Potentiation of Methotrexate Toxicity by Dipyridamole

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ABSTRACT

Dipyridamole, an inhibitor of facilitated transport systems for purines and pyrimidines, was shown to enhance the toxicity of methotrexate (MTX) against cells in culture and in mice. Under certain incubation conditions, the availability of preformed purines and pyrimidines in undialyzed serum appeared to render Chinese hamster ovary cells insensitive to MTX. Addition to the culture of nontoxic levels of dipyridamole conferred sensitivity to MTX. Inhibition of \[^{3}H\]hypoxanthine uptake also paralleled the enhanced MTX toxicity in a comparison of the dose-effect relationships. Inhibition of \[^{3}H\]hypoxanthine uptake also occurred, although approximately 10-fold higher levels of dipyridamole were required. In vivo dipyridamole enhanced MTX toxicity in mice; however, the antitumor activity of MTX toward Ridgway osteogenic sarcoma and L1210 leukemia was not dramatically improved.

INTRODUCTION

MTX, one of the earliest developed anticancer agents, produces a number of biochemical events in treated cells by interfering with folic acid metabolism. Among these, the drug inhibits de novo biosynthesis of purine and pyrimidine nucleotides. Since salvage of preformed purines and pyrimidines represents a means by which MTX toxicity can be "bypassed," it appears logical to assume that some tissues (and tumors) may be refractory to MTX due to their ability to salvage such precursors (4, 6). There is little evidence to support this premise, perhaps due to the fact that potent and specific inhibitors of purine and pyrimidine salvage enzymes are not, in general, yet available. In his Clowes lecture, Weber (18) presented data that dipyridamole [an inhibitor of facilitated transport systems such as that for nucleosides (10, 11)] enhanced the chemotherapeutic effect of acivicin, a glutamine antagonist and inhibitor of nucleotide biosynthesis de novo (19). We have examined the ability of dipyridamole to enhance the effects of MTX in vitro and in vivo. The data suggest that salvage of preformed purines and pyrimidines modulates response to MTX; however, in 2 mouse tumor model systems relatively refractory to MTX (Ridgway osteogenic sarcoma and leukemia L1210), dipyridamole failed to dramatically improve the antitumor activity of MTX. A preliminary report of these data has been presented (7).

MATERIALS AND METHODS

Tissue Culture. The mammalian cell lines used were cultured in McCoy's 5a Media plus 15% fetal calf serum as described previously (3). For viability determinations, 100 cells were allowed to attach to microtiter plates by overnight incubation at 37° in an atmosphere of 100% humidity, 95% air, and 5% CO2. Drugs were then added, and macrocolonies were determined 5 to 10 days later. ECso values were determined from log dose-response curves prepared from at least 2 separate experiments, each performed in triplicate.

The use of \[^{3}H\]thymidine and \[^{3}H\]hypoxanthine by CHO cells was determined in serum-free media after a 1-hr incubation of the attached cells with 1.0 μCi per ml of either precursor. After decanting the incubation media, the cells were extracted with cold perchloric acid. The acid-soluble material was neutralized with potassium hydroxide, and the insoluble potassium perchlorate was removed by centrifugation. The nucleotides were separated using high-pressure liquid chromatography with a Partisil SAX anion-exchange column (Whatman, Inc., Clifton, NJ) as described previously (9). Two-min aliquots of the eluting material were collected directly into 20-ml liquid scintillation vials (Model 2112 Redirac Fraction Collector; LKB Instruments). After addition of 15 ml of Scintiverse (Amerham-Searle Corp., Arlington Heights, IL), the samples were counted in a Beckman Model LS7500 Liquid Scintillation Instrument.

Animal Experiments. Chemotherapy experiments using Ridgway osteogenic sarcoma in male AKR mice and L1210 leukemia in C57BL x DBA/2 Fi male mice (Jackson Laboratories, Bar Harbor, ME) were performed using procedures described previously (8, 13, 16). Animals were observed for a minimum of 35 days after drug administration.

Materials. Dipyridamole (Persantin) was obtained from Sigma Chemical Co., St. Louis, MO. It was solubilized in HCl, and the pH was adjusted to 3 (limit of solubility) with NaOH. In animal experiments, controls were treated with similarly prepared solutions with Tris base substituted for dipyridamole. MTX and DON for injection were supplied by the National Cancer Institute. \[^{2}H\]Hypoxanthine (15 Ci/mmol) was obtained from Moravek Biochemicals (City of Industry, CA) and [methyl-\[^{3}H\]]thymidine (6 Ci/mmol) was a product of Schwarz/Mann (Orangeburg, NY). The CHO wild-type and mutant cell lines were provided by Dr. Priscilla Saunders (12) of this institution. Dr. Jorgen Fogh of the Sloan-Kettering Institute for Cancer Research, Rye, NY, kindly provided the 2 human osteosarcoma cell lines (5).

RESULTS

When CHO cells were cultured in 15% fetal calf serum, viability was not reduced by levels of MTX as high as 10^{-4} M (Table 1). Deficiency of TK (CHO/TK-) or of HGPRTase (CHO/hypoxanthine phosphoribosyltransferase-) in mutant cells conferred sensitivity of the CHO cells to MTX even in the presence of 15% fetal calf serum (i.e., ECso = 3 \times 10^{-4} M for either cell line). Deficiency of the phosphoribosyl transferase (CHO/hypoxanthine phosphoribosyltransferase-) increased the sensitivity to DON, an irreversible glutamine antagonist and an inhibitor of de novo purine biosynthesis (1), approximately 30-fold when compared with the parent or TK- cell lines (Table 1).

Preincubation of fetal calf serum with bovine milk xanthine
Table 1

Effect of MTX or DON on the viability of CHO cells deficient in thymidine kinase or HGPRTase

Wild-type CHO and mutant CHO cells were incubated in the presence or absence of the drugs shown. The concentrations of MTX or of DON which reduced cloning efficiency by 50% are given.

<table>
<thead>
<tr>
<th>Drug</th>
<th>CHO</th>
<th>CHO/TK-</th>
<th>CHO/HPRT-*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>&gt;100</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>DON</td>
<td>0.09</td>
<td>0.3</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*HPRT*, hypoxanthine phosphoribosyltransferase.

Table 2

Effect of treatment of fetal calf serum with xanthine oxidase on the toxicities of MTX or DON

Wild-type CHO cells were incubated in the presence of various concentrations of MTX using 15% fetal calf serum which had or had not been previously treated with xanthine oxidase (bovine milk). The xanthine oxidase treatment reduced the hypoxanthine concentration in the 15% fetal calf serum from 15 μM to less than 0.2 μM as determined by high-pressure liquid chromatography.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Without xanthine oxidase treatment</th>
<th>With xanthine oxidase treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>&gt;100</td>
<td>0.5</td>
</tr>
<tr>
<td>DON</td>
<td>0.09</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 3

Potentiation of MTX or DON toxicity by dipyridamole in CHO cells

CHO cells were incubated in the presence or absence of the drugs shown. The values given are the drug concentrations which reduced the cloning efficiency by 50% when compared with simultaneous controls.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Without dipyridamole</th>
<th>With dipyridamole (10^-6 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>&gt;100</td>
<td>0.1</td>
</tr>
<tr>
<td>DON</td>
<td>0.09</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Table 4

Potentiation of MTX toxicity toward human osteosarcoma cells in the presence of various concentrations of dipyridamole

The human osteosarcoma cells were grown in the presence or absence of various concentrations of dipyridamole and MTX. The concentrations of MTX which reduced cloning efficiencies to 50% of control values are given.

<table>
<thead>
<tr>
<th>Dipyridamole (μM)</th>
<th>MTX EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^-7</td>
<td>&gt;100</td>
</tr>
<tr>
<td>10^-6</td>
<td>0.2</td>
</tr>
<tr>
<td>10^-5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 5

Distribution of radioactivity in CHO cells incubated with [3H]thymidine

CHO cells were incubated for 1 hr in serum-free medium with radioactive thymidine in the presence or absence of dipyridamole as described in "Materials and Methods." The cold, acid-soluble radioactivity in the cells was resolved into its components by anion-exchange high-pressure liquid chromatography (9). The total uptake of [3H]thymidine in the presence of dipyridamole was 11 ± 2% (average value ± range) of control values. The results shown represent the average distribution of the radioactivity as determined in 2 separate experiments, each performed in duplicate. The total uptake of [3H]thymidine was 25,000 cpm per 10^6 cells in controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thymidine</th>
<th>dTMP</th>
<th>dTDP</th>
<th>dTTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.6</td>
<td>4.3</td>
<td>2.1</td>
<td>85</td>
</tr>
<tr>
<td>Dipyridamole (10 μM)</td>
<td>76</td>
<td>1.9</td>
<td>1.6</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 6

Distribution of radioactivity in CHO cells incubated with [3H]hypoxanthine

Incubation of CHO cells for 1 hr in serum-free medium with radioactive hypoxanthine was performed as described in "Materials and Methods." Distribution of radioactivity was determined by high-pressure liquid chromatographic analysis of the cold, acid-soluble extracts of the cells. The results shown are the mean values for 2 separate experiments, each performed in duplicate. The total uptake of radioactivity into the cells in the presence of hypoxanthine was 41 ± 7% (average value ± range) of control values in these experiments. The total uptake of [3H]hypoxanthine was 1000 cpm per 10^6 cells in controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HX</th>
<th>IMP</th>
<th>GDP</th>
<th>GTP</th>
<th>ADP</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23</td>
<td>2.4</td>
<td>1.0</td>
<td>6.1</td>
<td>3.8</td>
<td>36</td>
</tr>
<tr>
<td>Dipyridamole (10 μM)</td>
<td>68</td>
<td>1.4</td>
<td>0.1</td>
<td>2.4</td>
<td>2.2</td>
<td>24</td>
</tr>
</tbody>
</table>

radioactivity was also modified by dipyridamole. With either precursor, the relative amount of unaltered nucleoside or base associated with the cells was increased, whereas the phosphorylated derivatives were decreased. That is, dipyridamole produced apparent inhibition of the conversion of thymidine to dTMP (TK) or of hypoxanthine to IMP (HGPRTase) in intact CHO cells. Dipyridamole at 10^-4 M was without effect on either TK or HGPRTase in broken CHO cell homogenates (data not shown).

Since 10^-3 M dipyridamole reduced the use of both hypoxanthine and thymidine (Tables 5 and 6), experiments were performed to determine which may be more directly related to the potentiation of MTX toxicity toward CHO cells (i.e., Table 2). The dose-effect curves for dipyridamole inhibition of these parameters indicated that reduction of cell viability more closely paralleled the inhibition of thymidine use (Chart 1). That is, the EC50 for inhibition of thymidine use was about 10^-4 M, whereas 50% inhibition of hypoxanthine uptake occurred with about 10^-3 M dipyridamole.

Since dipyridamole potentiated MTX toxicity toward cells grown in tissue culture (Tables 2 and 3), it was of interest to ascertain possible interaction of the 2 agents in intact animals. There was an increase in the toxicity of MTX in mice treated with dipyridamole.
was measured. •, hypoxanthine uptake; D, thymidine uptake; •, cell viability.

The total uptake into acid-soluble material was measured. •, hypoxanthine uptake; D, thymidine uptake; •, cell viability.

Administration of dipyridamole alone at 8 a.m. did not produce deaths or significant body weight loss in 7 treated mice.

Dipyridamole alone was not toxic at 10 mg/kg.

When dipyridamole (10 mg/kg at 8 a.m. and 4 p.m.) was combined with MTX (5 mg/kg at 12 noon) on a 5-day schedule, the growth of Ridgway osteogenic sarcoma was impaired to a greater extent than was observed with either drug alone (Chart 2). There were 2 of 8 apparent drug deaths observed with the drug combination, whereas none of the other animals died during the period of observation. Also, there were no partial remissions in any case (i.e., reduction of tumor mass to less than 50% of the initial mass). The ability of dipyridamole alone to moderately slow the growth of Ridgway osteogenic sarcoma was confirmed in a repeat experiment using doses up to 42 mg/kg on a 9-day schedule (data not shown). Again, however, there were no partial remissions, and the therapeutic effect of a large single dose of MTX (50 mg/kg) was not markedly enhanced by dipyridamole.

Dipyridamole (10 mg/kg, i.p.) also failed to enhance significantly the chemotherapeutic effect of simultaneously administered MTX (5 or 10 mg/kg, i.p.) against murine leukemia L1210 (Table 8). In a repeat experiment not shown, dipyridamole (100 mg/kg, i.p.) was given at 8 a.m., 12 noon, and 4 p.m. alone or in combination with MTX (4 mg/kg, i.p., 8 a.m. only) for 4 days beginning 1 day after inoculation of $10^6$ L1210 cells i.p. This combination regimen approximated the 10% lethal dose and was not significantly different from the effect of MTX (25 mg/kg) alone (approximately 10% lethal dose). Administration of 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine, 5'-monophosphate (10 mg/kg, i.p.; 8 a.m., 12 noon, and 4 p.m.) alone or in combination with MTX failed to increase the toxicity or chemotherapeutic effect of MTX in the L1210 leukemia model (data not shown). These doses of the nucleoside transport inhibitors were shown to maximally reduce thymidine uptake (20 sec) into mouse whole blood cells for approximately 4 hr in vivo in separate experiments (data not shown).

### DISCUSSION

MTX inhibits the de novo biosynthesis of purines and pyrimidines, and these effects appear responsible for its cytotoxic effects. Dipyridamole and MTX Toxicity

#### Table 7

Lethality of MTX and dipyridamole to normal male BD2F, mice

<table>
<thead>
<tr>
<th>MTX + dipyridamole Dose of MTX</th>
<th>MTX alone</th>
<th>Pretreatment</th>
<th>Simultaneous</th>
<th>Posttreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mg/kg</td>
<td>0/7 (3)*</td>
<td>0/7 (6)</td>
<td>0/6 (1)</td>
<td>0/7 (1)</td>
</tr>
<tr>
<td>4 mg/kg</td>
<td>0/7 (3)</td>
<td>1/7 (12)</td>
<td>0/6 (5)</td>
<td>0/7 (6)</td>
</tr>
<tr>
<td>6 mg/kg</td>
<td>0/6 (5)</td>
<td>3/7 (22)</td>
<td>1/6 (12)</td>
<td>3/6 (12)</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>5/5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Numbers in parentheses, maximum animal body weight loss as a percentage of initial weight.

with dipyridamole (10 mg/kg, i.p.) whether the transport inhibitor was given before, at the same time, or after MTX (Table 7). Animal weight loss during these drug treatments also indicated that dipyridamole plus MTX is more toxic than either drug alone. Dipyridamole alone was not toxic at 10 mg/kg.

Male BD2F, mice were given i.p. implants of $10^6$ L1210 cells on Day 0. The animals were then treated for 5 days beginning on Day 17 as follows: •, control; D, dipyridamole, 10 mg/kg i.p. at 8 a.m. and 4 p.m.; •, MTX, 5 mg/kg i.p. at 12 noon; •, combination of dipyridamole and MTX. The results shown are mean values for groups containing 4 to 6 animals; bars, S.E. (where indicated).

#### Table 8

Effects of the combination of MTX and dipyridamole on L1210 leukemia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7 (6-8)*</td>
</tr>
<tr>
<td>Dipyridamole (10 mg/kg)</td>
<td>7 (5-7)</td>
</tr>
<tr>
<td>MTX (5 mg/kg)</td>
<td>11 (10-12)</td>
</tr>
<tr>
<td>MTX (5 mg/kg) + dipyridamole (10 mg/kg)</td>
<td>11 (10-15)</td>
</tr>
<tr>
<td>MTX (10 mg/kg)</td>
<td>11 (10-20)</td>
</tr>
<tr>
<td>MTX (10 mg/kg) + dipyridamole (10 mg/kg)</td>
<td>12 (11-14)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, range.
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action in many cell types. Thus, salvage of preformed purines and pyrimidines is a means by which the toxicity can be circumvented. Under the tissue culture conditions described herein, inability of CHO cell mutants to salvage preformed purines via HGPRTase or thymidine via TK was adequate to confer sensitivity of the cells to MTX (Table 1). Collateral sensitivity to MTX has been demonstrated in L1210 cells resistant to 6-mercaptopurine due to reduced levels of HGPRTase (2, 15). The possibility that hypoxanthine salvage plays a role in protecting the wild-type CHO cells was supported by the observation that depleting the media of hypoxanthine (i.e., xanthine oxidase treatment) also conferred sensitivity to MTX (Table 2). An alternative to using mutants of these salvage enzymes is to use inhibitors; however, potent and specific inhibitors of these mammalian enzymes are not readily available. On the other hand, potent inhibitors of nucleoside transport are available (11). One such inhibitor, dipyridamole, was shown to enhance MTX toxicity toward CHO cells (Table 3) and 2 human osteosarcoma cell lines (Table 4). Inhibition of thymidine uptake by dipyridamole may be related to the potentiation, since the dose-effect relationships are similar (Chart 1). In a related experiment, proliferation of L5178Y cells made dependent upon exogenous thymidine (i.e., MTX-treated) was blocked by the transport inhibitor, 6-[4-nitrobenzylthio]-9-β-D-ribofuranosylpurine (17).

The intracellular distribution of metabolites in CHO cells incubated with hypoxanthine, thymidine, and dipyridamole suggested apparent inhibition by the transport inhibitor of HGPRTase and TK (Tables 5 and 6). Dipyridamole failed to inhibit these enzymes in a broken-cell preparation.4 In the presence of dipyridamole, the intracellular levels of the radioactive substrates (hypoxanthine or thymidine) were unchanged, whereas the phosphorylated metabolites were decreased (Tables 5 and 6). These results suggest that a role for the transport system is to facilitate salvage of preformed purines and pyrimidines by the kinase or HGPRTase. That is, the carriers appear to present the substrates to the enzymes, thereby increasing the efficiency of the phosphorylation or phosphoribosyl transferase reactions (11). Alternatively, these effects of dipyridamole may have occurred due to changes in the endogenous pools of hypoxanthine and thymidine.

Dipyridamole also enhanced the toxicity of MTX toward mice (Table 7). The chemotherapeutic activity of MTX was not improved markedly when the combination was tested against Ridgway osteogenic sarcoma (Chart 2) or L1210 leukemia (Table 8). These results suggest that the refractoriness of these tumors to MTX may be related to factors other than their ability to salvage preformed purines and pyrimidines. The doses and schedules used in these preliminary experiments may not have been optimal, and it is possible that other doses and schedules may provide greater benefit in the treatment of other tumor types which may depend heavily on transport and/or salvage mechanisms.

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REFERENCES


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