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Effects of 9-(*trans*-2′,*trans*-3′-Dihydroxycyclopent-4′-enyl)-Adenine and -3-Deazaadenine on the Metabolism of *S*-Adenosylhomocysteine in Mouse L929 Cells

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SUMMARY

Neplanocin A [(−)-9-[*trans*-2′,*trans*-3′-dihydroxy-4′-(hydroxymethyl)-cyclopent-4′-enyl]-adenine] and 9-[*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl]-adenine (**1**) and -3-deazaadenine (**2**) are potent inhibitors of *S*-adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1) in mouse L929 cells. When cells were treated for 15 min with varying concentrations of the drugs, the IC_{95} values (concentration needed to produce 95% inhibition of AdoHcy hydrolase) for neplanocin A, **1**, and **2** were determined to be 0.2 μM, 0.5 μM, and 0.5 μM, respectively. Incubation of L929 cells with 1.0 μM concentrations of neplanocin A, **1**, or **2** produced rapid inactivation of AdoHcy hydrolase (within 30 min the enzyme was 95% inhibited), which persisted for at least 72 hr. At lower concentrations (0.032 μM), substantial recovery of AdoHcy hydrolase activity was noted after 48 and 72 hr in cultures treated with neplanocin A but not in cultures treated with **1** or **2**. L929 cells treated with neplanocin A, **1** or **2** showed a rapid increase in intracellular levels of AdoHcy (as well as the ratio of AdoHcy/*S*-adenosylmethionine). Cells treated with neplanocin A also contained significant amounts of *S*-neplanocylmethionine, whereas cells treated with **1** or **2** showed no evidence of the formation of a similar metabolite. When neplanocin A and adenosine were incubated in cell lysates, rapid conversion to neplanocin D and inosine, respectively, were observed, illustrating the affinity of these nucleosides for cellular adenosine deaminase. In contrast, when **1** and **2** were incubated in cell lysates, no evidence for deamination was observed. These data illustrate that compounds **1** and **2** retain the inhibitory activity of neplanocin A toward cellular AdoHcy hydrolase, producing elevated cellular levels of AdoHcy. However, by removing the 4′-hydroxymethyl group from neplanocin A, analogs **1** and **2** are no longer substrates for adenosine deaminase and adenosine kinase.

Neplanocin A [(−)-9-[*trans*-2′,*trans*-3′-dihydroxy-4′-(hydroxymethyl)-cyclopent-4′-enyl]-adenine] (Fig. 1) is a cyclopentenyl analog of adenosine that has been isolated from the culture filtrate of the microorganism *Ampullariella regularis* (1–3). This novel antibiotic was initially observed to be cytotoxic against cultured L5178 Y lymphoma cells (2) and exhibited significant antitumor activity in mice bearing L1210 leukemia (2, 4). Neplanocin A has also been shown to have antiviral activity (5, 7) and antimalarial activity (8).

Based on the structural similarity to adenosine, our laboratory investigated the possibility that some of the pharmacological activity of neplanocin A involves an alteration in cellular transmethylation mediated through an effect on AdoHcy hydrolase (EC 3.3.1.1). This enzyme, by catalyzing the reversible hydrolysis of AdoHcy to adenosine and homocysteine, plays a pivotal role in regulating cellular levels of AdoHcy, a potent product inhibitor of AdoMet-dependent methylation reactions (9). We observed that neplanocin A produced both concentration-dependent and time-dependent inhibition of purified bovine liver AdoHcy hydrolase, having a K_i value of 8.39 nM as determined by an Ackermann-Potter analysis (5).

Subsequently, neplanocin A has been shown to inactivate AdoHcy hydrolase by a mechanism that involves reduction of the enzyme-bound NAD^+ to NADH (10, 11). Recently, we have also shown that neplanocin A is a potent inhibitor of AdoHcy hydrolase isolated from a prokaryotic source (*Alcaligenes faecalis*) having a mechanism of inactivation identical to that in the eukaryotic enzyme (12).

Inhibition of AdoHcy hydrolase by neplanocin A has also been demonstrated in a number of cultured cell lines including L929 fibroblasts (5, 6) and N2a neuroblastoma cells (13). Coinciding with the inhibition of cellular AdoHcy hydrolase activity is an intracellular accumulation of AdoHcy leading to a 10- to 12-fold increase in the cellular ratio of AdoHcy/AdoMet.

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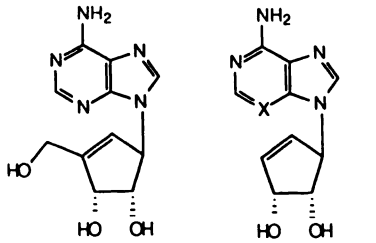
ABBREVIATIONS: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; HPLC, high performance liquid chromatography; NpcMet, *S*-neplanocylmethionine; CHO, Chinese hamster ovary; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine.



Neplanocin A

1 X=N

2 X=CH

Fig. 1. Structures of neplanocin A, **1**, and **2**.

Other co-workers have reported similar effects in human red blood cells (8) and HL-60 promyelocytic leukemia cells (14).

In addition to the inhibitory effects of neplanocin A on AdoHcy hydrolase, it also serves as a substrate for adenosine deaminase (4), being deaminated to the biologically inactive neplanocin D. Neplanocin A also serves as a substrate for adenosine kinase, being converted to the triphosphate derivative, which is subsequently a substrate for AdoMet synthetase, generating the corresponding AdoMet derivative, NpcMet. This metabolic conversion of neplanocin A has been shown to occur in L929 cells (15), N2a neuroblastoma cells (13), HT-29 human colon carcinoma cells (16), red blood cells (8), HL-60 promyelocytic leukemia cells (14), and CHO cells (17).

The conversion of neplanocin A to a nucleotide metabolite or to NpcMet appears to be responsible for the inhibitory effects of the drug on RNA synthesis (14, 16, 18, 19).

Thus, neplanocin A, like many other AdoHcy hydrolase inhibitors, is a "multifunctional" drug, having effects on a variety of molecular targets (e.g., AdoHcy hydrolase, adenosine kinase, and adenosine deaminase). Because the antiviral effects of neplanocin A appear to be mediated by inhibition of cellular AdoHcy hydrolase (5–7, 20) and the cytotoxic effects appear to be mediated by adenosine kinase (14, 16, 18, 19), analogs with a higher affinity for AdoHcy hydrolase and minimal substrate activity for adenosine kinase should have antiviral effects with minimal cytotoxicity. This approach has been successfully employed by Montgomery *et al.* (21) and Glazer *et al.* (22, 23) in their design of the carbocylic analog of 3-deazaadenosine and the 3-deaza derivative of neplanocin A, respectively.

In this study we describe the effects of some analogs of neplanocin A, which lack the 4′-hydroxymethyl group, on L929 cell AdoHcy hydrolase activity, cellular levels of AdoHcy and AdoMet, and cellular RNA and DNA synthesis. These analogs include 9-(*trans*-2′, *trans*-3′-dihydroxycyclopent-4′-enyl)-adenine (**1**) and 9-(*trans*-2′, *trans*-3′-dihydroxycyclopent-4′-enyl)-3-deazaadenine (**2**) (Fig. 1) (24), which have been shown to be potent inhibitors of bovine liver AdoHcy hydrolase (25).

Experimental Procedures

Materials. Standard chemicals and supplies were purchased from the following commercial suppliers: calf intestinal adenosine deaminase, SP-Sephadex C-25, (Sigma Chemical Co., St. Louis, MO); 3a70 scintillation cocktail (Research Products International, Mt. Prospect, IL); DE-81 disks (Whatman, Clinton, NJ); Waymouth's 752/1 (low calcium) medium (Hazleton Research Products, Denver, PA); EHNA (Burroughs Wellcome, Research Triangle Park, NC); [2,8-^{3}H]adenosine (30 Ci/mmol), [5,6-^{3}H]uridine (51 Ci/mmol), [*methyl*-^{3}H]thymidine (20 Ci/mmol) (New England Nuclear, Boston, Mass); and Zorbax C-8 reverse phase HPLC column (Dupont, Wilmington, DE).

[2,8-^{3}H]AdoHcy (7.1 mCi/mmol) was synthesized enzymatically from [2,8-^{3}H]adenosine and D,L-homocysteine using partially purified bovine liver AdoHcy hydrolase according to the method of Chabannes *et al.* (26). Protein determinations were done according to the method of Bradford *et al.* (27).

Neplanocin A was kindly donated by Toyo Jozo Co., Tagata-gun, Shizuoka, Japan. Compounds **1** and **2** were synthesized according to procedures described earlier by our laboratory (24).

Cell culture. Stock cultures of clone 929 mouse cells, strain L (Earle), were grown in suspension at 37° in Waymouth's 752/1 medium supplemented with 4% bovine calf serum and 70 μg/ml gentamycin as described previously (5, 15). Experimental cultures of L929 cells plated in tissue culture dishes were cultured in a chemically defined, modified version of Waymouth's MD 705/1 medium (KU-1) containing 1% calf serum.

AdoHcy hydrolase assay. Cultures of mouse L-929 cells (approximately 6×10^6 cells/100-mm diameter dish or approximately 2×10^6 cells/60-mm diameter dish), which were treated with various concentrations (Fig. 2) or for various times (Fig. 3) with AdoHcy hydrolase inhibitors, were harvested with trypsin treatment and pelleted in Eppendorf tubes (1.5 ml). The cells were lysed in 400 μl of cold hypotonic buffer (10 mM Na_2HPO_4, 10 mM NaCl, 1.5 mM magnesium acetate, pH 7.6) by rapid freezing and thawing. The cell debris was removed by centrifugation in an Eppendorf microcentrifuge (12,400 rpm, 1 min) and the AdoHcy hydrolase activity in the supernatant was determined using a modification of the procedure of Chiang *et al.* (28). In a total volume of 500 μl, the incubation mixture contained 150 mM potassium phosphate (pH 7.6), 1.0 mM EDTA, 40 μM [2,8-^{3}H]AdoHcy, and 4 units of adenosine deaminase. The reaction was started by addition of 320 μl of cell supernatant (approximately 1.3 mg of protein) and incubated for 60 min at 37°. After the addition of 100 μl of 5 N formic acid to stop the reaction, the reaction mixtures were loaded onto columns (1.2 × 4.0 cm) of SP-Sephadex C-25, which were then washed with 5 ml of 0.1 N formic acid. The eluent containing [2,8-^{3}H]inosine was eluted with an additional 15 ml of 0.1 N formic acid per column and collected. Radioactivity in 1 ml of the eluent mixed with 10 ml of 3a70 scintillation cocktail was determined by liquid scintillation counting.

Determination of intracellular levels of AdoHcy and AdoMet. Cultures of mouse L929 cells (approximately 2×10^6 cells/60-mm diameter dish) were incubated at 37° with KU-1 medium containing 1% calf serum with or without neplanocin A, **1** or **2**. Cells were removed from the culture dishes at various times by trypsinzation and lysed in 100 μl of 0.25 N perchloric acid by rapid freezing on dry ice. The samples were stored at −70° before analysis. In preparation for HPLC analysis, the samples were rapidly thawed and the cell debris was removed by centrifugation in an Eppendorf microcentrifuge (12,400 rpm, 1 min). The supernatant (100 μl) was injected into a Perkin-Elmer Series 3 HPLC equipped with a 25 cm x 4.6 mm Zorbax C-8 reverse phase column. AdoHcy and AdoMet were separated by a two-step gradient program at a flow rate of 1.0 ml/min as follows: solvent A, acetonitrile; solvent B, 50 mM sodium phosphate (pH 3.2), 10 mM heptane sulfonic acid; program, 5–20% A in 15 min then 20–25% A in 10 min; quantitated by absorption at 254 nm.

Metabolism of adenosine, neplanocin A, 1, and 2 in lysates of L-929 cells. L-929 cell lysates were prepared by freezing and thawing as described above in "AdoHcy hydrolase assay." Compounds (**1**, **2**, neplanocin A, or adenosine; 600 μM final concentration) were mixed with cell supernatants (1.7 mg/ml protein content) containing 150 mM potassium phosphate (pH 7.6) and 1.0 mM EDTA to a final volume of 1.5 ml. The reaction mixtures were incubated at 37° and at appropriate intervals aliquots of the reaction mixture were removed and the reaction was stopped by the addition of formic acid (0.25 N final concentration). Levels of adenosine, **1**, **2**, neplanocin A, or metabolites were determined by HPLC analysis as described above in "Determination of intracellular levels of AdoHcy and AdoMet."

Determination of DNA and RNA synthesis in L929 cells. Confluent L929 cell monolayers were cultured at 37° in 24-well dishes (13-mm diameter) in KU-1 medium containing 1% calf serum with (10 μM) or without neplanocin A, **1**, or **2**. After 12 hr of incubation, the cultures were pulse-labeled for 2 hr with [5,6-^{3}H]uridine (1.0 μCi/ml) or [*methyl*-^{3}H]thymidine (0.5 μCi/ml) in medium with or without the appropriate compounds. The culture medium was then removed by aspiration and the monolayers were lysed with 50 μl of lysing solution (1% sodium dodecyl sulfate, 5 mM EDTA, 20 mM Tris, pH 9.0, and 10 mM vanadyl ribonucleoside complex (29)). The lysate was mixed and suspended by repeated pipetting with a Gilson Pipetman, and transferred onto a DE-81 paper disk. Each well was rinsed with an additional 20 μl of lysing solution, which was transferred onto the same DE-81 disk. After washing five times with 5% Na_2HPO_4, 5 ml/disk, and two times with distilled water, radioactivity remaining on the disks was measured in 3a70 scintillation fluid by liquid scintillation counting.

Results

Effects of neplanocin A, 1, and 2 on L929 cell AdoHcy hydrolase activity. When murine L929 cells were treated with neplanocin A, **1**, or **2** for 15 min, concentration-dependent inhibition of cellular AdoHcy hydrolase activity was observed (Fig. 2). Neplanocin A appears to be slightly more active as an inhibitor of L-929 cell AdoHcy hydrolase than analogs **1** and **2** under the conditions of the experiments described in Fig. 2. For example, to achieve 95% inhibition of AdoHcy hydrolase activity (after 15 min of incubation) required approximately 0.5 μM concentrations of **1** and **2**, compared with approximately 0.2 μM concentration of neplanocin A. Neplanocin A, **1**, or **2** also showed time-dependent inactivation of cellular AdoHcy hydrolase (Fig. 3, A and B). When cells were treated with 1 μM concentrations of neplanocin A, **1**, or **2**, AdoHcy hydrolase was inhibited at least 95% for up to 72 hr (Fig. 3A). Cells treated with a lower concentration (0.032 μM) of neplanocin A, **1**, or **2** showed at least 80% inhibition of AdoHcy hydrolase up to 24 hr (Fig. 3B). In cells treated with **1** or **2** this level of AdoHcy hydrolase inhibition was maintained for up to 72 hr. In contrast, cells treated with 0.032 μM of neplanocin A showed partial recovery (50% inhibition) of AdoHcy hydrolase activity after 72 hr.

Metabolism of adenosine, neplanocin A, 1, and 2 in lysates of L929 cells. In lysates of L929 cells, adenosine and neplanocin A are deamidated by adenosine deaminase to inosine and neplanocin D, respectively (Fig. 4). When EHNA, an adenosine deaminase inhibitor, was added to the cell lysate, the metabolism of adenosine was significantly suppressed (data not shown). In sharp contrast to the data observed with adenosine and neplanocin A, compounds **1** and **2** were stable in L929 cell lysates up to 9 hr (Fig. 4), confirming our hypothesis that these analogs, which are devoid of the 4′-hydroxymethyl group, would be poor substrates for adenosine deaminase. The poor substrate activity of **1** and **2** for pure adenosine deaminase has been confirmed in separate studies (25).

Effect of neplanocin A, 1, and 2 on intracellular levels of AdoHcy and AdoMet. To determine the effects of neplanocin A, **1**, and **2** on intracellular levels of AdoHcy and AdoMet, we selected drug concentrations (1 μM, 10 μM, and 10 μM, respectively) that produced maximal inhibition of AdoHcy hydrolase (see data presented in Figs. 2 and 3) and minimal cytotoxic effects. Our laboratory has reported earlier (30) that these concentrations of neplanocin A, **1**, and **2** produce some cytostatic effects on murine L-929 cells but not cytocidial effects. During a 72-hr incubation, neplanocin A (1 μM), **1** (10 μM), and **2** (10 μM) produced approximately equal reductions in the rate of cell growth as determined by trypan blue staining of viable cells (30). No obvious morphological changes were observed in the cultures treated with these concentrations of the AdoHcy hydrolase inhibitors. In L929 cells treated with **1** (10 μM) or neplanocin A (1 μM) the cellular levels of AdoHcy increased rapidly during the first 12 hr (Fig. 5A). In the neplanocin A-treated cells, the elevated level of AdoHcy was maintained for 24 hr, after which time the level of AdoHcy steadily declined to near control values by 72 hr. In contrast, in cells treated with **1** (Fig. 5A) or **2** (data not shown), the level of AdoHcy steadily increased throughout the duration (72 hr) of the experiment.

In cells treated with neplanocin A or **1**, AdoMet levels paralleled control values, rising slightly over the 72-hr time course of the experiment (Fig. 5B). Similar results were observed with compound **2** (data not shown). This increase in AdoMet levels is most likely due to cell growth, because the protein content of all cultures increased similarly over the 72-hr time course of the experiment (Fig. 5C).

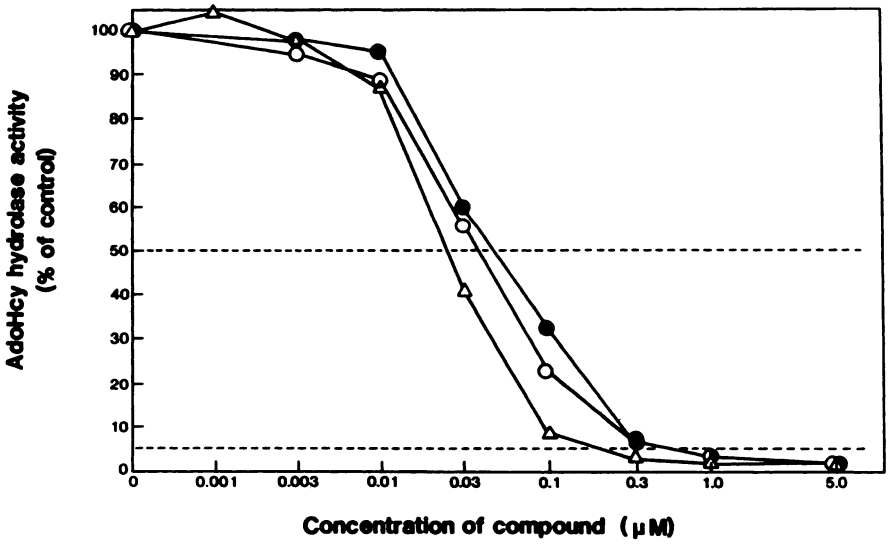


Fig. 2. Inhibition of mouse L929 cell AdoHcy hydrolase by neplanocin A, **1**, or **2**. Confluent cultures of mouse L929 cells (approximately 6 × 10^6 cells/100-mm diameter dish) were incubated for 15 min at 37° with the indicated concentrations of compound **1** (○), compound **2** (●), or neplanocin A (△) in KU-1 medium containing 1% calf serum. Cells were harvested and then lysed in cold hypotonic buffer as described in Materials and Methods. The cell debris was removed by centrifugation and AdoHcy hydrolase activity was determined in the supernatant (320 μl; 1.3 mg of protein) using [2,8-^{3}H]AdoHcy as the substrate as described in Materials and Methods. In control samples not treated with a AdoHcy hydrolase inhibitor, the AdoHcy hydrolase activity was approximately 327 pmol/mg/min.

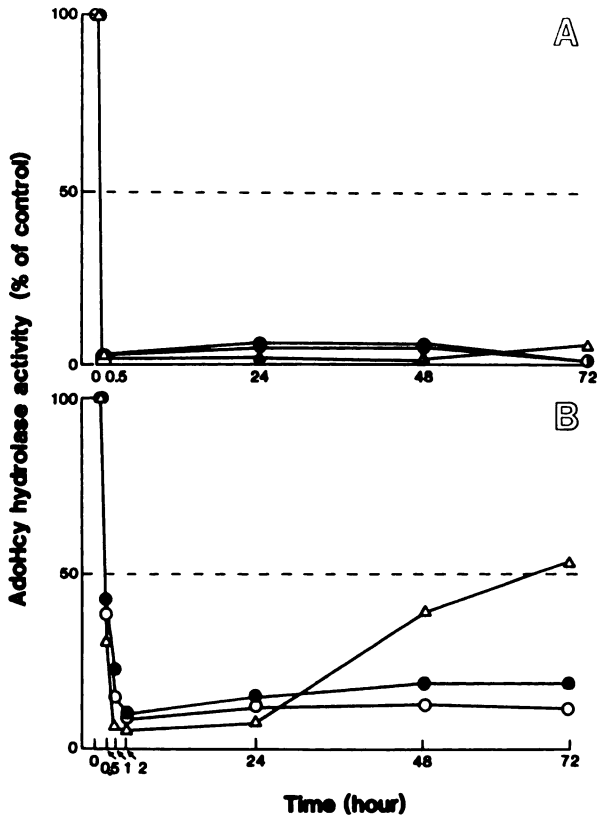


Fig. 3. Time-dependent inhibition of AdoHcy hydrolase in mouse L929 cells pretreated with neplanocin A, 1 or 2. Cultures of mouse L929 cells (approximately 2×10^6 cells/60-mm diameter dish) were incubated with 1.0 μM (A) or 0.032 μM (B) of compound 1 (○), 2 (●), or neplanocin A (△) in KU-1 medium containing 1% calf serum. Cells were harvested at the indicated times and lysed in cold hypotonic buffer as described in Materials and Methods. AdoHcy hydrolase activity in the cell lysate supernatant was measured by the method described in Materials and Methods. In control samples not treated with a AdoHcy hydrolase inhibitor, the AdoHcy hydrolase activity was approximately 238 pmol/mg/min.

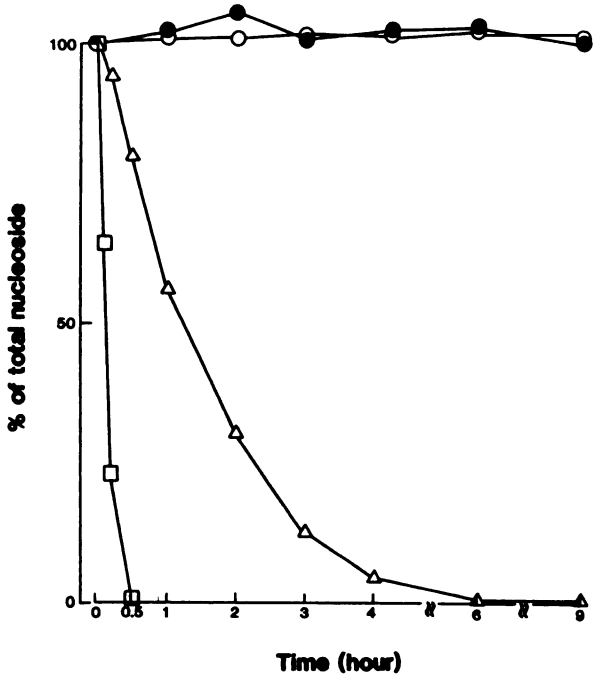


Fig. 4. Stability of 1, 2, or neplanocin A in mouse L929 cell lysate. Cell lysate was prepared with cold hypotonic buffer by the method described in Materials and Methods. The incubation mixture contained 150 mM Na_2HPO_4 (pH 7.3) 1 mM EDTA, 600 μM 1 (○), 2 (●), neplanocin A (△), or adenosine (□), and 1.7 mg of protein/ml of supernatant in the cell lysate in a total volume of 1.5 ml. The samples were incubated at 37° and aliquots (50 μl) were removed at the indicated times and acidified with 50 μl of 0.5 N formic acid. The supernatant was centrifuged and diluted 20 times with distilled water. Aliquots (100 μl) of the diluted supernatant were analyzed by HPLC as described in Materials and Methods. Retention times were 16.30 min, compound 1; 19.30 min, compound 2; 13.0 min, neplanocin A; and 14.15 min, adenosine.

When the changes in AdoHcy and AdoMet levels are expressed as the ratio of AdoHcy/AdoMet, the profiles shown in Fig. 6 are observed. Control cultures showed a consistent low ratio (approximately 0.05) of AdoHcy/AdoMet, whereas cultures treated with a 10 μM concentration of compound 1 showed an increase in the ratio of AdoHcy/AdoMet, reaching a maximum value of 1.1 at 24 hr and maintaining that ratio for up to 72 hr. Similar results were observed with compound 2 (data not shown). Cells treated with a 1 μM concentration of neplanocin A also showed an increase in the ratio of AdoHcy/AdoMet, reaching a maximum value of 1.9 at 12 hr but then steadily decreasing to near control values by 72 hr.

Previously, our laboratory has reported (15) that L929 cells treated with 1 μM of neplanocin A accumulate NpcMet to levels equal to or slightly greater than AdoMet (approximately 500 pmol/10^7 cells) within 12–24 hr. Fig. 7 shows HPLC chromatograms of cell lysates from L929 cells treated with 1 (10 μM) (Fig. 7A), 2 (10 μM) (Fig. 7B), or neplanocin A (1 μM) (Fig. 7C). The presence of NpcMet is evident in the neplanocin A-treated cells, whereas a similar metabolite is not detected in the cells treated with 1 or 2.

Effects of neplanocin A, 1, and 2 on L929 cell RNA and DNA synthesis. To assess the effects of these AdoHcy hydrolase inhibitors on other metabolic processes, [^{3}H]uridine and [^{3}H]thymidine incorporation were measured in L929 cells exposed to the inhibitors to evaluate RNA and DNA synthesis, respectively. As shown in Table 1, DNA and RNA synthesis were not affected in cells treated with 10 μM concentrations of compounds 1 or 2. In contrast, cells treated with 10 μM concentrations of neplanocin A showed a 66% inhibition of RNA synthesis but no effect on DNA synthesis.

Discussion

Current evidence suggests that neplanocin A has multiple molecular sites of action within a target cell. The molecular sites, which appear to mediate the pharmacological effects of the drug (e.g., antitumor, antiviral, and antimalarial), include AdoHcy hydrolase and adenosine kinase (Fig. 8). Neplanocin A also serves as a substrate for adenosine deaminase, generating neplanocin D, a biologically inactive metabolite (4). However, adenosine deaminase-catalyzed deamination of neplanocin A appears to be a minor pathway of metabolism (14, 16).

One of the molecular sites that appears to mediate some of the pharmacological effects of neplanocin A is AdoHcy hydrolase. This enzyme, by catalyzing the reversible hydrolysis of AdoHcy to adenosine and homocysteine, plays a pivotal role in regulating cellular levels of AdoHcy, a potent product inhibitor of AdoMet-dependent methylation reactions (9) (Fig. 8). Earlier studies in our laboratory showed that neplanocin A is a potent inhibitor of AdoHcy hydrolases isolated from bovine liver (5) and *A. faecalis* (12). The inactivation of these hydro-

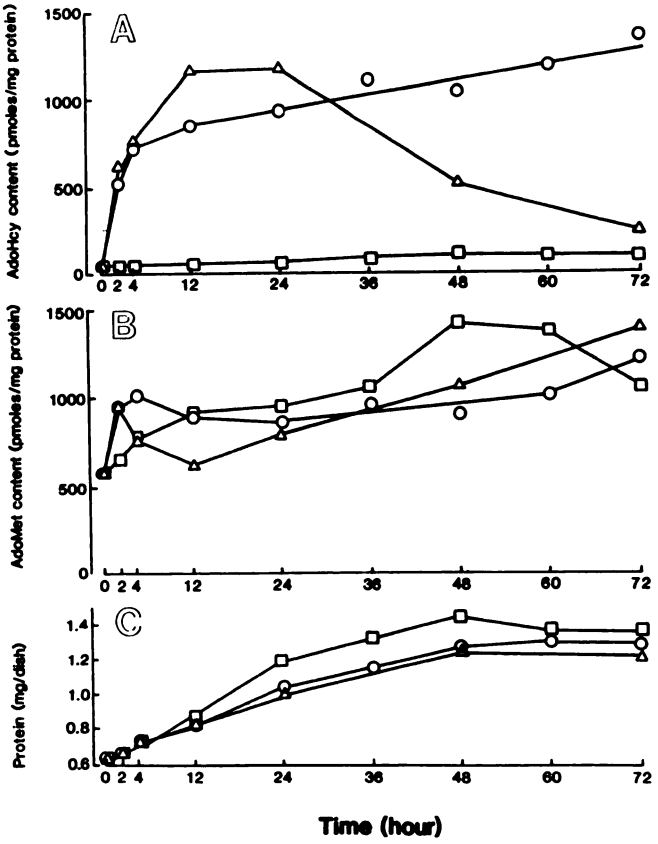


Fig. 5. Endogenous AdoHcy and AdoMet contents of L929 cells treated with compound 1 or neplanocin A. Cultures of mouse L929 cells (approximately 2 × 10^6 cells/60-mm diameter dish) were incubated at 37° with 10 μM 1 (○) or 1 μM neplanocin A (△) or without an AdoHcy hydrolase inhibitor (□) in KU-1 medium containing 1% calf serum. At various times, the cells were harvested and the protein AdoHcy and AdoMet concentrations were quantitated as described in Materials and Methods. A, AdoHcy content; B; AdoMet content; C, protein content.

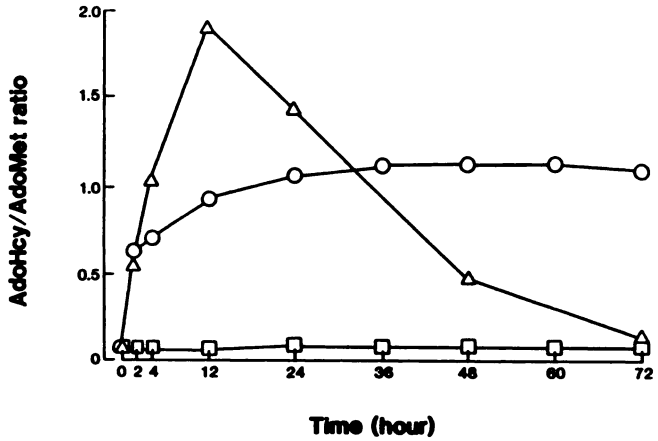


Fig. 6. AdoHcy/AdoMet ratios in L929 cells treated with compound 1 or neplanocin A. The AdoHcy/AdoMet ratios were calculated from the results shown in Fig. 5. △, Neplanocin A-treated cells; ○, compound 1-treated cells; □, controls (no drug treatment).

lases by neplanocin A occurs through a common mechanism involving reduction of enzyme-bound NAD^+ to NADH (10–12). Thus, neplanocin A is a K_{cat} type inhibitor of AdoHcy hydrolase. No evidence exists for the AdoHcy hydrolase-catalyzed conversion of neplanocin A to *S*-neplanocylhomocysteine, as has been demonstrated for some other nucleosides such as 3-deazaadenosine (9).

Neplanocin A also functions to inhibit AdoHcy hydrolase in cultured cell lines including L929 fibroblasts (5, 6), N2a neuroblastoma cells (13), red blood cells (8), and HL-60 promyelocytic leukemia cells (14). Inhibition of cellular AdoHcy hydrolase causes an intracellular accumulation of AdoHcy, leading to a significant increase in the cellular ratio of AdoHcy/AdoMet and subsequent inhibition of AdoMet-dependent methylations.

Recently, evidence has been presented that suggests that neplanocin A's inhibition of cellular AdoHcy hydrolase mediates its antiviral effects (5, 6, 30, 31). For example, De Clercq and Cools (31) have established a close correlation between the antiviral potency of several adenosine analogs, including neplanocin A, and their relative inhibitory effects on AdoHcy hydrolase. Our laboratory has recently shown that neplanocin A analogs (**1** and **2** in Fig. 1), which have inhibitory effects on AdoHcy hydrolase but are devoid of substrate activity for adenosine kinase and adenosine deaminase, still retain potent antiviral activity (30). Thus, it appears that an appropriate strategy for designing antiviral agents using neplanocin A as a prototype structure would involve optimizing the interaction of synthetic analogs with AdoHcy hydrolase and minimizing their interaction with adenosine deaminase and adenosine kinase.

The other major molecular site that appears to mediate some of the pharmacological effects of neplanocin A is adenosine kinase. In 1984 our laboratory (15) showed that neplanocin A could be converted in murine L929 cells to NpcMet, presumably through reactions catalyzed by adenosine kinase (to form neplanocin A triphosphate) and AdoMet synthetase (Fig. 8). Subsequently, this bioconversion of neplanocin A has also been shown to occur in N2a neuroblastoma cells (13), HT-29 human colon carcinoma cells (16), red blood cells (8), HL-60 promyelocytic leukemia cells (14), CHO cells (17), and L1210 leukemia cells (18). Glazer and Knode (16) were the first to propose that the cytocidal effects of neplanocin A on HT-29 human colon carcinoma cells were mediated by the formation of a nucleotide metabolite and/or NpcMet and their subsequent effects on RNA synthesis and RNA methylation. Additional evidence in support of this hypothesis has subsequently been obtained in human promyelocytic leukemia cells (14) and in L1210 leukemic cells (18). However, a report by Saunders *et al.* (17) suggests that the high cytotoxicity of neplanocin A to CHO cells was not related to the formation of the nucleotide metabolites of neplanocin A, because an adenosine kinase-deficient mutant (which did not produce the nucleotides) was only slightly more resistant than the wild type cell line (17).

Our laboratory (32) has recently purified NpcMet and characterized some of its metabolic effects. The results of these studies suggest that NpcMet is inactive as a substrate (or inhibitor) of AdoMet decarboxylase and it is a poor substrate (or inhibitor) for AdoMet-dependent methylations (e.g., protein carboxymethylation and phospholipid methylation) in lysates of L929 cells. In addition, we have been unable to detect the formation of *S*-neplanocylhomocysteine and 5′-methylthioneplanocin when neplanocin A is incubated with L929 cells in culture, further confirming the low metabolic activity of NpcMet toward the decarboxylation and methylation pathways (Fig. 8). Thus in cultured L929 cells, the metabolic conversion to NpcMet seems to have little, if any, consequence on cellular AdoMet-dependent processes.

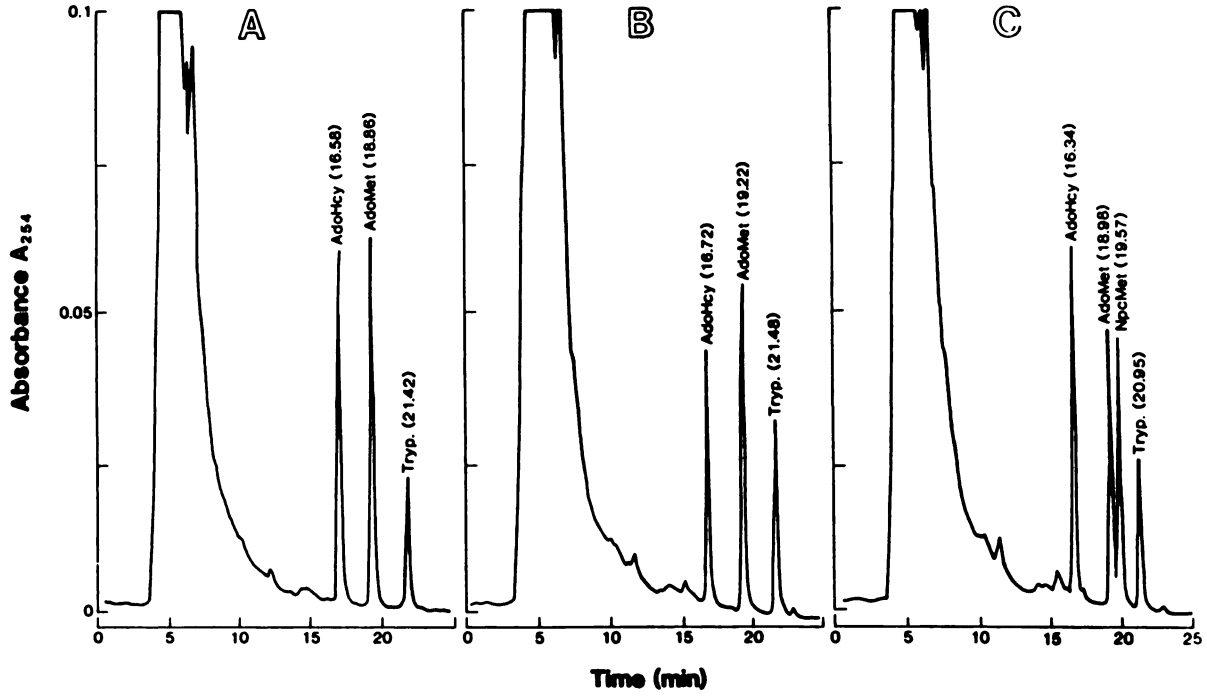


Fig. 7. Reverse phase HPLC profiles of L929 cell extract exposed to compounds **1**, **2**, or neplanocin A. Cultures of mouse L929 cells (approximately 2×10^6 cells/60-mm diameter dish) were incubated with 10 μM of analog **1** (A), 10 μM of analog **2** (B), and 1.0 μM of neplanocin A (C) in KU-1 medium containing 1% calf serum for 12 hr. The cells were harvested and the lysate was chromatographed as described in Materials and Methods.

TABLE 1

Effects of 1, 2, and neplanocin A on mouse L929 cell DNA and RNA synthesis

Confluent L929 cell monolayers were cultured at 37° in multiwell dishes (13-mm diameter) in KU-1 medium containing 1% calf serum with (10 μM) or without AdoHcy hydrolase inhibitors. After 12 hr of incubation at 37°, the cultures were pulse-labeled for 2 hr with [5,6-^{3}H]uridine or [*methyl*-^{3}H]thymidine in medium with or without the hydrolase inhibitor. The monolayer was lysed and applied to DE-81 paper disks. After washing five times with 5% Na_2HPO_4, 5 ml/disk, and two times with distilled water, radioactivity remaining on the disks was measured by liquid scintillation counting. Assays were done in duplicate.

Compound	Radioactivity remaining on DE-81 paper			
	[^{3}H]Uridine		[^{3}H]Thymidine	
	cpm/paper	*% of control*	*cpm/paper*	*% of control*
1	43,736	87	72,390	93
2	56,993	96	87,420	111
Neplanocin A	23,522	34	85,012	107
Control	50,094	100	77,624	100

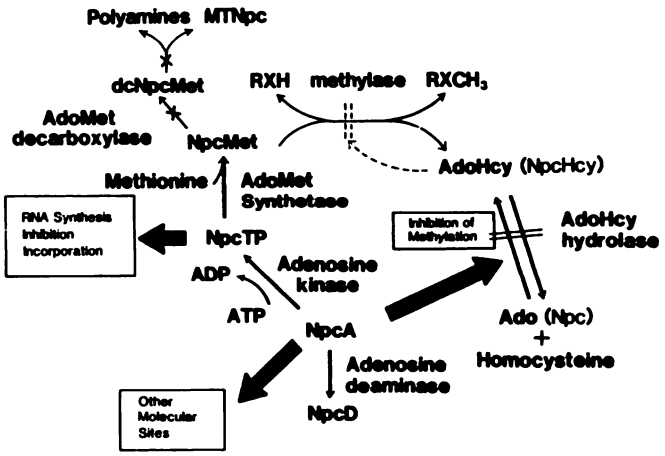


Fig. 8. Metabolic scheme for neplanocin A in mouse L-929 cells. *dcNpcMet*, decarboxylated *S*-neplanocylmethionine; *MTNpc*, 5′-methylthioneplanocin; *Npc A*, neplanocin A; *NpcTP*, neplanocin A triphosphate; *NpcMet*, *S*-neplanocylmethionine; *NpcHcy*, *S*-neplanocylhomocysteine; *RXH*, unmethylated acceptor molecule; *$RXCH_3$*, methylated acceptor molecule; *NpcD*, neplanocin D.

From the results reported in this paper and those reported by other workers (14, 16, 18, 19), it appears that neplanocin A preferentially suppresses RNA synthesis. Recently, we have observed that the synthetic analogs **1** and **2** have substantially lower toxicity toward L929 cells than neplanocin A itself (30) and that, unlike neplanocin A, these analogs do not inhibit RNA synthesis (Table 1). Because these analogs are not converted to nucleotide analogs, this observation supports the hypothesis by Glazer and Knode (16) that the cytotoxic effects of neplanocin A and its ability to inhibit RNA synthesis are, in part, due to its conversion to nucleotides and/or NpcMet. Further studies are ongoing in our laboratory to clarify this mechanism.

Therefore, because our laboratory has been primarily interested in the antiviral effects of neplanocin A and because this effect appears to be mediated through inhibition of cellular AdoHcy hydrolase, we have designed and synthesized analogs of this carbocylic nucleoside that retain the inhibitory effects towards AdoHcy hydrolase but that are devoid of substrate activity with adenosine kinase and adenosine deaminase. Analogs **1** and **2** have been shown in separate studies to be potent inhibitors of bovine liver AdoHcy hydrolase and to function by a K_{cat} mechanism involving reduction of enzyme-bound NAD^+ to NADH (25).

Using double-reciprocal plots of the initial rate of bovine liver AdoHcy hydrolase inactivation versus inhibitor concen-

tration, apparent K_i values (and K_{cat} values) were determined to be 3.8 nM (2.0 min^{-1}), 41 nM (0.5 min^{-1}), and 35 nM (0.5 min^{-1}) for neplanocin A, **1**, and **2**, respectively (25). In addition, we have also shown in a separate study that neplanocin A, **1**, and **2** are potent inhibitors of vaccinia virus replication in L929 cells (IC_{50} values: neplanocin A, 0.08 μM; analog **1**, 0.29 μM; and analog **2**, 0.95 μM) and that analogs **1** and **2** have substantially lower toxicity to the host cells than neplanocin A (ID_{50} values, neplanocin A, 0.5 μM; analog **1**, 17 μM; and analog **2**, 56 μM) (30). The antivaccinia effectiveness (ID_{50}/IC_{50}) for neplanocin A, **1**, and **2** was determined to be 6, 61, and 59, respectively (30). Like neplanocin A (7), analogs **1** and **2** have been shown in preliminary studies to have broad spectrum antiviral activity (i.e., vaccinia, vesicular stomatitis, and parainfluenza).[1] Thus, by removing the 4′-hydroxymethyl group of neplanocin A, we have retained the compound's inhibitory effects toward AdoHcy hydrolase as well as its antiviral effects but, in the process, have developed analogs devoid of substrate activity toward adenosine kinase and adenosine deaminase, exhibiting minimal cellular toxicity.

In an earlier study using purified bovine liver AdoHcy hydrolase (25) and in the current study (using L929 cellular AdoHcy hydrolase) (Fig. 2), we have shown that these analogs (**1** and **2**) of neplanocin A are potent inhibitors of these enzymes, but they are less active than the parent compound. However, analogs **1** and **2** have several advantages over neplanocin A, particularly their longer duration of inhibition of cellular AdoHcy hydrolase (Fig. 3) and their reduced cytotoxicity (30). This longer duration of action is probably due to the metabolic stability of the analogs compared with neplanocin A, which can be rapidly phosphorylated by adenosine kinase. As a result of this sustained inhibition of the hydrolase, analogs **1** and **2** can be used to maintain elevated cellular levels of AdoHcy for up to 72 hr (Figs. 5 and 6) without producing cytotoxic effects. Another advantage of analogs **1** and **2** is that they are not converted to nucleotides and, subsequently, to NpcMet, which suggests that they are "monofunctional" inhibitors (AdoHcy hydrolase) rather than compounds with multifunctional activity (AdoHcy hydrolase inhibitors and adenosine kinase substrates). Comparing analogs **1** and **2**, they appear to have similar potencies as inhibitors of AdoHcy hydrolase (Ref. 27 and Fig. 2) and subsequently produce similar effects on cellular levels of AdoHcy. However, the adenine analog **1** is a more potent antiviral agent than the 3-deazaadenine analog **2**, but it also shows higher cellular toxicity (30).

These analogs of neplanocin A have the potential of being useful in elucidating the importance of various metabolic sites in mediating the pharmacological effects of neplanocin A. This can be illustrated by the observation that these analogs have potent antiviral effects but are substantially less cytotoxic, indicating that the antiviral effects are in large part mediated by inhibition of AdoHcy hydrolase, whereas the cytotoxic effects are primarily mediated by the phosphorylations catalyzed by adenosine kinase (27).

Many inhibitors of AdoHcy hydrolase identified to date have had multiple sites of action within target cells. Therefore, the availability of "pure" inhibitors of this enzyme will help to clarify the role of this enzyme in regulating cellular methylations and enhance its attractiveness as a target for drug design.

[1] Masahide Hasobe, David R. Borcherding, Erik DeClercq, and Ronald T. Borchardt, unpublished data.

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Erratum

Volume 31, No. 6 (1987), in the article "Ligand Dissociation Constants from Competition Binding Assays: Errors Associated with Ligand Depletion," by Avram Goldstein and Ronald W. Barrett, pp. 603–609: on p. 605, equation 3 is printed incorrectly.

The correct equation 3 is:

$$K_x = \{1/[(2L/L_0) + (L/K_L) - 1]\}X_{50}$$