2,6-Diaminopurinedeoxyriboside as a Prodrug of Deoxyguanosine in L1210 Cells

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ABSTRACT

The mode of action of the antiproliferative nucleoside analogue 2,6-diaminopurinedeoxyriboside (DAPdR) has been characterized in cultured L1210 cells. A marked concentration-dependent decrease in DNA synthesis and ribonucleotide reductase activity occurred in L1210 cells exposed to 0.05 to 1.0 mM DAPdR. Concomitantly, dGTP levels increased as much as 1100-fold as compared to untreated controls. Adenosine deaminase efficiently catalyzed DAPdR conversion to deoxyguanosine in vitro. In a comparative study, DAPdR and deoxyguanosine gave similar results. A 50% inhibition of cell growth during a 72-h incubation was achieved with 0.14 mM DAPdR or 0.26 mM deoxyguanosine. Deoxycoformycin rescued the L1210 cells from DAPdR and deoxyguanosine toxicity to the same extent. DAPdR and deoxyguanosine counteracted the toxic effects of mycophenolic acid with the same efficiency.

While DAPdR was not metabolized to its 5'-triphosphate, 2,6-diaminopurine was converted to 2,6-diaminopurinenucleoside 5'-triphosphate in L1210 cells; accordingly 50% inhibition of cell growth occurred at 0.015 mM 2,6-diaminopurine. Combinations of DAPdR with erythro-9-(2-hydroxy-3-nonyl)adenine or deoxycoformycin resulted in antagonism in lieu of an expected synergism.

INTRODUCTION

The base-modified analogue DAPdR inhibits growth of various cell types including human lymphoblastic and KB cells (1), RPMI 6410 cells (2), and as shown in preliminary experiments, L1210 cells in culture (3). A biochemical mechanism for this antiproliferative effect, however, has not been reported. The chemical structure of DAPdR resembles that of deoxyadenosine or deoxyguanosine, and each of these deoxynucleosides is phosphorylated to the respective 5'-monophosphate in a reaction catalyzed by deoxyctydine kinase from calf thymus (4). This would suggest that a formation of nucleotide metabolites of DAPdR might occur in cells exposed to the analogue. Chemically synthesized 2,6-diaminopurinedeoxyriboside 5'-triphosphate was incorporated into DNA using Pol I Klenow DNA polymerase (5) or terminal deoxynucleotidyltransferase (6).

In preliminary work, DAPdR was shown to be converted to deoxyguanosine in the presence of adenine deaminase (3). This result indicated that DAPdR might lead to an elevation of dGTP, which is a well known inhibitor of ribonucleotide reductase, the rate limiting enzyme in DNA synthesis and hence of cell growth (7).

DAP is the parent compound of DAPdR and has been extensively characterized as an antineoplastic agent with additional bacteriostatic and antiviral properties (8-10). To allow comparison with its deoxyriboside derivative, DAP is included in this study. In the present paper, effects of DAPdR on cell growth, nucleic acid synthesis, ribonucleotide reductase activity, and cellular nucleotide contents are reported.

MATERIALS AND METHODS

Chemicals. DAPdR was purchased from US Biochemical Co. (Cleveland, OH). Sigma Chemical Co. (St. Louis, MO) supplied DAP, deoxyadenosines, deoxycytides, mycophenolic acid, alkaline phosphatase (bovine intestinal mucosa), and adenosine deaminase (calf intestinal mucosa). 2'-Deoxycoformycin was a generous gift from Warner-Lambert Co. (Ann Arbor, MI). EHNA was purchased from Burroughs Wellcome Co. (Research Triangle Park, NC). [U-14C]Cytidine (450 Ci/mol) came from Research Products Int. Co. (Mount Prospect, IL).

In Vitro Culture. L1210 cells were supplied by American Type Culture Collection (Rockville, MD). They were cultured in RPMI 1640 medium supplemented with 10% horse serum and sodium bicarbonate (2 g/liter) provided by Grand Island Biological Co. (Grand Island, NY). The medium contained gentamycin (50 mg/liter). For 72-h cell growth studies, drugs dissolved in 0.9% NaCl solution were added to 24-well tissue culture plates. Medium and cells were added to give a seeding concentration of 1.5 x 10^6 cells/ml in a final volume of 1 ml. After the incubation at 37°C in a humidified atmosphere of 90% air/10% CO_2, the cell density was determined in a Model ZBI Coulter Counter. For 5-day growth curves, drugs, medium, and cells were sequentially added to 6-well tissue culture plates and incubated as described above. The seeding concentration was 1.5 x 10^6 cells/ml in a final volume of 9 ml. Cells were counted in 0.5-ml aliquots withdrawn at 24-h intervals (11).

Incorporation of [14C]Cytidine into L1210 Cells. The cells were pelleted during log phase growth and resuspended in fresh medium to a final concentration of 5 x 10^5 cells/ml (2-h incubations) or approximately 1 x 10^7 cells/ml (24-h incubations). Drugs and cell suspensions were pipetted into plastic flasks and incubated at 37°C. During the last 30 min of the incubation, the cells were pulsed with [14C]Cytidine (0.1 μCi/ml). Incorporation of label into the acid-soluble fraction, nucleic acids, and deoxycytidine was analyzed using a modified Schmidt-Thannhauser procedure (11, 12). The cells were harvested at 4°C by centrifugation and extracted 3 times with 1 ml 6% perchloric acid by homogenization in the cold. The protein-free extracts were combined, neutralized with 5% KOH plus solid KC104, and, after removal of KC104, analyzed by HPLC. One-half of the extract was lyophilized overnight, and the residue was dissolved in 25 mM Tris buffer (pH 9.0) for a 4-h incubation at 37°C in the presence of 4 mM magnesium chloride, 1 mM dCMP and crude snake venom (Crotalus atrox; 4 mg/ml). The heat-deproteinized reaction mixture was applied to a 1-mm column of Dowex-1X-8 (400 borate form), and 14C-deoxycytidine was eluted with 4 ml H2O. The ratio of labeled deoxycytidine to total acid-soluble radioactivity reflected the ribonucleotide reductase activity in intact cells (13).

The pellet remaining after acid extraction was dissolved in 1 ml of 0.5 M NaOH by incubation at 37°C overnight. DNA was repainticated by addition of 0.16 ml of 60% perchloric acid, separated by centrifugation and reextracted with 6% perchloric acid. The pellet obtained after centrifugation was dissolved in 0.5 M NaOH for counting radioactivity incorporated into DNA. RNA labeling was determined by counting radioactivity in the combined acid extracts.

HPLC Analysis. In System A, nucleotides were separated on a Partisil 10 SAX column (Whatman, Clifton, NJ) using a 35-min linear gradient of ammonium phosphate with a flow rate of 2 ml/min (14). The starting buffer was 10 mM, pH 2.8, and the final buffer was 500 mM, pH 4.8. In System B, deoxynucleotides were analyzed by anion-exchange HPLC according to Tanaka et al. (15). The mobile phase consisted of 0.4 M ammonium phosphate/acetonitrile (10/1, v/v; pH 3.35) and was pumped at a rate of 2 ml/min through a Partisil 10 SAX column. Samples were cleaved with NaIO4 prior to analysis (15). With
TOXICITY OF DAPdR IN L1210 CELLS

System C, nucleosides were chromatographed on a Spherisorb 10 octadecyl silane column (Phenomenex, Palos Verdes, CA). The mobile phase consisted of 50 mM potassium phosphate, pH 4.6, containing 5% methanol (16). The flow rate was 2 ml/min. Nucleotides and nucleosides were monitored at 254 nm and quantitated on the basis of response factors obtained with standard compounds run in the same systems.

Enzymatic Measurements. Adenosine deaminase activity (calf intestine mucosa) was determined in a continuous spectrophotometric assay at 25°C. The test solution (1.2 ml) contained 0.1 mM sodium phosphate, pH 7.5, and varying concentrations of DAPdR, adenosine, or deoxyadenosine (1/3 Kᵦ to 5 Kᵦ). Reactions were initiated by addition of enzyme (0.4 mg/ml). The conversion of DAPdR was measured by the increase of absorbance at 252 nm using an experimentally determined ΔEₘ of 4.65 × 10⁴. Reactions of adenosine and deoxyadenosine were quantitated by the decrease of absorbance at 265 nm using a ΔEₘ of -7.8 × 10⁴.

Crude extract of Ehrlich ascites tumor cells served as a source of different enzymes. The preparation of the extract started with washing the cells twice in 0.0513 M NaCl solution (17). The pellet obtained by centrifuging at 5,000 rpm for 5 min was resuspended in H₂O containing 0.2 mmol of dihydrocorticosterone/100 ml of cells and was homogenized in a mixer for 2 min. After centrifugation at 10,000 rpm for 75 min the supernatant (20 ml) was put over 2-ml columns of Dowex-1X8-400 (acetate form) to bind nucleotides. Aliquots of the eluate were quick-frozen in dry ice/acetone and stored at -90°C. All assays were carried out at 25°C in 12 mM Tris buffer, pH 7.0, and were initiated by addition of crude extract. Test mixtures for adenine phosphoribosyltransferase contained 1.2 mM phosphoribosylpyrophosphate, 0.5 mM DAP, and 6 mM magnesium acetate. Assays for purine nucleoside phosphorylase contained 1.5 mM ribose 1-phosphate, 0.5 mM DAP, and 0.025 mM EHNA. Test solutions for deoxycytidine kinase consisted of 0.6 mM ATP, 0.2 mM DAPdR, 6 mM magnesium acetate, and 0.025 mM EHNA. Reactions were followed up to 5 h by HPLC analysis of heat-deproteinized 0.2-ml aliquots.

Formation of Uridine Nucleotides from [³⁴C]Cytidine. The acid-soluble fractions (150 µl) obtained from the labeling experiments were subjected to snake venom treatment as described above. After heat deproteinization, these samples were brought to pH 3.5 and passed over 1-ml columns of Dowex-50 (H⁺-form). Under this condition, 98.3% of the total cytidine was bound to the resin, while labeled uridine was counted in the eluate.

Other Procedures. For analysis of elevated GTP, the respective fraction from the anion-exchange HPLC system was collected. The eluate was diluted 10-fold with H₂O to lower the concentration of phosphate prior to adjusting the pH to 9.5 and addition of alkaline phosphatase (1000 units/mg specific activity; 10 units/ml) as well as magnesium acetate (1.2 mM). After an incubation at 37°C overnight, the solution was heat deproteinized and analyzed by reverse-phase HPLC.

Fluorescence spectra were obtained using an Amino-Bowman spectrofluorometer (American Instrument Co., Silver Spring, MD).

Protein was determined using the Bradford reagent of Bio-Rad (Richmond, CA).

RESULTS

Inhibitory Effects and Metabolism of 2,6-Diaminopurinedeoxyriboside in L1210 Cells. To identify the target sites of DAPdR, its effects on nucleic acid synthesis and ribonucleotide reductase activity were investigated. Exposure of L1210 cells to DAPdR for 2 h inhibited the incorporation of [³⁴C]cytidine into DNA in a concentration-dependent manner with 50% inhibition occurring at 0.1 mM (Fig. 1). The activity of ribonucleotide reductase as measured by the formation of deoxycytidine nucleotides from [³⁴C]cytidine was inhibited to a similar extent (Fig. 1). However, labeling of RNA, which paralleled the acid-soluble radioactivity was almost unaffected up to 0.5 mM DAPdR.

For analysis of DAPdR effects on the cellular nucleotide profile, L1210 cells were incubated for 2 hr at 0.1 mM DAPdR, extracted with perchloric acid and analyzed by HPLC (System A). The GTP pool was expanded by 150%, and the levels of ATP, UTP, and CTP deviated only slightly from control values. To rule out that the formation of DAPdR 5′-triphosphate was mistaken for a GTP elevation, the GTP peak from the DAPdR-treated cells was isolated, hydrolyzed in the presence of alkaline phosphatase, and rechromatographed in reverse-phase HPLC (Fig. 2). Under these conditions, only guanosine was detectable. This result was in line with the facts that periodate treatment completely destroyed the elevated GTP and that GTP isolated from DAPdR-treated and untreated L1210 cells gave the same fluorescence spectra with an emission maximum at 420 nm when the excitation wavelength was fixed at 296 nm.

The data so far implied a metabolic conversion of DAPdR to GTP involving the intermediate formation of deoxyguanosine.
Biological and Biochemical Effects of 2,6-Diaminopurinedeoxyriboside in Comparison with Deoxyguanosine. For comparison of the antiproliferative effect of DAPdR and deoxyguanosine, L1210 cells were exposed to increasing concentrations of both drugs, and cells were counted in 24-h intervals. The inhibition pattern was comparable for DAPdR and deoxyguanosine. In the presence of 0.25 mM of either drug, cell growth reached almost control values after an initial delay; 0.5 mM suppressed cell growth up to 3 days, and 1.0 mM was required to maintain the cytostatic effect over 4 days (Fig. 3, top and middle).

Nucleic acid synthesis and ribonucleotide reductase activity were analyzed after a 24-h exposure of L1210 cells to DAPdR or deoxyguanosine (Table 1). The labeling of DNA and the deoxycytidine pool was almost completely suppressed at 1.0 mM of the drugs. Inhibitory effects of 0.1 mM of either drug were essentially abolished after 24 h. The formation of radioactive uridine nucleotides from [14C]cytidine during the labeling amounted to less than 4% of the total acid-soluble radioactivity. By use of HPLC (System A), radioactive CTP, CDP, and CMP were completely separated in the same samples. The amount of radioactivity of CTP plus CDP in the control was 78% of the total acid-soluble radioactivity as compared to 89% measured in cells after a 24-h treatment with 0.1 or 1.0 mM of DAPdR or deoxyguanosine. Thus, neither a conversion of cytidine to uridine nor a decreased formation of cytidine nucleotides contributed to the decreased labeling of DNA and the deoxycytidine pool. In an experiment comparing alternative labeling techniques, L1210 cells were exposed to 0.25 mM DAPdR or deoxyguanosine for 24 h and labeled with [14C]uridine, [14C]thymidine, or [14C]cytidine. The degree of inhibition of DNA, measured by the incorporation of [14C]thymidine or [14C]cytidine, and of RNA, measured by the incorporation of [14C]uridine or [14C]cytidine, varied by not more than 9% between the tracers used.

Ribonucleotide patterns were compared in L1210 cells after incubation with DAPdR or deoxyguanosine for 2 and 24 h (Table 2). After the short incubation period, GTP was elevated up to 5-fold as compared to control, while after the extended exposure time GTP levels returned to normal values. DAPdR and deoxyguanosine influenced the ribonucleotide pattern in the same way.

The similar toxicity of DAPdR and deoxyguanosine suggested an accumulation of dGTP in DAPdR-metabolizing cells. In Table 3, the deoxynucleotide patterns as a result of drug treatment are shown. After 24 h, the level of dGTP was still 1000-fold higher than control levels when 1.0 mM DAPdR was used. By that time, the initially elevated dGTP levels under treatment with 0.1 and 0.25 mM DAPdR were returning back to normal values. The increase in dGTP was accompanied by a drastic expansion of the dATP pool. dTTP and dCTP levels were decreased at 0.25 and 1.0 mM DAPdR; however, the concentration of dCTP increased within the first 8 h upon treatment with 0.1 mM DAPdR. The time- and concentration-dependent shifts in the deoxynucleoside triphosphate pattern were very similar in cells treated with DAPdR or deoxyguanosine.

Reversal Experiments. Since deoxycytidine is known to counteract toxic effects of deoxyguanosine (18), it was tested in combination with DAPdR or deoxyguanosine with respect to cell growth inhibition (Fig. 4). The I50 values were increased approximately 2-fold in the presence of deoxycytidine, being 0.34 mM for the combination with DAPdR and 0.39 mM for the one with deoxyguanosine. Higher deoxycytidine concentrations (0.25, 0.3, and 1.0 mM) failed to enhance the rescue effect in this system. Using deoxyadenosine or deoxycytidine in

### Table 1 Inhibition by DAPdR and deoxyguanosine of [14C]cytidine metabolism

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of control*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of control*</td>
</tr>
<tr>
<td>DNA</td>
<td>RNA</td>
</tr>
<tr>
<td>DAPdR</td>
<td></td>
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<tr>
<td>0.1</td>
<td>87.5</td>
</tr>
<tr>
<td>1.0</td>
<td>17.4</td>
</tr>
<tr>
<td>2'-Deoxyguanosine</td>
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</tr>
<tr>
<td>1.0</td>
<td>6.2</td>
</tr>
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</table>

*Control values (cpm/10⁶ cells): 2'-deoxycytidine, 870; DNA, 3,800; RNA, 31,030.

### Table 2 Cellular contents of ribonucleoside triphosphates in drug-treated L1210 cells

<table>
<thead>
<tr>
<th>Exposure time</th>
<th>Treatment (mM)</th>
<th>UTP</th>
<th>CTP</th>
<th>ATP</th>
<th>GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAPdR</td>
<td>0.1</td>
<td>11</td>
<td>79</td>
<td>253</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>83</td>
<td>83</td>
<td>422</td>
</tr>
<tr>
<td>24 h</td>
<td>DAPdR</td>
<td>0.1</td>
<td>99</td>
<td>87</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>104</td>
<td>97</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>2'-Deoxyguanosine</td>
<td>99</td>
<td>87</td>
<td>92</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>91</td>
<td>301</td>
<td>53</td>
</tr>
</tbody>
</table>

*Control values (nmol/10⁶ cells): UTP, 0.90; CTP, 0.36; ATP, 2.81; GTP, 0.58.
stead of deoxycytidine increased the inhibitory effects of DAPdR or deoxyguanosine.

Mycophenolic acid toxicity is due to an inhibition of the de novo GTP synthesis and can be reversed by guanine supplied via the salvage pathway (19). Exposure of L1210 cells to mycophenolic acid inhibited nucleic acid synthesis almost completely, depleted purine nucleoside triphosphates, especially GTP, and increased pyrimidine nucleoside triphosphates (Table 4). Both DAPdR and deoxyguanosine counteracted these effects with similar efficacy; e.g., the incorporation of [14C]cytidine into DNA of L1210 cells which were treated with mycophenolic acid plus either nucleoside, was 12- to 15-fold higher than in cells exposed to mycophenolic acid alone.

Effects of 2,6-Diaminopurinedeoxyriboside in Comparison with 2,6-Diaminopurine. As shown in Fig. 3, DAP is by an order of magnitude (I50 = 0.015 mM) more inhibitory to L1210 cell growth than is DAPdR or deoxyguanosine. Correspondingly, incubation of L1210 cells for 24 h in the presence of 0.05 mM DAP inhibited cell growth by 98%, formation of [14C]deoxycytidine from [14C]cytidine by 81%, and labeling of DNA and RNA with radioactive cytidine by 98 and 92%, respectively. The nucleotide profile of these L1210 cells is shown in Fig. 5B. As shown by HPLC the predominant compound was the 5'-triphosphate of 2,6-diaminopurineriboside (shaded peak). It was completely destroyed by periodate treatment and reached a cellular content of 2.4 nmol/10^6 cells. GTP was essentially unchanged under DAP treatment, while ATP dropped to 33% of control. Under these conditions [14C]cytidine phosphates amounted to 98% of all intracellular radioactivity derived from [14C]cytidine as compared to 80% in control cells. Although a 2-h incubation in the presence of 0.1 mM DAP left the labeling of nucleic acids with [14C]cytidine unchanged in comparison to control values (cpm/10^6 cells): RNA, 7700; DNA, 570; (nmol/10^6 cells): GTP, 0.75; ATP, 3.25; CTP, 0.39; UTP, 0.97.

<table>
<thead>
<tr>
<th>Compound</th>
<th>No addition</th>
<th>DAPdR</th>
<th>2'-deoxyguanosine</th>
</tr>
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<tbody>
<tr>
<td>RNA</td>
<td>14.8</td>
<td>60.1</td>
<td>52.6</td>
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<tr>
<td>DNA</td>
<td>4.2</td>
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<td>48.1</td>
</tr>
<tr>
<td>GTP</td>
<td>26.9</td>
<td>48.7</td>
<td>46.2</td>
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<tr>
<td>ATP</td>
<td>68.1</td>
<td>89.6</td>
<td>89.2</td>
</tr>
<tr>
<td>CTP</td>
<td>145.8</td>
<td>107.5</td>
<td>110.9</td>
</tr>
<tr>
<td>UTP</td>
<td>170.5</td>
<td>120.2</td>
<td>128.1</td>
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</tbody>
</table>

* Control values (cpm/10^6 cells): RNA, 7700; DNA, 570; (nmol/10^6 cells): GTP, 0.75; ATP, 3.25; CTP, 0.39; UTP, 0.97.

Fig. 5. HPLC profiles of drug-treated L1210 cells. After a 24-h exposure of the cells to 0.05 mM DAP (B), 1 mM DAPdR (C), or 1 mM deoxyguanosine (deGuO) (D), perchloric acid extracts were prepared and analyzed by anion-exchange HPLC (System A). The amount of nucleotides in the chromatograms corresponds to 10^6 cells.
with untreated cells, a marked formation of 2,6-diaminopurineriboside 5'-triphosphate (1.2 nmol/10⁶ cells) was already detectable.

Substrate Properties of 2,6-Diaminopurine-deoxygenoside and 2,6-Diaminopurine. In order to define the biochemical basis for the fact that DAPdR is similar to deoxyguanosine and different from DAP regarding metabolic and toxic effects, enzymic studies were performed. In the presence of adenosine deaminase DAPdR was rapidly converted to deoxyguanosine. The substrate efficiency (Vₘₐₓ/Kₘₐₓ) was comparable to that of adenosine or deoxyadenosine (Table 5). Accordingly, DAPdR was completely deaminated to deoxyguanosine in a time-dependent manner during incubation with crude extract from Ehrlich ascites tumor cells (data not shown). EHNA inhibited this reaction by 99%. Phosphorylation of DAPdR, however, was not detectable when the analogue was incubated with ATP/magnesium, crude extract, and EHNA.

The conversions of DAP were determined using the same crude extract. In the purine nucleoside phosphorylase reaction 47 nmol 2,6-diaminopurineriboside/h/mg protein was formed from DAP and ribose 1-phosphate. DAP was also activated in a phosphoribosyl-pyrophosphate-dependent reaction yielding 95 nmol of 2,6-diaminopurineriboside 5'-monophosphate/h/mg protein. The latter was completely dephosphorylated upon incubation with alkaline phosphatase.

Inhibitors of Adenosine Deaminase and 2,6-Diaminopurine-deoxygenoside. Since EHNA completely inhibited deamination of DAPdR in vitro the effect of DAPdR combined with adenosine deaminase inhibitors on LI210 cells was characterized. Exposure of LI210 cells for 2 h to 0.05 mM DAPdR alone or deoxycoformycin prevented cell growth inhibition up to 0.3 mM DAPdR.

DISCUSSION

The data presented provide a mechanistic basis for the mode of action of DAPdR in L1210 cells. Characterization of biological and biochemical properties of DAPdR was achieved by studying the drug alone, in combination with modulating agents like deoxycytidine, or in comparison with related drugs like DAP.

Short-term incubations (2 h) in the presence of DAPdR concentrations up to 0.4 mM had opposing effects on RNA and DNA synthesis (Fig. 1). The increased labeling of RNA by [¹⁴C]cytidine under these conditions is in line with an elevated mRNA synthesis in B-lymphoblasts treated with deoxyguanosine plus 8-aminoguanosine (20). In both instances, exposure of the respective cell type to the drugs is associated with a marked elevation of GTP levels. The incorporation of [¹⁴C]cytidine into DNA was strongly diminished in DAPdR-treated L1210 cells (Fig. 1) and paralleled the reduced activity of ribonucleotide reductase, the enzyme that provides the precursors, dNTPs, for DNA synthesis. After a 24-h exposure, 0.1 mM DAPdR or deoxyguanosine had only slightly inhibitory effects (Table 1). However, ribonucleotide reductase activity and DNA synthesis were still suppressed at 1.0 mM of these drugs. The differential effects of short and extended periods of exposure to the deoxy-nucleosides are probably caused by the uptake and metabolism (Tables 2 and 3) of the drugs entailing their depletion in the medium and, hence, the cessation of their action.

The drastic change in the cellular dGTP content (Table 3) appears to be the decisive event in the toxic mechanism of DAPdR. With dGTP building up in DAPdR-metabolizing cells, ribonucleotide reductase, which is feedback-regulated by dGTP, becomes less active (Fig. 1; Table 1) (7). Inhibition of ribonucleotide reductase leads to decreased DNA synthesis which explains the antiproliferative effect of DAPdR. The data shown in Tables 2 and 3 strongly support the hypothesis of DAPdR acting as a precursor of deoxyguanosine since the drug-induced shifts of ribonucleotides and deoxyribonucleotides were almost identical in cells treated with DAPdR or deoxyguanosine.

Metabolism of DAPdR to its 5'-triphosphate was not detectable in the concentration range of DAPdR used (Figs. 2 and 5; Table 2). Deoxycytidine kinase, however, catalyzes the 5'-phosphorylation of DAPdR in vitro (4), a prerequisite for the occurrence of the 5'-triphosphate. This discrepancy might be due to different properties of the kinase from calf thymus (4) and that from L1210 cells, or it might reflect a rapid deamination of DAPdR 5'-monophosphate to dGMP catalyzed by AMP deaminase. To increase the probability of 5'-phosphorylation of DAPdR, L1210 cells were exposed to 2'-deoxycoformycin as an inhibitor of DAPdR deamination. The decreased antiproliferative effect of DAPdR in this combination (Fig. 6) might reflect the crucial role of dGTP formation from DAPdR for DAPdR toxicity. This result was surprising since DAPdR resembles, e.g., arabinofuranosyladenine, which is totally inactivated in L1210 cells by adenosine deaminase but acts as a strong antitumor agent when combined with deoxycoformycin (21). L1210 cells have high levels of adenosine deaminase (21), which together with the favorable substrate properties of DAPdR for this enzyme (Table 5) provide for an extensive formation of deoxyguanosine from DAPdR. All cell experiments were carried out in medium supplemented with 10%
horse serum which is known to have little, if any, adenosine deaminase activity (22).

The growth inhibitory effect of DAP was achieved with one-tenth of the DAPdR concentration and correlated with the generation of high cellular levels of DAP riboside 5'-triphosphate. The formation of DAP-derived nucleotides is in line with the activation of DAP by adenosine phosphoribosyltransferase and purine nucleoside phosphorylase from a crude extract of Ehrlich ascites tumor cells. Both enzymes of other sources have also been demonstrated to activate DAP (8, 23). As shown in Fig. 5C, L1210 cells treated with 1.0 mm DAPdR produce small amounts of a new compound eluted between ATP and GTP (shaded peak). It is tentatively named 2,6-diaminopurinriboside 5'-triphosphate since it coelutes with the respective compound of DAP-treated cells and is completely destroyed by periodate. This compound was not detectable after a 24-h exposure to 0.1 or 0.25 mm DAPdR. The contribution of this compound to the mode of action of DAPdR appears to be small since the IC₅₀ values of DAPdR and deoxyguanosine were quite similar, deoxycoformycin counteracted cell-growth-inhibiting effects of DAPdR (Fig. 6), and deoxyctydine relieved the antiproliferative effects of both DAPdR and deoxyguanosine. The deoxyctydine rescue (Fig. 4) could be due to replenishment of dCTP pools, which are depleted because of ribonucleotide reductase inhibition, or it might be explained by competition of deoxyctydine with deoxyguanosine for deoxyctydine kinase (24).

In conclusion, the present paper provides evidence that DAPdR acts as precursor of deoxyguanosine in mouse leukemia L1210 cells. Based on our data the following metabolic conversions are suggested to occur. DAPdR is deaminated to deoxyguanosine which is predominantly cleaved to guanine and activated to GMP and further to GTP. Additionally, deoxyguanosine is phosphorylated by deoxyctydine kinase and further converted to dGTP. At high concentrations of DAPdR, a third reaction sequence also takes place: DAPdR is cleaved to a small extent to DAP, the precursor of 2,6-diaminopurinriboside and the respective 5'-phosphates. Direct phosphorylation of DAPdR does not appear to occur.

As an anticancer drug DAPdR could be promising with tumors that possess high adenosine deaminase activity and are located in host tissue with low activity of this enzyme. Under these conditions, DAPdR could exert selective antitumor activity. To increase the growth-inhibiting effects of DAPdR it can be used in combination with other drugs. For example, DAPdR plus pyrazolomidazole and Desferal exert a synergistic anti-neoplastic effect on L1210 cells (3). The rationale for this combination is the inhibition of the non-heme iron subunit of ribonucleotide reductase by pyrazolimidazole plus Desferal and the simultaneous generation of dGTP from DAPdR to block the effector binding subunit of the reductase. The evaluation of DAPdR in combination chemotherapy is in progress.

REFERENCES

2,6-Diaminopurinedeoxyriboside as a Prodrug of Deoxyguanosine in L1210 Cells

Gisbert Weckbecker and Joseph G. Cory


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