Improved Methotrexate Therapy of Murine Tumors Obtained by Probenecid-mediated Pharmacological Modulation at the Level of Membrane Transport

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ABSTRACT

Our earlier studies showed that both carrier-mediated influx and efflux of \(^{3}H\)methotrexate in murine tumor cells was inhibited by probenecid. The concentration of probenecid required for inhibition of influx was 10-fold greater than that required to inhibit efflux. As a consequence, the intracellular exchangeable level of \(^{3}H\)methotrexate at steady state was markedly increased. Since these effects were observed at concentrations of probenecid which were pharmacologically achievable, studies were initiated in mice to evaluate the adjuvant use of this agent during therapy with folate analogs. The simultaneous administration of probenecid (125 mg/kg s.c.) with methotrexate (9 to 18 mg/kg s.c.) on a schedule of every 2 days for five doses to tumor-bearing mice resulted in an increased antitumor effect over that obtained with methotrexate alone. Against the L1210 leukemia, >213% increased life span was obtained compared to 152% increased life span with methotrexate alone. Against the Sarcoma 180 ascites tumor, >179% increased life span was obtained compared to 82% with methotrexate alone. Long-term survivors (10 to 20%) were obtained only with methotrexate plus probenecid. Data on plasma levels of \(^{3}H\)methotrexate following this schedule of administration showed a 2-fold higher initial level in probenecid-treated mice versus mice given \(^{3}H\)methotrexate alone, a cross-over in levels in these mice 2 to 3 hr later, and a 2-fold lower level sustained for at least 24 hr. Initial rate and maximum level of accumulation in probenecid-treated mice were reduced 2-fold in liver, kidney, bone marrow, and small intestine. Otherwise, overall persistence of \(^{3}H\)methotrexate in these tissues was minimally affected. In Sarcoma 180 cells, the initial rate but not the maximum level of \(^{3}H\)methotrexate accumulation was reduced 2-fold by probenecid. However, the overall persistence of \(^{3}H\)methotrexate in these cells was increased 2-fold with probenecid. The effect on plasma and tissue levels of \(^{3}H\)methotrexate was dependent on the dosage of probenecid but did not depend on the dosage of the antifolate itself, at least within the dosage range used. These results show that the effects of probenecid on methotrexate transport, documented for tumor cells in vitro studies, were favorably and selectively expressed in these cells at a pharmacological level and as such may have clinical implications.

INTRODUCTION

In earlier studies (21) from our laboratory, we examined the effect of the organic ion, probenecid, on carrier-mediated transport of methotrexate by murine tumor cells. These studies were prompted by the earlier suggestion (1, 11, 22) that this agent might be useful in inducing blockade of carrier-mediated methotrexate clearance from either blood plasma or cerebrospinal fluid during treatment of human cancers with this antifolate. Prior to such use, however, it seemed important to obtain information on the effects of probenecid on carrier-mediated transport of methotrexate (reviewed in Refs. 4 and 11) in both neoplastic and normal proliferative tissues.

Probenecid inhibits renal tubular transport and biliary excretion of methotrexate in both animals and humans (1, 7–9). Increased toxicity to methotrexate has been associated (7, 8) with the concurrent administration of probenecid. Other data have been reported (10, 22) showing that probenecid inhibits transport of methotrexate from rabbit cerebrospinal fluid. In one of these studies (22), evidence was derived for an inhibition, by probenecid, of transport of this folate analog in vitro in isolated choroid plexus. In our own studies (21), it was found that both carrier-mediated influx and efflux of \(^{3}H\)methotrexate by L1210, Ehrlich, and Sarcoma 180 cells were inhibited by probenecid. However, in each case, the concentration of probenecid required for inhibition of influx (1.35 to 1.8 mM) was markedly greater than that required to inhibit efflux (0.11 to 0.19 mM). As a consequence, the level of intracellular exchangeable \(^{3}H\)methotrexate at steady state was also markedly increased in the presence of probenecid. Since net accumulation of exchangeable \(^{3}H\)methotrexate is a determinant (reviewed in Refs. 4, 5, and 14) of cytotoxicity, it was not unexpected to also find that probenecid potentiated (21) the inhibition by methotrexate of tumor cell growth in culture.

As the above effects on \(^{3}H\)methotrexate transport were observed at concentrations of probenecid which appeared to be pharmacologically achievable, studies were initiated to evaluate the adjuvant use of this agent during therapy with folate analogs against murine ascites tumors. In companion pharmacokinetic studies, we also examined the effect of probenecid on the accumulation and persistence of methotrexate in normal and tumor tissue, as well as on the accumulation and clearance in blood plasma. Our results show that the effects of probenecid on methotrexate transport, documented during in vitro studies (21), were favorably expressed at a pharmacological level. The therapeutic index of methotrexate was improved, at times markedly, by probenecid administration, and the basis of this improvement appears to be explained by the derived pharmacokinetic data.

MATERIALS AND METHODS

Only a brief outline of the experimental methodology will be presented, since a very detailed description has already been given in several previous reports (14) from our laboratory. Ascitic forms of the L1210 leukemia and Sarcoma 180 tumor were maintained (14, 16–
20) by i.p. transplantation of $10^6$ cells in female C57BL/6 × DBA/2 F1 (hereafter called BD2F,) mice (Sprague-Dawley, Madison, Wis.). For pharmacokinetic experiments, tumor cells were harvested from the peritoneal cavity of sacrificed mice after s.c. administration of $[^{3}H]$-methotrexate (usually 3 to 4 days after implanting $10^6$ cells). Cells were washed twice with buffered NaCl solution (0.14 M NaCl-0.01 M potassium phosphate, pH 7.4) and drug was extracted by heat treatment (2, 14, 16–20). The removal of contaminating RBC's prior to washing and heat extraction has also been described (14, 16–20). After tumor cells were harvested, the small intestine was surgically removed and washed in cold buffered NaCl solution, as described previously (14, 16–20) to remove drug from interstitial space and blood vessels. After a blotting to remove excess liquid, sections of tissue were weighed, homogenized, and heat extracted. Liver and kidney were processed in the same manner. Marrow was collected by aspiration from each femur after surgical removal from the animal. Cold ($0^\circ$) buffered NaCl solution was forced through one end of the femur with the aid of a syringe, and the cell suspension was collected from the opposite end. Marrow from 4 to 8 mice were pooled, and RBC's were removed as described previously (20) before heat extraction. Blood was removed through the orbital sinus of anesthesized mice by means of a micropipet. Samples were allowed to clot, and serum was collected following centrifugation. The amount of $[^{3}H]$-methotrexate in replicate samples was determined by scintillation spectrophotometry of radioactivity. The dihydrofolate reductase content in cell extract was determined by a titration inhibition assay with methotrexate (2,13,14,16-20) studies using direct measurements of plasma and tissue content of unlabeled methotrexate.

For all therapy experiments, new cell lines were initiated by BD2F, mice from a bank of frozen cell lines prepared earlier from a line obtained. Estimations of log,$_{10}$ tumor cell kill during therapy were made according to the method of Schabel (12) using correlations between experimentally determined survival times obtained following transplantation of different numbers of tumor cells. Samples of methotrexate and probenecid (4-[[dipropylamino]sulfonyl]benzoic acid) were provided by the Drug Procurement and Synthesis Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. $[^{3},^{5},^{9},^{10}][^{3}H]$Methotrexate was purchased from Moravek Biochemicals, City of Industry, Calif. Radiolabeled purity of this material was determined by chromatographic (15) and spectrophotometric analysis to be $>97\%$ pure.

RESULTS AND DISCUSSION

Preliminary Toxicity Studies. In a series of experiments in which mice were given varying doses of probenecid s.c., the LD$_{50}$ dosage for acute lethality was determined to be $1.6 \pm 0.2$ g/kg. Death from acute intoxication occurred within 30 min of injection of a lethal dose. The rapidity of death and the almost immediate onset of neurological symptoms in treated mice are in agreement with the known central nervous system (10) actions of this agent in the acute lethal dosage range. Neurological symptoms were observed in mice treated with probenecid at dosages above 500 mg/kg. For this reason, dosages of 500 mg/kg or below were used during the in vivo experiments described here.

The effect of probenecid on methotrexate toxicity using a schedule of s.c. administration of every 2 days for 5 doses is shown in Chart 1. The data are expressed in terms of the LD$_{10}$ dosage for methotrexate (15.3 ± 3 mg/kg for methotrexate alone) and without s.c.-administered probenecid. An effect on the toxicity of methotrexate was observed at a dosage of probenecid of 125 mg/kg and above. At 125 mg/kg, probenecid administered simultaneously with methotrexate resulted in a small increase in toxicity (LD$_{10}$ = 13.4 ± 2 mg/kg). However, there was no increase in toxicity when administration of probenecid was delayed for at least 4 hr after methotrexate. Also, prior administration of probenecid (1 to 4 hr before methotrexate) appeared to have little effect on toxicity. At higher doses of simultaneously administered probenecid, toxicity was markedly increased (LD$_{10}$ = 6.7 ± 2 mg/kg with a 250-mg/kg dose of probenecid and LD$_{10}$ = 4.8 ± 1 mg/kg with a 500-mg/kg dose of probenecid, respectively). With a 250-mg/kg dose of probenecid, a delay of 8 hr was necessary before the LD$_{10}$ dosage was at control level (methotrexate alone), whereas with probenecid, 500 mg/kg, a delay of 24 hr appeared to be necessary to avoid an increase in toxicity.

Studies on the Antitumor Activity of Methotrexate with Probenecid. Our initial therapy trials were carried out with the L1210 leukemia. In these studies, dosages and schedules of administration of probenecid were used with methotrexate, which appeared in the studies described previously to be well tolerated. A dosage range for methotrexate of 6 to 18 mg/kg was tested on a schedule of every 2 days for 5 doses with s.c. administration of each agent. The data obtained are given in Chart 2. Probenecid alone had no antitumor effect (data not given) in any of the tumor models used for these studies. Also, simultaneous administration of probenecid, 65 mg/kg, with any dosage of methotrexate did not increase (data not shown) the antitumor effect of methotrexate when given alone. At a dosage of 125 mg/kg when probenecid was given either simultaneously with methotrexate or with a 4-hr delay, there...
was a greater antitumor effect compared to methotrexate alone. The increased antitumor effect was greatest with a schedule of simultaneous administration. Within the range of the LD10 dosage (12 to 18 mg/kg), antitumor effects on this schedule varied from 200 to 209% ILS compared to 152 to 154% ILS with methotrexate alone. The administration of probenecid, 125 mg/kg, 2 hr before methotrexate gave (data not shown) essentially the same result. A similar result was obtained with a schedule using probenecid, 125 mg/kg, simultaneously with methotrexate and again 4 hr later. However, on this schedule, the best result occurred at a 12-mg/kg dose of methotrexate, since appreciable toxicity was observed at 18 mg/kg. No increase in antitumor effect was observed when this same dosage of probenecid was given with an 8-hr delay. With methotrexate alone, the level of antitumor effectiveness of the best schedule depended on the age (12 to 18 mg/kg), antitumor effects on this schedule varied from 200 to 209% ILS compared to 152 to 154% ILS with methotrexate alone. Also, a substantial number (20%) of long-term survivors were observed at the 2 highest dosages. No long-term survivors were obtained with methotrexate alone. Finally, no increased antitumor effect was observed at this probenecid dosage when an 8-hr delay was used.

Additional experiments were carried out to further document the level of antitumor effectiveness of the best schedule devised in the previous experiment, i.e., coadministration of probenecid, 125 mg/kg, with methotrexate, 9 to 18 mg/kg. These results are given in Table 1. In these studies, a similar improvement in the percentage of ILS against the L1210 leukemia was obtained with this schedule. Moreover, some long-term survivors were obtained on this schedule. None were seen with methotrexate alone. When the same schedule of administration of methotrexate with probenecid was used against the Sarcoma 180 ascites tumor, a marked improvement in antitumor effect was observed (Table 1). The ILS obtained with probenecid within the range of 9 to 18 mg/kg was twice that obtained with methotrexate alone. Also, a substantial number (20%) of long-term survivors were observed at the 2 highest dosages. No long-term survivors were obtained with methotrexate alone.

The data derived in these experiments take on greater significance when expressed as log10 tumor cell kill, according to the method of analysis used by Schabet (12). In the case of the L1210 leukemia, the further increase in survival time obtained with probenecid represents an additional 1- to 2-log cell kill at the highest dosage of methotrexate or a total of 5 to 6 logs of cell kill. In the Sarcoma 180 tumor, the difference in log tumor cell kill with and without probenecid is striking. The maximum of 3 log cell kill obtained with methotrexate alone was increased.

![Chart 2. Effect of probenecid on the antitumor effect of methotrexate given to L1210 leukemic mice. Different dosages of methotrexate and probenecid were given s.c. on various schedules of administration. The data obtained from 3 different experiments are expressed as percentage of ILS. S.E. <±12% of the mean. pbcd, probenecid.](chart2.png)

### Table 1

**Effect of methotrexate alone and with simultaneously administered probenecid against the L1210 leukemia and Sarcoma 180 ascites tumor**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Methotrexate (mg/kg)</th>
<th>Probenecid (mg/kg)</th>
<th>No. of mice</th>
<th>MSTb (days)</th>
<th>ILSc (%)</th>
<th>60-day survivors</th>
<th>Terminal wtd (g)</th>
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<tr>
<td>L1210</td>
<td>9</td>
<td>5 x 6</td>
<td>5 x 6</td>
<td>7.2 ± 0.6a</td>
<td>136/30</td>
<td>23.4 ± 0.6b</td>
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<td>12</td>
<td>5 x 6</td>
<td>5 x 6</td>
<td>17.0 ± 0.6</td>
<td>136/30</td>
<td>21.7 ± 1.1</td>
<td>21.2 ± 1.3</td>
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<td>16</td>
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<td>18.1 ± 1.9</td>
<td>152/30</td>
<td>21.6 ± 1.4</td>
<td>21.5 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>125</td>
<td>5 x 6</td>
<td>&gt;21.4 ± 1.2</td>
<td>&gt;198/1</td>
<td>21.9 ± 1.3</td>
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<tr>
<td></td>
<td>12</td>
<td>125</td>
<td>5 x 6</td>
<td>&gt;22.5 ± 1.6</td>
<td>&gt;213/2</td>
<td>20.6 ± 1.1</td>
<td>20.6 ± 1.1</td>
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<td>Sarcoma 180</td>
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<td>5 x 3</td>
<td>5 x 3</td>
<td>11.9 ± 0.8</td>
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<td>24.6 ± 2.1</td>
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<td>5 x 3</td>
<td>&gt;25.2 ± 2.3</td>
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<td>25.5 ± 2.2</td>
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<td>&gt;29.9 ± 2.7</td>
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<td>24.2 ± 1.7</td>
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<tr>
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<td>125</td>
<td>5 x 3</td>
<td>&gt;34.4 ± 3.2</td>
<td>&gt;179/3</td>
<td>22.6 ± 2.8</td>
<td>22.6 ± 2.8</td>
</tr>
</tbody>
</table>

* Number of mice x number of experiments.

b MST, median survival time.

c Does not include long-term survivors.
d Initial weight, 20 ± 0.5 g.

e Median ± S.E.

f Mean ± S.E.
to approximately 6 logs. With both tumors, the incorporation of probenecid with methotrexate on this schedule of administration resulted in a partially curative form of therapy. Although the calculations for log tumor cell kill appear to predict a higher fraction of surviving animals with probenecid than actually obtained, it should be noted that the method of calculation uses values for average survival and does not take into consideration the number of methotrexate-resistant cells present in each tumor cell population.

Pharmacokinetic Studies. The data presented in this report show that coadministration of probenecid with methotrexate enhances the antitumor effect of this antifolate against 2 murine tumor models. However, the relationship of this enhancement to effects of probenecid documented in our prior report (21) on [3H]methotrexate transport in tumor cells in vitro is unestablished. Although no information is available on the effect, if any, of probenecid on pharmacological behavior of methotrexate in either tumor or normal tissues. With these questions in mind, a series of in vivo experiments were carried out to determine if there is indeed any effect of probenecid on the time course for accumulation and persistence of methotrexate in different tissues. Prior studies from our laboratory (14, 16-20) have already documented differences among tissues in these pharmacokinetic parameters, which appear to reflect kinetic differences at the level of membrane transport of methotrexate and correlate with both relative responsiveness among different tumors and selective antitumor action.

In these experiments, [3H]methotrexate, 12 mg/kg, s.c., was administered alone and with probenecid, 125 mg/kg s.c., to mice bearing the Sarcoma 180 ascites tumor. The dosage and schedule of administration selected for these studies were in the optimal range for maximum antitumor effects as shown during therapy experiments. At different times after [3H]methotrexate administration, mice from each group were sacrificed and blood and various tissues were removed for analysis of total tritium content. The results obtained on clearance of methotrexate in blood plasma are shown in Chart 3. The effect of probenecid on the kinetics of clearance were more complex than anticipated in the light of prior studies (7, 8) of this drug combination. Although the initial plasma level of [3H]methotrexate was higher in probenecid-treated animals, a cross-over occurred between 2 and 3 hr after [3H]methotrexate administration; therefore, by 6 hr, levels in probenecid-treated animals were 2-fold lower than in animals not receiving probenecid. This difference was sustained for the 24-hr period during which measurements were made.

Data on intracellular [3H]methotrexate levels with time after administration are shown for liver, kidney, and bone marrow in Chart 4. In each case, the initial rate and maximum level of accumulation of [3H]methotrexate was reduced approximately 2-fold in probenecid-treated animals. In contrast, the persistence of drug over and above the dihydrofolate reductase binding equivalence was only minimally affected; i.e., persistence of [3H]methotrexate was increased only slightly. A similar result was obtained in the case of small intestine (Chart 5), the site of drug-limiting toxicity in this rodent species. Initial uptake and the maximum level of accumulation in this organ were also reduced about 2-fold by probenecid. Again, there was only a minimal effect (increase) in the persistence of the antifolate. The results obtained in Sarcoma 180 cells (also in Chart 4), however, are in sharp contrast to that obtained with all of the other tissues examined. Although the same reduction in initial uptake was observed in probenecid-treated animals, the maximum level of accumulation was essentially unaffected. More important, the persistence of [3H]methotrexate (not bound to dihydrofolate reductase) was maintained for 24 hr in probenecid-treated animals as compared to 10 to 12 hr for control animals (3H]methotrexate alone).

In additional experiments, [3H]methotrexate-probenecid dosage relationships were further examined on a schedule of simultaneous administration. Levels of [3H]methotrexate were measured 4 hr after s.c. administration with and without probenecid in Sarcoma 180 cells, small intestine, and plasma. The data are given in Table 2. It can be seen that the relative effect of probenecid, 125 mg/kg, on tissue levels of [3H]methotrexate was the same for each dose (3 to 48 mg/kg) of [3H]methotrexate examined. Intracellular exchangeable levels of [3H]methotrexate at each dose were increased 2- to 3-fold in Sarcoma 180 cells and elevated only slightly in the small...
The skillful technical assistance of Lydia J. Gourtas and Lynn Kelleher are gratefully acknowledged.

ACKNOWLEDGMENTS

The pharmacokinetic effect seen in Sarcoma 180 cells and probably in L1210 cells following coadministration of probenecid with [3H]methotrexate would appear to be unrelated to probenecid-mediated alterations in the plasma pharmacokinetics for [3H]methotrexate. In the first place, elevated plasma levels of [3H]methotrexate were observed only for the first 2 to 3 hr after coadministration of probenecid with the antifolate. Also, the effect of probenecid on tissue levels of [3H]methotrexate was selective in terms of the magnitude of the effect on the overall persistence of the antifolate observed in different tissues. Only minimal effects on this parameter were seen in normal tissues in comparison to effects seen with Sarcoma 180 cells. Although data have not been obtained on the effects of probenecid on carrier-mediated cellular membrane transport in these normal tissues, it seems probable that the interaction between these 2 agents demonstrated for murine tumor cells is different in magnitude in these other tissues.

The complexity of the effects mediated by probenecid on the plasma pharmacokinetics of [3H]methotrexate are only partially explained by the results. The higher levels of [3H]methotrexate seen initially in the plasma of probenecid-treated mice would seem to reflect the much lower rate of accumulation observed in all of the tissues examined. The reasons for the more rapid decrease in plasma level seen during later times in probenecid-treated mice which results in a sustained 2-fold reduction in [3H]methotrexate levels are less apparent. Inhibition of resorption in kidney and/or small intestine may be one possible explanation for this effect. It is interesting to note that in earlier studies (1) in monkeys, simultaneous doses of probenecid and methotrexate given i.v. by bolus injection gave a similar result. Plasma levels of methotrexate in probenecid-treated animals were approximately 2-fold higher initially and by 4 hr the level approached that seen in the control.

When interpreted in the light of the documented (21) effects of probenecid on methotrexate transport, the results of the current studies also provide additional support to conclusions derived from our earlier studies (14, 16-20), namely, that the pharmacokinetics for methotrexate observed in different tissues are primarily an expression of membrane transport properties of those tissues.

ACKNOWLEDGMENTS

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