Deamination of Arabinosyladenine by Adenosine Deaminase and Inhibition by Arabinosyl-6-mercaptopurine

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SUMMARY

6-Mercapto-9-(β-D-arabinofuranosyl)purine (ara-6-MP) was found to be a competitive inhibitor of L1210 adenosine deaminase when β-D-arabinosyladenine (ara-A) was used as substrate (pseudoproduct inhibition). The phosphorylation of adenosine, ara-A, and β-D-ribosyl-6-methylthiopurine did not appear to be affected by ara-6-MP. Studies on the kinetic parameters of adenosine deaminase from two L1210 lines, ara-6-MP-sensitive L1210 and ara-6-MP-resistant L1210 (L1210/ara-6-MP), indicated that the L1210/ara-6-MP subline had mutated. Mice bearing L1210 cells survived longer when treated with ara-A plus ara-6-MP than when treated with either agent alone. No such advantage of the combination drug treatment was seen with mice bearing the line resistant to ara-6-MP (L1210/ara-6-MP).

INTRODUCTION

ara-A is carcinostatic for a number of experimental tumors (2-4, 10, 16). ara-ATP, the inhibitory form of ara-A, was found to inhibit DNA polymerase and, to a lesser extent, CDP reductase (6, 16). ara-A is converted by adenosine deaminase to arabinosylhypoxanthine, an inactive compound (2-4). Potential inhibitors of adenosine deaminase have been investigated (10) as a means of preventing the rapid deamination of ara-A. However, adenosine deaminase inhibitors also compete for the phosphorylation of ara-A or interfere with the transport of ara-A into the cell (10). ara-6-MP was reported to inhibit the CDP reductase (9, 11) without prior conversion to nucleotide (11). Since ara-6-MP is an inosine (or deoxyinosine) analog and is not a substrate for kinases, it was investigated as a possible pseudoproduct inhibitor of adenosine deaminase. This study describes the results obtained.

MATERIALS AND METHODS

Biochemicals

ara-6-MP and ara-A were purchased from Pfamstiehl Laboratory, Inc., Waukegan, Ill. Adenosine-8-14C and ara-A-2-3H were obtained from New England Nuclear, Boston, Mass. Adenosine deaminase (calf intestinal mucosa), pyruvate kinase, phosphoenolpyruvate, and ATP were bought from Sigma Chemical Co., St. Louis, Mo. The S was prepared by exchange with sulfur-35S (New England Nuclear)

In Vitro Studies

Cell Preparations

L1210 ascites cells were grown in BDF1 female mice by the i.p. implantation of 10^6 cells/mouse. Tumor cells were harvested 7 days after implantation by flushing of the peritoneal cavity with ice-cold 0.9% NaCl solution, followed by centrifugation at 1500 X g for 2 mm. Red blood cells contaminating the L1210 cells were eliminated by the suspension of the L1210 cells in 5 volumes of ice-cold distilled water for 30 sec. Isotonicity was restored immediately by the addition of an equal volume of 1.8% NaCl solution, the cells were packed by centrifugation at 1500 X g for 2 mm, and the red supernatant was discarded. The tumor cells were resuspended in 3 volumes of Robinson's medium (14) for in vitro whole-cell experiments. Cell-free enzyme extracts were prepared as follows. Cells were suspended in 10 parts of ice-cold distilled water for 10 min and then were homogenized in a Potter-Elvehjem homogenizer with 30 strokes. The cell homogenates were brought to 10 mM with potassium phosphate buffer (pH 7.4) and were centrifuged at 16,000 X g for 90 mm at 4. The supernatants were decanted for use as enzyme extracts.

Enzyme Assays

Adenosine Deaminase. The adenosine deaminase activity of L1210 enzyme extracts and the calf intestinal enzyme were followed spectrophotometrically at 265 nm, with the use of a molar extinction coefficient of 7.0 X 10^4, as described by Kalckar (8). Activity in whole-cell preparations was followed by the chromatographic separation of products as described below. Cell suspensions (1.25 to 2.5 X 10^6 cells) were added to 4 ml of Robinson's medium (14) containing 4.8 μmoles of adenosine-8-14C or ara-A-2-3H and various concentrations of ara-6-MP. The reaction mixtures were incubated at 37° in air for 15 min in a metabolic shaker. The reactions were terminated by the addition of ice-cold perchloric acid. Protein was removed by centrifugation, and the supernatants were neutralized with KOH in the cold. Insoluble KClO4 was...
removed by centrifugation, and the supernatants were chromatographed on Whatman No. 3MM papers in an isobutyric acid:concentrated NH₄OH:water (66:33:1) solvent. The UV-absorbing spots were cut from the developed and dried papers and were counted directly in a toluene fluor in a Packard Model 3380 scintillation spectrometer.

**Kinase Assays.** Two procedures were followed. In the 1st, the assay system of Schnebli et al. (15) was followed. The incubation mixtures each contained in 0.4 ml the following: 1 mM adenosine-8-¹⁴C, 0.5 µCi/µmole or 1 mM ara-A-2-³H, 0.83 µCi/µmole; 2.5 mM ATP; 0.25 mM MgCl₂; 50 mM potassium phosphate buffer, pH 7.0; and cell-free enzyme extract, 0.2 ml. Incubations were carried out at 25° for 30 min and were stopped by boiling for 2 min. In the 2nd procedure, the assay method of Pierre and LePage (13) was used, with slight modification, in that an ATP-regenerating system composed of phosphoenolpyruvate, ATP, and pyruvate kinase was used, instead of creatine phosphate, creatine phosphokinase, and ATP. Incubations were carried out at 37° for 5 min and were stopped as described above.

Protein concentration of enzyme extracts was determined by the biuret method as modified by Cleland and Slater (5), with the use of bovine serum albumin as standard.

**Survival Studies**

BDF₁ female mice (The Jackson Memorial Laboratories, Bar Harbor, Maine), 20 to 25 g, each received i.p. implantations of 10⁸ L1210 cells or L1210/ara-6-MP cells. Drug therapy was started 24 hr later. We dissolved ara-6-MP and ara-A in 0.9% NaCl solution by gently heating the solutions. Injections (i.p.) were given twice daily (a.m. and p.m.) in a volume of 0.2 ml for 6 days. Control mice received 0.9% NaCl solution injections i.p. Mice were given drinking water containing streptomycin sulfate, 50 mg/liter, ad libitum.

**RESULTS AND DISCUSSION**

**Enzyme Studies.** ara-6-MP was found to be a competitive inhibitor of the adenosine deaminase from calf intestine (Table 1) with either adenosine or ara-A as substrate. A Kᵢ value for ara-6-MP of about 2 x 10⁻⁴ M was found. The Kₘ and Vₑₐₓ values obtained were in good agreement with values reported for extracts of another murine tumor (16). The Kₘ values were 4.5 x 10⁻⁵ M and 8.4 x 10⁻⁵ M for adenosine and ara-A, respectively. Vₑₐₓ for ara-A as substrate was about one-tenth that of the natural substrate. The adenosine deaminase from L1210 and L1210/ara-6-MP cells was also inhibited competitively by ara-6-MP. Also, the adenosine deaminase from L1210 cells gave slightly different Kᵢ values (Kᵢ apparent) than those given by the calf intestinal enzyme. These were 1.6 x 10⁻⁵ M for adenosine and 1.3 x 10⁻⁴ M for ara-A. The apparent Kᵢ values for ara-6-MP with adenosine and ara-A as substrate were 6.3 x 10⁻⁴ M, and 7.8 x 10⁻⁴ M, respectively. Interestingly, the adenosine deaminase of L1210/ara-6-MP may have mutated from the L1210 enzyme. It is known that the CDP reductase of a ara-6-MP-resistant tumor is no longer inhibited by ara-6-MP (9). Comparison of absolute values of apparent Kₘ and apparent Kᵢ between the

<table>
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<tr>
<th>Enzyme source</th>
<th>Substrate</th>
<th>Kₑₐₓ x 10⁻⁴ M</th>
<th>Vₑₐₓ (nmoles/µg/min)</th>
<th>Kᵢ x 10⁻⁴ M</th>
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<tr>
<td>Calf intestinal mucosa</td>
<td>Adenosine</td>
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<tr>
<td>L1210</td>
<td>ara-A</td>
<td>0.82</td>
<td>84</td>
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<tr>
<td>L1210/ara-6-MP</td>
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<td>0.16</td>
<td>70</td>
<td>6.27</td>
</tr>
<tr>
<td></td>
<td>ara-A</td>
<td>1.25</td>
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<td>7.80</td>
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<tr>
<th>ara-6-MP: ara-A ratio</th>
<th>ara-H₆⁸ formed (nmoles/10⁸ cells/15 min)</th>
<th>Inhibition of deamination (%)</th>
<th>ara-A nucleotides formed (nmoles/10⁸ cells/15 min)</th>
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<tr>
<td>2</td>
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*ara-H₆⁸, β-D-arabinosylhypoxanthine.*

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L1210 and L1210/ara-6-MP enzymes may not have much meaning here, since the enzymes were assayed in crude extracts. However, the ratios of $K_m$ to $K_i$ for each substrate differ between the 2 enzymes. Also, while the affinity of the L1210 enzyme for ara-A is about 1 order of magnitude less than for the natural substrate, the affinities of the L1210/ara-6-MP enzyme are the same and are decreased. It would be of interest to compare the kinetic parameters of the purified L1210 and L1210/ara-6-MP enzymes.

The question of whether the inhibitory activity of ara-6-MP on adenosine deaminase activity would hold in the L1210 cells (in vitro) was examined. It was found (Table 2) that the ara-6-MP had a pronounced inhibitory effect on the deamination of ara-A in whole cells in vitro. A 50% inhibition was obtained at a 2:1 molar ratio of ara-6-MP to ara-A. Also, the net yield of ara-A nucleotides did not appear to be affected by the ara-6-MP (Table 3). This suggested that ara-6-MP was without overall effect on the phosphorylation of ara-A. For this reason, studies of ara-A kinases were initiated, with adenosine and ara-A as substrates. Since the pH optimum for adenosine kinase ranges from 5.4 to 6.8, depending on the tissue studied (7, 12, 15), it was necessary to determine the pH optimum for the L1210 enzyme. It was found that in the L1210, adenosine kinase had a broad pH profile, and our
The $K_m$ values for adenosine kinase with adenosine or ara-A as substrate were $1.9 \times 10^{-4}$ and $5.6 \times 10^{-4}$ M, respectively. The values for $V_{max}$ for adenosine and ara-A as substrates were $3.8 \times 10^{-5}$ and $2.5 \times 10^{-5}$ moles/5 min/mg protein, respectively. The $K_m$ for adenosine was 2 orders of magnitude higher than that reported by Lindberg et al. (12) and Schnebli et al. (15). However, it is close to the $K_m$ reported by Ho et al. (7) for human and mouse red blood cell adenosine kinase. The $K_m$ value for ara-A as substrate was about the same as that reported by Lindberg et al. (12).

ara-6-MP had no apparent inhibitory effect on the phosphorylation of either adenosine, ara-A, or MMPR, even when the ratio of ara-6-MP to substrate was 12:1 (Table 3).

Survival Studies. The results of survival studies that used combinations of ara-6-MP and ara-A in the therapy of BDF$_1$ mice bearing the L1210 ascites tumors (L1210 or L1210/ara-6-MP) are shown in Table 4. ara-A at the low dosages used did not give significant increases in survival times, which is consistent with earlier observations (2—4). When ara-A was used at 12.5 mg/kg twice daily with ara-6-MP at 100 mg/kg twice daily (Table 4, Experiment 1) in combination therapy of the ara-6-MP-sensitive L1210 tumor, there was no increase in survival over that obtained with ara-6-MP alone. However, when the dosage of ara-A was increased to 25 mg/kg twice daily in combination with ara-6-MP, 100 mg/kg twice daily, it increased the survival of BDF$_1$ mice (Table 4, Experiment 2). These experiments with the L1210 tumor appeared to substantiate the enzyme experiments, in that ara-6-MP inhibited the deamination of ara-A but did not block its phosphorylation. Thus, ara-A would be spared to be phosphorylated to the inhibitory form (16), ara-ATP. However, the results of Experiment 3 (Table 4) indicate that this explanation does not suffice to explain the effects of combination therapy of the L1210 tumor. When ara-A at 25 mg/kg was used in combination with ara-6-MP at 100 mg/kg in the treatment of BDF$_1$ mice bearing the ara-6-MP-resistant L1210 tumor, no increases in survival of mice occurred beyond that obtained with ara-A alone. This indicates that the combined effects of ara-6-MP (and possibly ara-A) on the ribonucleotide reductase and of ara-A on DNA polymerase are required for the increased survival times shown by the combination. The adenosine deaminase activity, with ara-A as substrate, is very high in the L1210 lines. The inhibition of deamination by ara-6-MP was evidently not sufficient to enhance ara-ATP formation. This inhibition might become significant in other tumors with more moderate levels of deaminase.

REFERENCES


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