Biochemical Mode of Cytotoxic Action of Neplanocin A in L1210 Leukemic Cells

Makoto Inaba, Kyoko Nagashima, Shigeru Tsukagoshi, and Yoshio Sakurai
Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Kami-iikebukuro, Toshima-ku, Tokyo 170, Japan

ABSTRACT

Neplanocin A, a novel antitumor antibiotic, was investigated to determine the biochemical mode(s) of its cytotoxic action. The molecule is an adenosine analogue with a unique cyclopentane structure in its ribose moiety. Both sublines of L1210 and P388 leukemia resistant to neplanocin A were cross-resistant in vitro to bre dinin and 9-β-o-arabinofuranosyladenine, which have been reported to be activated by adenosine kinase. The adenosine kinase activity was markedly reduced in the resistant sublines as compared with that of the respective sensitive lines. Furthermore, neplanocin A competitively inhibited the phosphorylation reaction of adenosine in a cell-free system. The results indicate that neplanocin A is activated by adenosine kinase. Regarding the target site for neplanocin A, the antibiotic suppressed RNA synthesis to a significantly greater extent than DNA synthesis. This RNA-preferential effect is unique among common antineoplastoic antitumor agents.

INTRODUCTION

Neplanocin A is an antibiotic produced by the actinomycete Ampullariella regularis A11079 (1) and possesses antitumor activity against murine leukemias such as L1210 and P388 (2). In addition, neplanocin A is resistant to B16 melanoma, but is inactive against B16 melanoma. NPA3 exhibits significant antitumor activity on the CD8F, mammary tumor, but is inactive against B16 melanoma. NPA4 is activated by adenosine kinase. Regarding the biochemical antitumor agents.

MATERIALS AND METHODS

**Chemicals.** Neplanocin A, bredinin, and 2′-deoxycoformycin kindly were supplied by Toyo Jozo Co., Ltd. (Shizuoka, Japan); and 1-β-o-arabinofuranosytosine, by Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). 5-Fluorouracil, 6-mercaptopurine, and 9-β-o-arabinofuranosyladenine were purchased from Sigma Chemical Co. (St. Louis, MO), and methotrexate from Lederle Japan, Ltd. (Tokyo, Japan). The radioactive compounds [methyl-3H]thymidine (43 Ci/mmol), deoxyc[5-3H]ytidine (22 Ci/mmol), and [5-6-3H]uridine (40 Ci/mmol) were purchased from Amersham International, plc (Amersham, England), and [2,8-3H]adenosine (32.2 Ci/mmol) from New England Nuclear (Boston, MA).

**Tumors.** Original L1210 and P388 leukemic lines were supplied by the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, MD. Each P388 subline resistant to 5-FluRa, 6-MP, or MTX was developed in our laboratory by an in vivo procedure (6). L1210 and P388 sublines resistant to NPA were developed recently for the present study by inoculating mice with 2×106 L1210 or P388 leukemia cells and treating the animals with NPA (3 mg/kg) daily for 5 days in each transplant generation. Complete resistance in vivo to such treatment was acquired on the third or fourth transplant generation. The resistance phenotype of some sublines was stable even in the absence of NPA. Original cell lines and all drug-resistant sublines were passaged weekly through DBA/2 and BALB/c × DBA/2 (CDF1) mice (Charles River Japan Inc., Atsugi, Japan), respectively.

**In Vitro Culture.** RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (M. A. Bioproducts), 2 μM 2-hydroxyethyl disulfide (Aldrich Chemical Co., Inc., Milwaukee, WI), and kanamycin, 100 μg/ml (Meiji Seika Kaisha, Ltd., Tokyo, Japan) was used as culture medium. Cells were harvested from tumor-bearing mice 5 to 6 days after transplantation and suspended in the culture medium to a concentration of 5×10^6 cells/ml. Various concentrations of the drug were added to the culture medium throughout the 48-h culture period. For those cells exposed to the drug for 3 h, cell suspensions were diluted with 4 ml complete medium after incubation with the drug for 3 h and centrifuged at 4×10^5 g for 5 min. The cells were resuspended in fresh medium and cultured in a CO2 incubator at 37°C for 48 h. The number of cells was counted in a Model ZBI Coulter Counter after a 5-min incubation with 0.25% trypsin to disaggregate the cells.

**Assay of Adenosine Kinase.** Adenosine kinase activity was assayed according to the procedure of Rabin and Gottesman (7). Approximately 5×10^6 cells harvested from the i.p. cavity of mice were suspended in 1 ml of 20 mM sodium phosphate (pH 8.5) containing 0.5% Triton X-100, stirred, and centrifuged at 20,000×g for 30 min. The supernatant was used as the enzyme source, and its protein content was determined by the method of Lowry et al. (8) with bovine serum albumin solution as a standard. The reaction mixture (90 μl) contained 50 mM sodium phosphate (pH 6.5), 2.5×10^{−4} ATP, 2.5×10^{−4} MgCl2, 2.5×10^{−4} M [3H]adenosine (0.5 μCi), and cell-free extract. Unless otherwise stated, 2.5×10^{−4} M 2′-deoxycoformycin was also present. The reaction was carried out at 37°C for predetermined periods of time (usually up to 5 min) and stopped by adding 1×10^{−4} M lanthanum chloride. The products were collected on Whatman GF/C filters and counted with 10 ml Aquasol (New England Nuclear) in a Beckmann Model LS-355 scintillation counter. The velocity of AMP production was determined by plotting the linear

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: NPA, neplanocin A; dCF, 2′-deoxycoformycin; ara-A, 9-β-o-arabinofuranosyladenine; ara-C, 1-β-o-arabinofuranosytosine; 5-FluRa, 5-fluorouracil; MTX, methotrexate; 6-MP, 6-mercaptopurine; L1210/NPA, L1210 cells resistant to neplanocin A.
relationship of AMP formed versus time of incubation.

**RNA and DNA Synthesis.** Cells (2 × 10⁵) were suspended in 1 ml culture medium containing a given concentration of the various antitumor drugs and cultured in a CO₂ incubator. For cultures containing NPA, ara-C, or 6-MP, 50 μl of [5,6-³H]uridine (2 μCi), or [methyl-³H]thymidine (1 μCi) were added at predetermined times and incubated for pulse labeling. For cultures containing 5-FUra or MTX, 50 μl of [5,6-³H]uridine or [5-³H]deoxycytidine (1 μCi) were added. After exposure to the radiolabel for 30 min the cells were immediately centrifuged at 1200 × g at 4°C for 5 min and suspended in 5 ml of 10% cold trichloroacetic acid solution. Trichloroacetic acid-insoluble fractions were collected on Whatman GF/C filters and washed with both 5% cold trichloroacetic acid and saline solution. After filters were kept in 1 ml of Protosol (New England Nuclear) overnight at room temperature, the radioactivity was counted with 10 ml of Econofluor (New England Nuclear) in a liquid scintillation counter.

**RESULTS**

To understand the mode of the cytotoxic action of NPA, NPA-resistant cells were examined for resistance in vitro to bredinin and ara-A. As illustrated in Fig. 2, the resistant cells had cross-resistance to both bredinin and ara-A. The degree of cross-resistance to bredinin was almost equivalent to that of resistance to NPA, whereas cross-resistance to ara-A was appreciably lower. Cross-resistance to bredinin and ara-A was examined also in P388 leukemic cells sensitive and resistant to NPA, respectively, with nearly identical results (data not shown).

The slope of the dose-response curve of cells exposed to NPA was significantly different from the slopes obtained when the cells were exposed to bredinin or ara-A. When growth inhibition induced by these drugs was compared at the level of that concentration that inhibited 50% of in vitro cell growth for the original sensitive cells, NPA was effective at a much lower concentration than bredinin or ara-A. In comparison with the concentrations necessary for complete growth inhibition, however, NPA and bredinin possessed approximately the same potency, whereas ara-A required a 10-fold greater concentration.

Using the same amount of supernatant protein (200 μg), adenosine kinase activities were compared in the presence or absence of dCF between NPA-sensitive and -resistant lines of both L1210 and P388 leukemias (Fig. 3). The activity of adenosine kinase was markedly increased by the addition of dCF, an inhibitor of adenosine deaminase. Its potentiating activity seemed to be much greater in the parent cells of both leukemias as compared to the respective NPA-resistant cells. In either the presence or absence of dCF, however, the activity of adenosine kinase was remarkably reduced in the resistant cells as compared with that of the original sensitive cells.

Kinetic parameters of the adenosine kinase activity of crude extracts from the sensitive and resistant cells were also compared, as shown in Table 1. In NPA-resistant cells of both L1210 and P388, one order lower V_max was observed, while the K_m suggested that affinity of the enzyme to its substrate was somewhat higher in the resistant cells. Because the above results indirectly support the hypothesis that NPA is phosphorylated by adenosine kinase in these tumor cells, the competitive influence of NPA on the phosphorylation reaction of adenosine in a cell-free system was examined. The resulting Lineweaver-Burk plot shown in Fig. 4 provides direct evidence that NPA is a competitive inhibitor of adenosine kinase;
MODE OF ACTION OF NPA

Table 1
Kinetic parameters of adenosine kinase activities in L1210 and P388 leukemic cells sensitive or resistant to neplanocin A

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>L1210</th>
<th>P388</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (pmol AMP formed/mg protein/min)</td>
<td>492</td>
<td>30</td>
</tr>
<tr>
<td>$K_m$ ($\times 10^{-4}$ M)</td>
<td>15.8</td>
<td>9.6</td>
</tr>
</tbody>
</table>

NPA itself is phosphorylated by adenosine kinase. Under the same experimental conditions with a supernatant of the cell homogenate of L1210, the relative affinities of adenosine kinase for adenosine and NPA were measured. From Fig. 4, the $K_m$ for adenosine was estimated to be 1.6 $\times 10^{-5}$ M, whereas the $K_i$ for NPA was found to be 3.1 $\times 10^{-5}$ M according to the Dixon plot shown in Fig. 5. These results show that at least in this experimental system adenosine kinase has a relatively stronger affinity for its natural substrate, adenosine, than for NPA, although not to any great extent.

To determine the target site(s) for NPA, the effect of this antibiotic on DNA and RNA synthesis was examined. To begin with, a dose-response relationship was measured by pulse labeling with radioactive thymidine or uridine for the last 30 min of a 3-h exposure to various concentrations of NPA. A significant RNA-selective inhibitory effect was observed, in which RNA synthesis inhibition gradually increased as the concentration of NPA increased from $10^{-7}$ to $10^{-5}$ M, reaching a maximum inhibition of 50% (Fig. 6A). Less than 20% inhibition was the maximum observed for DNA synthesis, even after exposure of the cells to an NPA concentration of $10^{-5}$ M, which is the concentration that inhibited 10% of in vitro cell growth for NPA after 3 h exposure. The same experiment was performed with L1210/NPA cells, in which inhibition of RNA synthesis was found to be significantly reduced, as compared with that in the sensitive cells, and no inhibition of DNA synthesis was observed (Fig. 6B).

DISCUSSION

Because the use of resistant sublines often aids in the elucidation of the cytotoxic action of a given compound, we initially examined NPA-resistant sublines of L1210 and P388 leukemia for cross-resistance in vitro to bredinin and ara-A. Both compounds are known to be activated by adenosine kinase (9, 10). From its chemical structure, one would expect NPA to be phosphorylated by adenosine kinase. Our results indicate that these sublines have cross-resistance to both bredinin and ara-A (Fig. 2). The lower degree of cross-resistance to ara-A may be ex-
thymidine (O) or [5,6-3H]uridine (•), respectively, 30 min before the end of each medium. DNA or RNA synthesis was measured every 3 h by adding [methyl-

comparison, the values for the drug concentration for 90% inhibition of in vitro cell growth were approximately 3 x 10^-6, 2 x 10^-5, 3 x 10^-5, and 3 x 10^-4 M for ara-

antitumor agents in L1210 cells. Cells were incubated with various concentrations of ara-C (A), MTX (B), 5-FUra (C), or 6-MP (D) at 37°C in complete culture medium. After 2.5 h [methyl-3H]thymidine or [5-3H]deoxycytidine in the case of MTX and 5-FUra (C) or [5,6-3H]uridine (W) was added and the cells were incubated for an additional 30 min for pulse labeling of DNA and RNA, respectively. For

models (Fig. 7; Table 1). These findings strongly suggest that
deoxyctydine kinase is involved in the phosphorylation of ara-A

expression. Another group also found that NPA specifically inhibited antitumor agents against RNA and DNA synthesis. With ara-C and MTX, a DNA-specific inhibition was observed. On the other hand, 5-FUra exerted a more potent

In terms of the target site(s) for the action on NPA, it has been recently reported that NPA is metabolized to S-neplanocymethionine, which inhibits S-adenosylhomocysteine hydrolase (15, 16). In addition, the same workers showed that NPA was a potent inhibitor of vaccinia virus multiplication in cultured mouse L-cells. At a concentration of 10^-6 M, NPA induced almost complete inhibition of both plaque formation of the virus and hydrolase activity in the infected L-cells but only relatively mild inhibition of growth in the L-cells themselves. These results suggest that the inhibitory effect of NPA on S-adenosylhomocysteine hydrolase is not the primary mechanism by which this antibiotic exerts toxicity against mammalian cells. In this connection, another group also found that NPA specifically inhibited RNA methylation because S-neplanocymethionine was a poor methyl donor for RNA methyltransferase (17).

In our preliminary study on the target site for NPA, we found that NPA selectively inhibited RNA synthesis (Fig. 6). Although the maximal inhibition of the rate of RNA synthesis was 50 to 60% even at 10^-4 to 10^-3 M NPA and incubation periods up to 3 h, prolongation of the exposure time caused almost complete inhibition of not only RNA but also of DNA synthesis (Fig. 7). For comparison, we examined the relative inhibitory activities of several typical antimetabolic antitumor agents against RNA and DNA synthesis. With ara-C and MTX, a DNA-specific inhibition was observed. On the other hand, 5-FUra exerted a more potent

The competitive action of NPA on the phosphorylation reaction of adenosine in a cell-free system provides direct evidence for an activation mode of NPA. Comparison of the Km for adenosine and the Ki for NPA revealed that adenosine kinase has a slightly lower affinity for NPA rather than for its natural substrate, adenosine (Figs. 4 and 5), although the Km's are significantly higher than the authentic value (11), probably because of the use of cell-free extracts as an enzyme source. Very recently, Saunders et al. (12) reported that Chinese hamster ovary cells deficient in adenosine kinase failed to phosphorylate NPA.

Initially, it seemed very difficult to identify the activation enzyme of NPA because adenosine was not able to reverse the growth-inhibitory action of NPA on L1210 cells grown in vitro. The presence of 10^-4 M adenosine did not prevent the growth-inhibitory effect on cells by 10^-3 M NPA in the presence of 10^-4 M dCF (data not shown). Yoshida et al. (13) also found similar results after culturing L5178Y cells in vitro. On the other hand, Sakaguchi et al. (14) and Koyama and Tsuji (10) failed to reverse the cytotoxic action of bredinin, which has been reported to be activated by adenosine kinase, by addition of adenosine in the culture medium for L5178Y and FM3A cells, respectively. These results suggest general difficulty in reversing the cytotoxic action of adenosine kinase-activated drugs by exogenous adenosine. This difficulty may be the result of limited intracellular utilization of exogenous adenosine as compared with other bases or nucleosides, probably due to the abundant endogenous adenosine pool. In spite of the absence of any reversal effect by adenosine, however, our findings indicate that adenosine kinase is the activation enzyme of NPA. Identification of an assumed active metabolite of NPA on a chromatogram is difficult still because of the unavailability of radioactive NPA.

In general, the nature of the activation enzyme of a given compound is integrally associated with the pattern of cross-resistance. Most probably cross-resistance occurs as a result of the sharing of activation and/or degradation enzymes between drugs. In terms of its activation enzyme, NPA appears to be unique among currently available antitumor agents because there is no major clinically available antitumor drug activated by adenosine kinase. Interestingly, P388 leukemia cells resistant to 5-FUra, ara-C, 6-MP, or MTX are not cross-resistant to NPA either in vitro or in vivo (data not shown).

plained by the fact that not only adenosine kinase but also deoxyctydine kinase is involved in the phosphorylation of ara-A (9). In subsequent experiments we found that the adenosine kinase activity of the resistant cells was significantly decreased as compared with that of sensitive cells in both L1210 and P388 systems (Fig. 2; Table 1). These findings strongly suggest that NPA is activated by adenosine kinase, regardless of the cyclopentane structure in its ribose moiety.

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Fig. 7. Kinetics of DNA and RNA synthesis inhibition by neplanocin A in L1210 cells. Cells were incubated without or with 10^-8 M NPA at 37°C in complete culture medium. DNA or RNA synthesis was measured every 3 h by adding [methyl-3H]thymidine (C) or [5,6-3H]uridine (W), respectively, 30 min before the end of each observation period.

Fig. 8. Comparison of DNA and RNA synthesis inhibition by typical antimetabolic antitumor agents in L1210 cells. Cells were incubated with various concentrations of ara-C (A), MTX (B), 5-FUra (5-FUra) (C), or 6-MP (D) at 37°C in complete culture medium. After 2.5 h [methyl-3H]thymidine or [5-3H]deoxycytidine in the case of MTX and 5-FUra (C) or [5,6-3H]uridine (W) was added and the cells were incubated for an additional 30 min for pulse labeling of DNA and RNA, respectively. For comparison, the values for the drug concentration for 90% inhibition of in vitro cell growth were approximately 3 x 10^-6, 2 x 10^-5, 3 x 10^-5, and 3 x 10^-4 M for ara-C, MTX, 5-FUra, and 6-MP, respectively, when L1210 cells were exposed to each of these agents for 3 h and then cultured for 45 h in drug-free medium.

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inhibitory effect on synthesis of DNA rather than RNA, and 6-MP suppressed both syntheses similarly (Fig. 8). Comparing the actions of NPA with each of these antitumor agents, we find the RNA-preferential action of NPA to be unique. We speculate that NPA is a poor substrate for ribonucleotide reductase because of its characteristic cyclopentane structure. If this proves to be the case, the chemical uniqueness of the ribose moiety may provide NPA with distinctive biochemical features. The precise nature of the inhibitory action of NPA on RNA synthesis remains to be determined.

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