–12969 (1984).
8. Bartel, R. L., and R. T. Borchardt. Effects of adenosine dialdehyde on *S*-adenosylhomocysteine hydrolase and *S*-adenosylmethionine-dependent transmethylations in mouse L929 cells. *Mol. Pharmacol.* **25**:418–424 (1984).
9. Chiang, P. K., H. H. Richards, and G. L. Cantoni. *S*-Adenosyl-L-homocysteine hydrolase: analogues of *S*-adenosyl-L-homocysteine as potential inhibitors. *Mol. Pharmacol.* **13**:939–947 (1977).
10. Montgomery, J. A., S. J. Clayton, H. J. Thomas, W. M. Shannon, G. Arnett, A. J. Bodner, I.-K. Kion, G. L. Cantoni, and P. K. Chiang. Carbocyclic analogue of 3-deazaadenosine: a novel antiviral agent using *S*-adenosylhomocysteine hydrolase as a pharmacological target. *J. Med. Chem.* **25**:626–629 (1982).
11. Landes, G. M. The pleiotypic effects of dexamethasone on mouse L cells in culture. Ph.D. dissertation, University of Kansas (1978).
12. Glazer, R. I., and A. L. Peale. Measurement of *S*-adenosyl-L-methionine levels by SP-sephadex chromatography. *Anal. Biochem.* **91**:516–520 (1978).
13. Hirata, F., O. H. Viveros, E. J. Diliberto, Jr., and J. Axelrod. Identification and properties of two methyltransferases in the conversion of phosphatidylethanolamine to phosphatidylcholine. *Proc. Natl. Acad. Sci. USA* **75**:1718–1721 (1978).
14. Diliberto, E. J., Jr., O. H. Viveros, and J. Axelrod. Subcellular distribution of protein carboxymethylase and its exogenous substrates in the adrenal medulla: possible role in excitation-secretion coupling. *Proc. Natl. Acad. Sci. USA* **73**:4050–4054 (1976).
15. Kim, S., S. Nochumson, W. Chin, and W. K. Paik. A rapid method for the purification of *S*-adenosylmethionine: protein-carboxyl O-methyltransferase by affinity chromatography. *Anal. Biochem.* **84**:415–422 (1978).
16. Aswad, D. W., and E. A. Deight. Purification and characterization of two distinct isozymes of protein carboxymethylase from bovine brain. *J. Neurochem.* **40**:1718–1726 (1983).
17. Pegg, A. E., and G. Jacobs. Comparison of inhibitors of *S*-adenosylmethionine decarboxylase from different species. *Biochem. J.* **213**:495–502 (1983).
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin reagent. *J. Biol. Chem.* **193**:265–275 (1951).
19. Pegg, A. E. The role of polyamine depletion and accumulation of decarboxylated *S*-adenosylmethionine in the inhibition of growth of SV-3T3 cells treated with α-difluoromethylornithine. *Biochem. J.* **224**:29–38 (1984).
20. Lombardini, J. B., A. W. Coulter, and P. Talalay. Analogues of methionine as substrates and inhibitors of the methionine adenosyltransferase reaction: deductions concerning the conformation of methionine. *Mol. Pharmacol.* **6**:481–499 (1970).
21. Borchardt, R. T. Mechanism of alkaline hydrolysis of *S*-adenosyl-L-methionine and related sulfonium nucleosides. *J. Am. Chem. Soc.* **101**:458–463 (1979).
22. Clarke, R. S. *S*-Adenosyl-L-methionine metabolism in polyamine biosynthesis in Friend erythroleukemia cells. *Fed. Proc.* **40**:1644, abstr. 606 (1981).
23. Farooqui, J. Z., H. W. Lee, S. Kim, and W. K. Paik. Studies on compartmentation of *S*-adenosyl-L-methionine in *Saccharomyces cerevisiae* and isolated rat hepatocytes. *Biochim. Biophys. Acta* **757**:342–351 (1983).
24. Zimmerman, T. P., R. P. Deeprose, G. Wolberg, and G. S. Duncan. Metabolic formation of nucleoside-modified analogues of *S*-adenosylmethionine. *Biochem. Biophys. Res. Commun.* **91**:997–1004 (1979).
25. Borchardt, R. T., Y. S. Wu, J. A. Huber, and A. F. Wycpalek. Potential inhibitors of *S*-adenosylmethionine-dependent methyltransferases. 6. Structural modifications of *S*-adenosylmethionine. *J. Med. Chem.* **19**:1104–1110 (1976).
26. Houston, D. M., B. Matuszewska, and R. T. Borchardt. Potential inhibitors of *S*-adenosylmethionine-dependent methyltransferases. 10. Base- and amino acid-modified analogues of *S*-aristeromycinyl-L-homocysteine. *J. Med. Chem.* **28**:478–482 (1985).

Send reprint requests to: Dr. Ronald T. Borchardt, Department of Pharmaceutical Chemistry, Room 3006 Malott Hall, University of Kansas, Lawrence, KS 66045.