Identification of 6-Azauridine Triphosphate in L1210 Cells and Its Possible Relevance to Cytotoxicity

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ABSTRACT

L1210 cells treated with 1 mM 6-azauridine (AzUrd) (concentration causing 50% inhibition of cell growth, 3 μM) continued to divide at a reduced rate for 72 h before stopping. However, a 24-h treatment was lethal to 99% of the cells, as determined by colony formation. To investigate the mechanism of cytotoxicity, the metabolism of AzUrd was studied. Cells incubated with AzUrd contained a new 254 nm-absorbing component, not found in control cells. It appeared to be 6-azauridine-5'-triphosphate, since it was the only peak in the triphosphate region of the chromatogram which contained [3H]AzUrd. Incorporation of [3H]AzUrd into the acid-insoluble fraction (nucleic acids) was also detected. A role for this incorporation in the mechanism of AzUrd cytotoxicity was strongly suggested by the observation that cordycepin (0.01 mmol) partially protected cells from the lethality of AzUrd, presumably by preventing its incorporation into RNA. The previously known inhibition of pyrimidine de novo synthesis by AzUrd was confirmed by a decrease in the intracellular contents of UTP and CTP in AzUrd-treated cells. Therefore, we propose that the inhibition of pyrimidine de novo synthesis and the incorporation into nucleic acid(s) may act in concert to produce the cytotoxic effects of AzUrd.

INTRODUCTION

AzUrd was originally isolated as a metabolite of 6-azauracil in Escherichia coli (1), and it was subsequently shown to have activity against experimental tumors in animals and in vitro (2, 3). Clinical trials showed significant antileukemic activity, although the remissions observed were partial and temporary (4). Investigation of the mechanism of this cytotoxic activity revealed that the primary biochemical effect of AzUrd was inhibition of orotidylate decarboxylase (5), a key enzyme in pyrimidine de novo synthesis. The resulting depletion of the pyrimidine nucleotide pools (6) appeared to account for the inhibition of cell growth by AzUrd, since the inhibition could be prevented by the addition of a natural pyrimidine nucleoside, uridine or cytidine, to the cell culture medium (2, 7, 8). The active metabolite of AzUrd responsible for the inhibition of orotidylate decarboxylase was shown to be AzUMP. In AzUrd-treated L5178Y murine leukemia cells, in fact, AzUMP was the only acid-soluble metabolite identified (9). Reports on the further metabolism of AzUrd in mammalian systems have been mixed. Incorporation of [14C]AzUrd into nucleic acids of Sarcoma 180 and L5178Y tumor cells occurred "to a very limited degree, if at all" (3). On the other hand, [14C]AzUrd was incorporated into RNA, but not into DNA (11). More recently, the metabolism of AzUrd was investigated in germating wheat embryonic axes (12). AzUTP was identified, and incorporation of AzUrd into RNA was demonstrated. Furthermore, in this system the maximal inhibition of growth and protein synthesis by AzUrd occurred after AzUrd had been removed from the cultures and the intracellular UTP level had returned to normal (13). These and additional observations led the authors to suggest that AzUrd inhibited growth of wheat embryonic axes by a mechanism independent of its inhibition of UTP biosynthesis, namely that the AzUrd-substituted RNA synthesized during AzUrd treatment was deficient in its ability to support protein synthesis (13).

In the present paper we report that the mono-, di-, and triphosphate derivatives of AzUrd appear to be formed in AzUrd-treated L1210 murine leukemia cells. We also address the question of whether AzUrd may exert its lethal effect on these cells through incorporation into RNA and/or DNA.

MATERIALS AND METHODS

Tissue Culture Supplies

Fischer's medium for leukemic cells of mice was purchased from GIBCO Laboratories, Grand Island, NY; and defined equine serum (donor) was from HyClone Laboratories, Logan, UT.

Chemicals

AzUrd was obtained from Aldrich Chemical Co., Milwaukee, WI; [3H]AzUrd, 14 Ci/mmol, was from Moravek Biochemicals, Brea, CA; cordycepin (3'-deoxyadenosine) was from Sigma Chemical Co., St. Louis, MO. Monobasic potassium phosphate for HPLC elution buffer was HPLC grade from Fisher Scientific, Fair Lawn, NJ. Other chemicals were reagent grade from commercial sources.

Cell Culture

L1210 murine leukemia cells were cultured in vitro in Fischer's medium for leukemic cells of mice supplemented with 10% heat-inactivated (56°C, 30 min) equine serum as described in detail previously (14), except that antibiotics were omitted. AzUrd (20 μM) or cordycepin (2 μM) was dissolved in tissue culture medium without serum and sterilized by filtration (Millipore, Bedford, MA) before being added to the cultures. Growth curves were obtained by determining the average number of cells per ml in two replicate tubes of L1210 cell suspension, at each time point by using a Coulter Model ZBI particle counter (Coulter Electronics, Hialeah, FL). Cell counts were plotted semilogarithmically as a function of time, and the growth rates were derived from the slopes of the exponential portions of the curves as described previously (14). Operationally, the growth rate for a drug-treated culture as a percentage of control is equal to the population doubling time of the control culture divided by the population doubling time of the drug-treated culture. The average control population-doubling time was 12 h.

Viability Determinations

Drug-treated cells (10⁶/ml) were separated from the medium by centrifugation (200 × g, 5 min), washed twice with and resuspended in nonmammalian systems. In Streptococcus faecalis, AzUrd was converted to its mono-, di-, and triphosphate derivatives, and was incorporated into RNA, but not into DNA (11). More recently, the metabolism of AzUrd was investigated in germating wheat embryonic axes (12). AzUTP was identified, and incorporation of AzUrd into RNA was demonstrated. Furthermore, in this system the maximal inhibition of growth and protein synthesis by AzUrd occurred after AzUrd had been removed from the cultures and the intracellular UTP level had returned to normal (13). These and additional observations led the authors to suggest that AzUrd inhibited growth of wheat embryonic axes by a mechanism independent of its inhibition of UTP biosynthesis, namely that the AzUrd-substituted RNA synthesized during AzUrd treatment was deficient in its ability to support protein synthesis (13).

In the present paper we report that the mono-, di-, and triphosphate derivatives of AzUrd appear to be formed in AzUrd-treated L1210 murine leukemia cells. We also address the question of whether AzUrd may exert its lethal effect on these cells through incorporation into RNA and/or DNA.
warm (38°C), drug-free culture medium with 10% equine serum. Colony formation was assayed in soft agar (0.115% Difco purified agar in Fischer’s medium with 15% equine serum) as described previously (15). In control cultures 72 ± 3% (SE) of the cells formed colonies (n = 19).

Nucleotide Pool Determinations

L1210 cells (10^6/ml) were incubated 2 h with 1 mM AzUrd or in drug-free medium as controls, and the neutralized perchloric acid extracts were prepared as described previously (16). The nucleotides were separated by anion exchange chromatography with a Beckman Model 346 HPLC system, including a Beckman Model 165 detector. Samples of 200 µl, each representing the extract of 2–3 x 10^6 cells, were injected on the 4.6-mm x 25-cm Beckman Ultrasil AX column, and eluted at a flow rate of 1 ml/min, first with a linear gradient of Buffer A (0.002 M KH2PO4, pH 4.7) to Buffer B (0.5 M KH2PO4, pH 4.4) over 40 min, then with 100% Buffer B for 20 min. The UV absorbance of the eluate was monitored at 254 nm, and the natural nucleotides were quantified by standardization with authentic samples.

Incorporation of [3H]AzUrd

Nucleotides. L1210 cells were incubated as for the nucleotide pool determinations (see above) with the addition of 1 µCi/ml [3H]AzUrd. The extracts were prepared and analyzed as described above. Fractions (1 ml) of the eluate were collected, and their 3H content was determined by liquid scintillation counting. The samples for counting contained 0.5 ml of HPLC eluate fraction, plus 0.5 ml of water per sample for fractions 1–25 or 1.0 ml of water per sample for fractions 26–60, and 10 ml 5 g/l PPO in toluene:Triton X-100 (2:1).

Acid-insoluble Fraction. L1210 cells (10^6/ml) were incubated with 1 mM AzUrd, including 100 µCi/ml [3H]AzUrd. At the indicated times, triplicate 100-µl samples were analyzed for incorporation of 3H into acid-insoluble material. The samples were applied to filter paper discs and were cold acid extracted in batches as described previously (15). A blank value, which was subtracted from each measurement, was determined from samples of tissue culture medium containing 1 mM AzUrd, including 100 µCi/ml [3H]AzUrd. These samples were placed on filters and extracted with the other samples.

RESULTS

AzUrd Cytotoxicity to L1210 Cells. Continuous incubation of L1210 cells with AzUrd inhibited cell growth in a concentration-dependent manner (Fig. 1A), and the concentration of AzUrd required to produce a 50% decrease in the final growth rate was 3 µM (Fig. 1B). At concentrations equal to or greater than 0.1 mM, AzUrd exerted its maximal inhibition of cell growth (Fig. 1A). In 0.1 or 1 mM AzUrd, the cells apparently divided twice at a reduced rate, with a population doubling time of about 36 h. Then the cell number remained essentially constant or declined slightly. These observations suggested that prolonged exposure to the drug was required to obtain the cytotoxic effect of AzUrd.

The effect of maximal growth inhibition on the viability of L1210 cells was investigated by soft agar cloning after various times of exposure to 0.1 or 1 mM AzUrd. The cells were killed more rapidly by 1 mM than by 0.1 mM AzUrd (Fig. 2), although as noted above, both concentrations produced a similar degree of growth inhibition. The gradual slope of both curves suggested that appreciable time may be required to attain the intracellular conditions for lethality, e.g., a critical concentration of a metabolite. These results further supported the conclusion that killing of L1210 cells by AzUrd was a gradual process. At 1 mM AzUrd, 24 h of treatment was required to kill 99% of the cells. During this time, the cell number in the population increased about 1.6-fold, indicating that at least 60% of the cells progressed through the cell cycle and divided once during the 24-h treatment. (More than 60% of the cells may have divided if cell lysis also occurred, but this possibility was not investigated.) Thus, the cytotoxicity of a 24-h exposure to 0.1 or 1 mM AzUrd was delayed in the sense that the cell number in the population continued to increase for another 48 h, to a total nearly 4-fold increase in cell number before stopping (Fig.
AzUrd was 0.73 ± 0.07 (n = 5) nmol/10^6 cells. The amounts of 3H in the four peaks, as a percentage of the summation of this peak represented AzUTP was well supported. The average amount of AzUTP formed after 2-h incubation with 1 mM AzUrd was 0.73 ± 0.07 (n = 5) nmol/10^6 cells. The amounts of 3H in the four peaks, as a percentage of the summation of

Table 1 Effect of AzUrd on Natural Nucleotide Content of L1210 Cells

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Cells</th>
<th>AzUrd</th>
<th>Control</th>
<th>AzUrd</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SE</td>
<td>n</td>
<td>Mean ± SE</td>
<td>n</td>
</tr>
<tr>
<td>UDP</td>
<td>6</td>
<td>0.105 ± 0.019</td>
<td>8</td>
<td>0.133 ± 0.062</td>
<td>8</td>
</tr>
<tr>
<td>CDP</td>
<td>7</td>
<td>0.165 ± 0.079</td>
<td>6</td>
<td>0.421 ± 0.247</td>
<td>255</td>
</tr>
<tr>
<td>ADP</td>
<td>8</td>
<td>0.617 ± 0.085</td>
<td>8</td>
<td>0.487 ± 0.070</td>
<td>79</td>
</tr>
<tr>
<td>GDP</td>
<td>8</td>
<td>0.223 ± 0.026</td>
<td>8</td>
<td>0.138 ± 0.0125</td>
<td>62</td>
</tr>
<tr>
<td>UTP</td>
<td>8</td>
<td>1.46 ± 0.191</td>
<td>7</td>
<td>0.42 ± 0.118</td>
<td>29</td>
</tr>
<tr>
<td>CTP</td>
<td>8</td>
<td>0.744 ± 0.109</td>
<td>8</td>
<td>0.322 ± 0.108</td>
<td>43</td>
</tr>
<tr>
<td>ATP</td>
<td>8</td>
<td>5.71 ± 0.354</td>
<td>8</td>
<td>4.98 ± 0.562</td>
<td>87</td>
</tr>
<tr>
<td>GTP</td>
<td>8</td>
<td>1.69 ± 0.070</td>
<td>8</td>
<td>0.579 ± 0.088</td>
<td>34</td>
</tr>
</tbody>
</table>

For a comparison of the means for control and AzUrd-treated cells, i.e., the probability that the percentage of control is not different from 100.

Fig. 4. Incorporation of [3H]AzUrd into the nucleotide pools of L1210 cells. Cells were incubated 2 h with 1 mM AzUrd and 1 µCi/ml [3H]AzUrd, extracted and analyzed as described in "Materials and Methods." The UV (254 nm) absorbance, and 3H, profiles of the HPLC eluates from a typical experiment are shown. One fraction (1 ml) was collected per min; where no point is shown, 3H cpm/fraction <10.

Effect of AzUrd on Natural Nucleotides in L1210 Cells. When L1210 cells were incubated 2 h with 1 mM AzUrd, their intracellular contents of GDP, GTP, UTP, and CTP were significantly decreased, while ATP content remained relatively constant (Table 1). These results are in qualitative agreement with the observations of Janeway and Cha (6) on the effects of 5 µM AzUrd on the natural nucleotides in L5178Y' cells.

Incorporation of AzUrd into Nucleotide Pools. Nucleotide analysis after AzUrd treatment (2 h, 1 mM) revealed a new 254 nm-absorbing peak that eluted in the triphosphate region of the chromatogram, between CTP and ATP (Fig. 3). Since this peak was not detected in extracts from control cells, it appeared that it was a metabolite of AzUrd. Its UV spectrum was obtained by activating the peak scan feature of the detector at the point in the elution where the new peak appeared. The spectrum was consistent with that obtained from an authentic sample of AzUrd in Buffer B with the use of a standard scanning UV spectrophotometer. To further test the possibility that the new peak was a metabolite of AzUrd, L1210 cells were incubated with [3H]AzUrd, and their nucleotides were extracted and analyzed. The HPLC eluate was monitored for absorbance at 254 nm and for 3H, and the resulting profiles are shown in Fig. 4. Four peaks of 3H-labeled material were detected. The first peak fell in the monophosphate region of the chromatograph and therefore was presumably the monophosphate derivative, AzUMP. The separation procedure used does not resolve the various components eluting in this region well enough to allow determination of the location of the presumptive AzUMP peak relative to the natural nucleotides. The third and fourth peaks of 3H-labeled material fell between CDP and ADP, and between CTP and ATP, respectively (Fig. 4). These peaks appeared to represent the diphosphate (AzUDP) and the triphosphate (AzUTP) derivatives of AzUrd. Since the only peak of 3H-labeled material in the triphosphate region coincided with the void volume of the column and therefore appeared to be the unaltered nucleoside, AzUrd. The second peak fell in the monophosphate region of the chromatogram and therefore was presumably the monophosphate derivative, AzUMP. The separation procedure used does not resolve the various components eluting in this region well enough to allow determination of the location of the presumptive AzUMP peak relative to the natural nucleotides. The third and fourth peaks of 3H-labeled material fell between CDP and ADP, and between CTP and ATP, respectively (Fig. 4). These peaks appeared to represent the diphosphate (AzUDP) and the triphosphate (AzUTP) derivatives of AzUrd. Since the only peak of 3H-labeled material in the triphosphate region coeluted with the new 254 nm-absorbing peak formed after incubation with AzUrd (Fig. 3), it appeared extremely unlikely that the 3H-labeled material in the triphosphate region could be accounted for by any nonspecific mechanism. Thus the conclusion that
all four peaks, were as follows: AzUrd, 45 ± 2.2; AzUMP, 34 ± 1.7; AzUDP, 1.9 ± 0.6; AzUTP, 19 ± 3.3.

Incorporation of [3H]AzUrd into the Acid-insoluble Fraction of L1210 Cells. Incorporation into the acid-insoluble material was measured after various times of exposure to 1 mM AzUrd, with 100 µCi [3H]AzUrd/ml, and the results are shown in Fig. 5. Between 1 and 6 h, the H content of the acid-insoluble material increased at the rate of 103 cpm/10^6 cells/h. On the basis of comparison with a standard, this rate was calculated to be equivalent to 7.05 pmol/10^4 cells/h. The high value of the zero-time intercept in Fig. 5, 710 cpm/10^6 cells, could be accounted for by residual nonspecific binding of only 0.05% of the [3H]-AzUrd in the medium to the filter after the acid and ethanol washes. A blank equal to 0.25% of the total H in the medium was subtracted. The presence of the cells may slightly enhance the nonspecific binding in the samples for measurement of incorporation, as compared to the blanks. Similar non-zero intercepts have been obtained for [3H]dThd incorporation in other studies. Thus, it appeared reasonable to conclude that the incorporation of H from [3H]AzUrd into the acid-insoluble fraction between 1 and 6 h represented incorporation of AzUrd into DNA and/or RNA.

Effect of Cordycepin on Killing of L1210 Cells by AzUrd. The conclusion that AzUrd was incorporated into cellular nucleic acid(s) (Fig. 5) suggested that it would be of interest to investigate the possibility that this incorporation might play a role in AzUrd-induced cytotoxicity. Since cordycepin (3'-deoxyadenosine) has been shown to inhibit RNA synthesis selectively (17), simultaneous addition of cordycepin to cell cultures along with AzUrd would be expected to prevent incorporation of AzUrd into RNA. If this incorporation plays a role in the cytotoxicity of AzUrd, the lethality of AzUrd would be decreased by the presence of cordycepin. The results of such an experiment are shown in Figs. 2 and 6. The fraction of the cells killed by AzUrd at either 0.1 or 1 mM, was less when cordycepin was present (Fig. 6; cf. Fig. 2). The effect was particularly striking after 24 h of treatment with 1 mM AzUrd, when 20% of the cells survived if cordycepin was added, as opposed to 1% if it was not. The Student's t test showed that 0.01 mM cordycepin significantly (P < 0.02) increased the viability of the cells treated with 0.1 mM AzUrd for 24 h, and of the cells treated with 1 mM AzUrd for 6, 12, and 24 h. In effect, the presence of cordycepin decreased the lethal potency of AzUrd about 10-fold, since the effect of 0.1 mM AzUrd alone was virtually identical with the effect of 1 mM AzUrd plus 0.01 mM cordycepin (Fig. 6; cf. Fig. 2). Cordycepin (0.01 mM) alone inhibited cell growth to a degree and in a manner similar to that of 0.1 mM AzUrd (data not shown), but growth inhibition by cordycepin was more reversible than that by AzUrd. Specifically, L1210 cells treated with 0.01 mM cordycepin for 6 h were 81 ± 10% viable; for 12 h, 66 ± 8% viable; and for 24 h, 50 ± 9% viable, as determined by colony formation, and expressed as percentage of the control without drug treatment. The inhibition of L1210 cell growth by 0.1 or 1 mM AzUrd was not significantly influenced by cordycepin (data not shown). Thus, while AzUrd inhibited cell growth regardless of the presence of cordycepin, the lethality of AzUrd was significantly decreased by cordycepin. These observations suggested that AzUrd was incorporated into RNA, and that this incorporation might at least partially account for its lethal effect on L1210 cells.

DISCUSSION

This paper presents several major findings regarding the metabolism and biological effects of AzUrd in L1210 cells. Formation of two new metabolites, most likely the di- and triphosphate derivatives of AzUrd, has been demonstrated (Figs. 3 and 4), as well as incorporation of AzUrd-derived metabolites into the acid-insoluble fraction, i.e., presumably into RNA and/or DNA (Fig. 5). Clearly, AzUTP could be an immediate precursor for RNA, acting as a substrate analogue for RNA polymerase. On the other hand, incorporation of AzUrd into DNA would require more extensive metabolism, to AzdCTP and/or AzdTTP to provide a DNA precursor. AzdThd has been shown to inhibit growth of bacteria and to decrease their viability (18, 19). Its biochemical effects in bacteria include inhibiting utilization of dThd for DNA synthesis (19), and being incorporated into DNA (20). AzdThd also inhibited DNA synthesis in tumor cells (21) and in bone marrow cells (22), though it appeared virtually devoid of antitumor activity in vitro (21, 23) or in vivo (20, 23). Studies on the incorporation of AzdThd into DNA in mammalian cells have not been reported. AzdCyd, on the other hand, has been reported to stimulate incorporation of cytidine into DNA, due to its allosteric activation of dCMP deaminase, and no information was presented on whether or not it was cytotoxic (24). Therefore, while it was plausible that AzUrd might be metabolized to AzdThd and/or AzdCyd nucleotides and be incorporated into DNA, it appeared unlikely that this mechanism would account for the cytotoxicity (Ref. 2; Figs. 1 and 2) and antitumor activity (3, 4) observed for AzUrd.

A role for the incorporation of AzUrd into RNA in the cytotoxicity of AzUrd was strongly supported by the protective
effect of cordycepin (Figs. 2 and 6). When incorporation of AzUrd into RNA was decreased by cordycepin, the lethal effect of AzUrd was partially prevented, i.e., more cells remained viable after the combined drug treatment than after AzUrd alone. As in studies reported previously (5, 6), it appeared that AzUrd also inhibited pyrimidine de novo synthesis, since the cellular content of the natural pyrimidine triphosphates was decreased in AzUrd-treated cells (Table 1). The inhibition of orotidylate decarboxylase by AzUMP is the well-established mechanism for this effect (5). It is proposed that the incorporation of AzUrd into RNA may represent a second biochemical effect which plays an important role in the lethality of AzUrd. It appears possible that these two effects might work in concert, since the decrease in the supply of natural pyrimidine nucleotides for RNA synthesis would enhance the incorporation of the analogue. Such a mechanism would be analogous to that reported for the enhancement of 1-β-D-arabinofuranosylcyto-
sine cytotoxicity by excess thymidine (25).

The protection of cells from the lethality of AzUrd by uridine or cytidine has previously been attributed to the preformed pyrimidines providing the end products of the blocked pyrimi-
dine de novo pathway (2, 7, 8). The exogenous preformed pyrimidines would also serve to expand the intracellular pools of UTP and CTP, effectively decreasing the ratio of AzUTP to UTP, thus decreasing the frequency of incorporation of AzUrd into RNA. Thus, uridine or cytidine would be expected to provide protection from both the inhibition of pyrimidine de novo synthesis by AzUrd and the incorporation of AzUrd into RNA.

The delay in the cytotoxicity of AzUrd (Fig. 1A; cf. Fig. 2) may be at least partially due to the requirements for metabolism of AzUrd to produce the cytotoxic metabolite(s) and/or be incorporated into nucleic acid(s). AzUrd already taken up into the cell, and possibly metabolized to nucleotides, may have been further metabolized, possibly by incorporation into nucleic acids, after the cells were removed from AzUrd-containing medium. This further metabolism may be required to cause cessation of growth, since the cells continued to divide at a reduced rate for about 48 h after 99% of them had lost the ability to form colonies if removed from the AzUrd (1 mm)-containing medium. The delay in the expression of cytotoxicity may also be due to time and/or cell division being required to "fix" the damage caused by incorporation into nucleic acids, i.e., to convert the damage to a form that causes permanent cessation of growth.

The delayed cytotoxicity, demonstrated here for the first time for AzUrd (Fig. 1A; cf. Fig. 2), is a well known feature of the cellular lethality of the thiopurines (26–28). TGua has long been known to be incorporated into the DNA of TGua-treated cells, and this incorporation had been indirectly implicated in the mechanism of TGua cytotoxicity (29). However, it was only more recently that the deleterious consequences of the presence of TGua in the DNA were demonstrated: DNA-protein cross-links (30), DNA strand breaks (31, 32), and cytologically observable chromatid damage (33). Current investigations into the molecular mechanisms of TGua lethality can be expected to offer insights into how substitution of structurally modified nucleotides into DNA affects its structure and function. Thus, although AzUrd did not appear promising as a clinical candidate (4), it is still of interest as a prototype antineoplastic agent. Since it appears that incorporation of AzUrd into RNA plays a role in its cytotoxicity, investigation of the molecular conse-

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AzutP IN L1210 CELLS


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