Neplanocin A
A POTENT INHIBITOR OF S-ADENOSYLMETHIONINE HYDROLASE AND OF VACCINIA VIRUS MULTIPLICATION IN MOUSE L929 CELLS*

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Neplanocin A, a novel cyclopentenyl analog of adenosine, is a naturally occurring antibiotic which exhibits significant antitumor activity against L1210 leukemia in mice (Yaginuma, S., Muto, N., Tsujino, M., Sudate, Y., Hayashi, M., and Otani, M. (1981) J. Antibiot. 34, 359-366). In the present study we demonstrate that neplanocin A is also a potent inhibitor of S-adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1) having a $K_i$ of 8.39 nM for the purified bovine liver enzyme. Analysis of the apparent irreversible inactivation of AdoHcy hydrolase by neplanocin A indicates that the drug is a tight binding inhibitor, exhibiting a stoichiometry of one molecule of inhibitor to one molecule (tetramer) of enzyme.

In addition, we show that neplanocin A is a potent inhibitor of vaccinia virus (WR) multiplication in monolayer cultures of mouse L-cells. Concentrations of the drug as low as 0.5 and 1.0 μM in the culture medium produce 84 and 95% inhibition of plaque formation, respectively, while exhibiting little toxicity to the host cells. The inhibition of virus multiplication by neplanocin A coincides with a rapid inhibition of AdoHcy hydrolase activity in the infected cells and a subsequent 10-fold increase in the intracellular AdoHcy/S-adenosylmethionine ratio. These findings suggest that the antiviral actions of this compound may be related to an inhibition of S-adenosylmethionine-dependent macromolecular methylation reactions which are essential to the production of new virus particles (e.g., viral messenger RNA).

In recent years, S-adenosyl-l-homocysteine hydrolase (EC 3.3.1.1) has emerged as a specific target for the design of potential chemotherapeutic agents (1-3). Such an approach has been prompted by the important role that this enzyme is known to play in regulating biological methylation reactions (i.e., modulating the intracellular AdoHcy/AdoMet ratio). AdoHcy hydrolase catalyzes the reversible hydrolysis of AdoHcy to adenosine and homocysteine. Although the equilibrium of the reaction favors synthesis, AdoHcy is efficiently hydrolyzed under physiological conditions because Ado and Hcy are simultaneously removed by several metabolic routes (4). Inhibition of AdoHcy hydrolase in intact cellular systems results in the accumulation of AdoHcy, a product inhibitor of AdoMet-dependent methylation reactions (5, 6). As a consequence of inhibiting AdoHcy metabolism cellular methylation reactions are perturbed, many of which are required for maintenance of the normal metabolic integrity of the cell.

An example of an essential methylation reaction is found in the maturation scheme of certain eukaryotic and viral messenger RNA molecules. It is known that, in many instances, these mRNA molecules must be both capped and methylated on their 5' terminus (e.g. mGpppmA'pppA'...). To promote active translation of the corresponding proteins (7). Methylation of the 5' cap structure has been demonstrated to enhance the efficiency of initiation of translation at the 5' end of the mRNA (8). Moreover, it has been shown that the vaccinia virus-specific enzymes which catalyze these reactions for viral mRNAs (i.e. guanine 7-methyltransferase; 2'-O-nucleoside methyltransferase) are susceptible to inhibition by AdoHcy (9, 10). It is not surprising, therefore, that potent inhibitors of AdoHcy hydrolase such as 3-dezaadenosine (11), 3-deazaaristeromycin (12, 13), and adenosine dialdehyde elicit significant antiviral activity against viruses requiring a methylated 5' cap structure on their mRNAs.

Recently, the isolation and characterization of neplanocin A ((-)-9-[(trans-2,trans-3-dihydroxy-4-(hydroxymethyl)cyclopent-4-enyl]adenine) has been reported (14, 15). This compound, a novel carbocyclic analog of adenosine in which the ribose moiety is replaced by a cyclopentene ring (Fig. 1), has been shown to possess antitumor properties with relatively low cytotoxicity. Considering its structural similarity to adenosine, it is conceivable that the pharmacological activity of neplanocin A may be mediated through interaction with an enzyme involved in adenosine metabolism, such as AdoHcy hydrolase. In this paper we report that neplanocin A is a potent inhibitor of AdoHcy hydrolase both in vitro and in vivo, and that it elicits potent antiviral activity against vaccinia virus (WR) in mouse L929 cells.

MATERIALS AND METHODS
Neplanocin A was kindly donated by the Toyo Jozo Co., Ltd., Japan. Purification of Bovine Liver AdoHcy Hydrolase—AdoHcy hydrolase was purified according to the procedure of Palmer and Abeles (18) with a final specific activity of 0.109 IU (1 IU is defined as 1 μmol of product formed per min/mg of protein). The protein concentration (0.46 mg/ml) was determined from the absorbance at 280 nm, $E_{1cm} = 14.2$, and by the procedure of Lowry et al. (17). The enzyme preparation was 50% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18).

In Vitro Assay of Purified AdoHcy Hydrolase—The assay of AdoHcy hydrolase activity in the hydrolytic direction was determined by a modified procedure of Richards et al. (19). In a total volume of 500 μl, the incubation mixture contained 150 mM potassium phosphate buffer (pH 7.6), 1.0 mM EDTA, 100 μM [2,8-3H]AdoHcy, and 4

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1 The abbreviations used are: AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine.

2 B. T. Keller, and R. T. Borchardt, unpublished observations.
units of intestinal adenosine deaminase. The reaction was started by the addition of AdoHcy hydrolase and incubated for 5 min at 37°C. The reaction was terminated by the addition of 100 μl of 5 N formic acid. The reaction mixture and a 500-μl wash of 0.1 N formic acid were layered onto a SP-Sephadex C-25 column (1.2 × 2.4 cm), previously equilibrated in 0.1 N formic acid. The column was eluted with 8.0 ml of 0.1 N formic acid and the eluant containing [2,8-3H]inosine (the product of the hydrolysis of [2,8-3H]AdoHcy and subsequent deamination of [2,8-3H]adenosine) was collected. A 1-ml aliquot was added to 10 ml of scintillation mixture (Research Products International, Mount Prospect, IL) and the amount of radioactivity determined by liquid scintillation spectrometry.

Cell Culture—Stock cultures of clone 929 mouse cells, strain L (Earle) were grown in suspension at 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 35-mm (plaque assay), 60-mm (AdoHcy/AdoMet determination), or 100-mm (AdoHcy hydrolase assay) polystyrene tissue culture dishes at the indicated density 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 (i.e. KU-1) (20).

Vaccinia Plaque Formation—Plaque assays were performed in 35-mm culture dishes containing 1.5 × 10⁶ cells/dish. The medium was removed by aspiration and fresh KU-1 medium containing vaccinia virus (WR) was added. The plates were incubated for 60 min, the viral inoculum was removed and fresh medium (3 ml) containing the indicated concentrations of neplanocin A was added. The infected cultures were then incubated for 72 h (37°C), after which the medium was discarded, the cells stained with crystal violet, and the plaques counted over a light box. All samples were carried out in quadruplicate.

L-cell Toxicity—[3H]Thymidine incorporation was measured using 60-mm culture dishes containing 3.0 × 10⁶ cells/dish. To start the experiment, the medium was removed by aspiration and replaced with fresh KU-1 medium (5 ml) containing the indicated concentrations of neplanocin A. At later times (36 and 72 h), the medium was recovered by dialysis for 24 h and counted on a hemocytometer.

Results

In Vitro Studies of Neplanocin A with Bovine Liver AdoHcy Hydrolase—It has been demonstrated that neplanocin A is a substrate for adenosine deaminase (22). Under in vitro conditions the conversion rate of neplanocin A was reported to be approximately 1.7% of that for adenosine, whereas in vivo neplanocin A was observed to be very rapidly converted to the deaminated form, neplanocin D (22). Since the AdoHcy hydrolase assay employed in this study is a coupled assay using calf intestinal adenosine deaminase to convert [3H]adenosine to [3H]inosine, we evaluated the effects of neplanocin A on the deamination of [3H]adenosine. At concentrations up to 0.2 mm, neplanocin A produced no significant inhibition of adenosine deaminase (data not shown).

An attempt was also made to determine if neplanocin A serves as a substrate for AdoHcy hydrolase. When AdoHcy hydrolase was incubated with neplanocin A (1 mM) and Hcy (1 mM) for 10 min at 37°C, we were unable to detect by high pressure liquid chromatography the corresponding neplanocin A-homocysteinyi derivative (data not shown). However, incubation of adenosine under identical conditions, led to the formation of detectable levels of AdoHcy.

When purified AdoHcy hydrolase was incubated with neplanocin A, both concentration-dependent (Fig. 2) and time-dependent inhibition of enzyme activity (hydrolytic direction: conversion of [3H]AdoHcy to [3H]adenosine) was observed. Our results also indicate that the inhibition of AdoHcy hydrolase by this analog appears to be irreversible. Following complete inactivation of the enzyme by incubation with 1 μM neplanocin A for 20 min (37°C), less than 10% of the activity was recovered by dialysis for 16 h (4°C) against 150 mM potassium phosphate buffer (pH 7.6), 5 mM dithiothreitol, and 1 mM EDTA. In contrast, dialysis of the untreated enzyme under identical conditions resulted in no detectable loss of enzymatic activity.

Neplanocin A exhibits the characteristics of a tight binding type inhibitor. Fig. 3A shows an Ackermann-Potter plot (23) of data generated by incubating varying amounts of the enzyme with several concentrations of neplanocin A for 5 min at 37°C, after which the residual hydrolase activity was determined by addition of [3H]AdoHcy (0.1 mM) and 4 units of adenosine deaminase. The results shown in Fig. 3A suggest a stoichiometry of one molecule of neplanocin A binding to one molecule (tetramer) of AdoHcy hydrolase. The Ackermann-Potter plot can be used to characterize a tight binding type inhibitor (24) and to determine both the molar equivalency (Fig. 3B) and the catalytic turnover (kₐ) (Fig. 3C) for the enzyme. The calculated molar equivalency of the AdoHcy
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Neplanocin A is a potent inhibitor of AdoHcy hydrolase. The affinity of neplanocin A for AdoHcy hydrolase (Fig. 3B) is 27 nM, which is in close agreement with the value of 29 nM determined knowing the protein concentration, enzyme purity, and enzyme molecular weight. The catalytic turnover ($k_\text{cat}$) can be calculated in two ways. Knowing that the slope of the Ackermann-Potter plot is equal to $k_\text{cat}$/$K_\text{m} + S$ and the values of $S$ (0.1 mM) and the $k_\text{m}$ of AdoHcy (0.01 mM), we calculated $k_\text{cat} = 35 \text{ min}^{-1}$. $k_\text{cat}$ can also be estimated from a plot (Fig. 3C) of the $v$ axis intercepts versus $[I]$ which gives a value of 33 min$^{-1}$.

Although the information obtained from the Ackermann-Potter plot is useful, one cannot readily estimate the inhibition constant, $K_\text{i}$, for neplanocin A. However, the data can be utilized to determine the $I_{50}$ values (24) (the total inhibitor concentration at which the enzyme reaction velocity is 50% of the uninhibited reaction) as seen in Fig. 4A. A plot of these $I_{50}$ values versus enzyme concentration (Fig. 4B) yields a straight line with an $I_{50}$ intercept equal to 8.39 nM which represents the $K_\text{i}$ value.

**Antiviral Activity of Neplanocin A**—Previous work from this laboratory as well as others (11–13) indicates that inhibitors of AdoHcy hydrolase often possess some antiviral activity. Our findings that neplanocin A is a potent inhibitor of the hydrolase enzyme prompted us to test this compound as a potential antiviral agent. The ability of neplanocin A to inhibit vaccinia virus plaque formation after 72 h in monolayer cultures of mouse L-cells was examined. As shown in Table I, significant inhibition of plaque formation was observed with concentrations of neplanocin A as low as 0.1 and 0.25 µM in the culture medium (46.9 and 71.9% of control). Virus multiplication was more severely suppressed by 1.0 and 5.0 µM neplanocin A which yielded 93.3 and 97.5% inhibition, respectively. Thus, neplanocin A like other AdoHcy hydrolase inhibitors, does exhibit antiviral activity against vaccinia virus (WR).

In evaluating the pharmacological potential of neplanocin A as an antiviral agent, an important consideration is the effect of the compound on the host cell system. To examine the aspect of cellular toxicity, [3H]thymidine incorporation into DNA of uninfected L-cell cultures was measured after 36- and 72-h exposures to the same concentrations of the drug used in the plaque inhibition assays (Table I). Although 5.0 µM neplanocin A caused significant inhibition of cellular DNA synthesis after 36 h (36.1% compared to untreated controls), 0.5 and 1.0 µM (concentrations which displayed good antiviral activity) produced substantially less inhibition (11 and 19.4%, respectively).

In contrast, the response to neplanocin A after a 72-h treatment appeared as a stimulation of DNA synthesis compared to the control. It is interesting that 0.5 and 1.0 µM exhibit the largest effects (202.6 and 209.0%, respectively) since, as mentioned, they elicit good antiviral activity. These results are similar to those we have observed for adenosine dialdehyde, another AdoHcy hydrolase inhibitor with antiviral activity, and suggest that neplanocin A is not toxic to
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**Fig. 4.** Determination of $K_i$ for neplanocin A inhibition of AdoHcy hydrolase. A, a plot of $V/V_i$ versus neplanocin A concentration where $V_i$ is the velocity in the absence of neplanocin A and $V$ is the velocity in the presence of various amounts of neplanocin A (from A). The $V/V_i$ values for different amounts of AdoHcy hydrolase ($\bullet$, 5 µL; ○, 10 µL; ▲, 20 µL; △, 30 µL; ■, 40 µL; □, 50 µL; △, 60 µL) were plotted versus the appropriate inhibitor concentrations. B, plot of $I_{50}$ values (from A) versus the amount of AdoHcy hydrolase.

**Table I**

<table>
<thead>
<tr>
<th>Concentration of neplanocin A (µM)</th>
<th>% inhibition of plaque formation at 72 h</th>
<th>% of control incorporation of $[^{3}H]$thymidine at 36 h and 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>46.9</td>
<td>94.6</td>
</tr>
<tr>
<td>0.25</td>
<td>71.9</td>
<td>88.5</td>
</tr>
<tr>
<td>0.50</td>
<td>84.3</td>
<td>89.0</td>
</tr>
<tr>
<td>1.0</td>
<td>93.9</td>
<td>80.6</td>
</tr>
<tr>
<td>5.0</td>
<td>97.5</td>
<td>65.9</td>
</tr>
</tbody>
</table>

L-cells, but rather acts as a cytostatic agent causing temporary growth inhibition. This hypothesis is supported by a comparison of the growth curves for untreated and neplanocin A-treated L-cells (Fig. 5) which demonstrate that the rate of increase in cell number is much slower for the neplanocin A-treated cells between 12 and 48 h after initial exposure to the drug.

Considering the potent inhibitory action of neplanocin A on bovine liver AdoHcy hydrolase in vitro, it was of interest to determine whether the drug is similarly inhibiting this enzyme in vaccinia virus-infected L-cells. As shown in Fig. 6, exposure of infected cells to 0.1 µM neplanocin A for 15 min resulted in 66.1% inhibition of L-cell hydrolase activity compared to the untreated control cultures. The extent of inhibition continued to increase over the next 45 min reaching approximately 85.0% within 30 min and 90.6% by 60 min. In contrast, 1.0 µM neplanocin A produced more dramatic inhibition within the first 15 min (89.2%) and appeared to have a maximal effect by 30 min (95.4%). Thus, neplanocin A is observed to be a potent inhibitor of AdoHcy hydrolase both in vitro and in vivo.

If, as we suspect, the antiviral activity of AdoHcy hydrolase inhibitors is due to an inhibition of AdoMet-dependent macromolecular methylation reactions, increases in the intracellular amount of AdoHcy and the AdoHcy/AdoMet ratio should be apparent in the neplanocin A-treated cells. Fig. 7 illustrates that exposure of L-cells to 1.0 µM neplanocin A following vaccinia virus infection results in a striking 10-fold increase in the intracellular AdoHcy/AdoMet ratio within 24 h. Subsequently, with the gradual recovery of AdoHcy hydrolase activity (data not shown) this ratio steadily declines so that by 72 h the value in the treated cultures is approximately 3-fold higher than the untreated controls. The antiviral effects of neplanocin A, therefore, would appear to be related to the large increase in the intracellular AdoHcy/AdoMet ratio which is known to cause inhibition of AdoMet-dependent methylation reactions in uninfected mouse L929 cells (25).
Therefore, classical kinetic methods of analysis, based on the steady state assumptions are not valid for analysis of the kinetics of either pseudo-irreversible (e.g. tight binding inhibitors) or truly irreversible (e.g. functional group reagents) inhibitors. Since neplanocin A appeared to be an irreversible inhibitor of AdoHcy hydrolase we employed the Ackermann-Potter approach to analyze the inactivation of the enzyme by neplanocin A. Analysis of the neplanocin A inactivation data showed a stoichiometry of one molecule of the inhibitor/tetramer of enzyme. The $K_i$ value for neplanocin A was calculated to be 8.39 nM.

In comparison to other reported inhibitors of AdoHcy hydrolase, our results indicate that neplanocin A is significantly more potent than 3-deazaadenosine ($K_i = 4 \mu M$) (1), 9-$\beta$-D-arabinofuranosyladenosine ($K_i = 5 \mu M$) (26), and 2'-deoxyadenosine ($K_i = 66 \mu M$ compared to $K_i = 24 \mu M$ for 9-$\beta$-D-arabinofuranosyladenosine) (26), the latter two of which also cause irreversible inactivation of the enzyme. However, the inhibitory activity of neplanocin A is quite comparable to that of aristeromycin ($K_i = 5 \mu M$) (27), 3-deazaaristeromycin ($K_i = 1 \mu M$) (12), and adenosine dialdehyde ($K_i = 3.5 \mu M$) (28), the most potent AdoHcy hydrolase inhibitors studied to date. In contrast to 3-deazaaristeromycin, aristeromycin and adenosine dialdehyde both inactivate the enzyme although inactivation by the latter is reversible upon dialysis against Tris buffer (29).

The chemical nature of the interaction between neplanocin A and AdoHcy hydrolase is still unclear. Our kinetic analysis could fit either a tight binding type mechanism or a covalent modification type mechanism. Robert H. Abeles has observed that the NAD$^+$ of AdoHcy hydrolase is reduced to NADH in the presence of neplanocin A, suggesting a $K_m$ mechanism similar to that reported for 2'-deoxyadenosine and 9-$\beta$-D-arabinofuranosyladenosine (30).

Although there is precedent for the antiviral activity of AdoHcy hydrolase inhibitors (11-13), the precise mechanism of their action is not well established. DeClercq and Montgomery (13) demonstrated that 3-deazaaristeromycin, another potent AdoHcy hydrolase inhibitor, is particularly effective against pox and (-)RNA viruses with little activity toward herpes or (+)RNA viruses, suggesting that an impairment of viral mRNA transcription and/or processing may be involved. The inhibition of vaccinia virus production by neplanocin A seems to be of the same nature. Although we have preliminary evidence that neplanocin A is phosphorylated in vivo and thus, may be incorporated into RNA or DNA, the lack of cellular toxicity and the similarity of antiviral activity to adenosine dialdehyde and 3-deazaaristeromycin (13) (which are not phosphorylated) indicate that the inhibition of AdoHcy hydrolase may be the common mechanism of action of these drugs.

It is known that methylation of the 5'-terminal cap structure of vaccinia virus mRNA is required for efficient translation of viral proteins and thus for viral replication (7). Moreover, it has been demonstrated that the virus specific enzymes responsible for these modifications, like other AdoMet-dependent methyltransferases, are susceptible to inhibition by AdoHcy (9, 10). Inhibiting AdoHcy hydrolase in vaccinia virus-infected cells would lead to an intracellular accumulation of AdoHcy and presumably inhibit the virus-specific enzymes as well as those of the host cell. In the present study we have confirmed that treatment of infected cells with neplanocin A does result in an inhibition of the hydrolase enzyme and a 10-fold increase in the AdoHcy/AdoMet ratio. Although these findings strongly implicate an inhibition of methylation as the mechanism of antiviral action of neplanocin A.

3 R. H. Abeles, personal communication.

**DISCUSSION**

The Ackermann-Potter approach has been used extensively to analyze "irreversible" reactions between enzymes and inhibitors (23). These irreversible reactions may be pseudo-irreversible (e.g. tight binding inhibitor, where the interaction of the enzyme and inhibitor is theoretically reversible but where the dissociation constant is so small that, for all practical purposes, the enzyme-inhibitor complex does not undergo dissociation) or truly irreversible (e.g. covalent modification where the enzyme is converted to a form which cannot be converted back to the native enzyme). In either case the amount of enzyme inactivated by the inhibitor will depend not only on the amount of inhibitor, but also on the amount of enzyme present. Therefore, classical kinetic methods of analysis, based on the steady state assumptions are not valid for analysis of the kinetics of either pseudo-irreversible
ocin A, direct analysis of the 5'-cap structure of viral mRNA after treatment with the inhibitor remains to be performed.

REFERENCES

Neplanocin A. A potent inhibitor of S-adenosylhomocysteine hydrolase and of vaccinia virus multiplication in mouse L929 cells.
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