Co-administration of Probenecid, an Inhibitor of a cMOAT/MRP-like Plasma Membrane ATPase, Greatly Enhanced the Efficacy of a New 10-Deazaaminopterin against Human Solid Tumors in Vivo


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INTRODUCTION

The internalization of folate analogues by tumor cells is mediated (reviewed in Refs. 1 and 2) by the one-carbon, reduced folate transporter. This transporter has recently been shown (3–8) to be encoded by the RFC-1 gene. Although this mobile carrier is capable of mediating (1, 2) bidirectional flux of these analogues, their net intracellular accumulation in tumor cells is limited (9–13) by their extrusion via one or more outwardly directed plasma membrane ATPases, most likely a member or members (14–27) of the cMOAT3/MRP family. The properties of ATPase-mediated efflux of folate analogues in L1210 cells have been described (13) in some detail in work from our laboratory. However, the exact identity of this ATPase within this family of ABC cassette transporters has yet to be revealed.

Earlier studies from our laboratory have also shown (12, 13, 28) that the ATPase in tumors cells, retrospectively found to be responsible for the outward extrusion of MTX through the plasma membrane, is inhibited by a number of structurally different pharmacological agents. One of these, the uricosuric agent PBCD, was shown to significantly increase net intracellular accumulation of MTX in tumor cells when added along with the folate analogue to the culture medium. Although PBCD will also inhibit (12, 28) internalization of MTX by the one-carbon, reduced folate transporter, it is a markedly better inhibitor of the plasma membrane ATPase operative in these cells that extrudes this folate analogue. As a consequence of this differential in inhibition, the cytotoxicity of MTX was increased...
severalfold by the addition of PBCD to the culture medium. Other earlier (29) studies extended these observations to in vivo systems, wherein the co-administration of PBCD with MTX was shown to significantly enhance the efficacy of this folate analogue against L1210 and Sarcoma 180 ascites tumors.

The current studies further extend these earlier findings in the following manner. Although our earlier (29) studies provided proof-of-principle for the notion that improved efficacy of folate analogues can be obtained by co-administration of PBCD, these studies were carried out with murine ascites tumor models. Growth of these tumors i.p. as ascites cell suspensions in mice may have been uniquely amenable to this type of pharmacological modulation. For this reason, the current studies focused on a variety of murine and human solid tumor models in mice, all of which express several members of the cMOAT/MPR family of ATPases. Also, these studies were carried out using the newest clinical candidate (PDX) among a group of 10-deazaaminopterin analogues with antitumor efficacy in these model systems substantially greater (30) than that produced by MTX or any other analogue in the group, including edatrexate (10-ethyl-10-deazaaminopterin). We now report on these studies, which used in vivo models of murine and human lung and human breast and prostate tumors.

MATERIALS AND METHODS

A549, TSU-PR1, and PC-3 tumor cells were maintained in culture in RPMI supplemented with fetal bovine serum. The methodology used for maintaining these cells in culture and for examining the effect of PBCD on the cytotoxicity of MTX and PDX during pulse exposure to these agents has been provided in our earlier reports (28, 30–32). The tumors used during the in vivo studies were obtained from the National Cancer Institute Developmental Therapeutics Program (human MX-1 mammary carcinoma and LX-1 lung tumor), the American Type Culture Collection (human A549 NSCL, TSU-PR1, and PC-3 prostate tumors), and the Southern Research Institute (Lewis lung carcinoma). The Lewis lung tumor and the human tumors were maintained by s.c. transplantation in BD2F1 and athymic NCR-nu mice, respectively. After tumor growth, a cell suspension in RPMI was used during the experiments. Although our earlier (29) studies provided evidence of this folate analogue have also been described (31, 36). A similar methodology was used for determining the plasma concentration of PBCD. Specific experimental details for both in vitro and in vivo experiments are provided in the legends of the appropriate figures.

The relative level of expression of the different MRP genes was determined by semiquantitative RT-PCR. Total cellular RNA was prepared from frozen tumors by the TRizol reagent (Life Technologies, Inc., Gaithersburg, MD). Ten µg of RNA were reverse transcribed in a 20-µl reaction using oligo-dT primers and Superscript II RT according to the manufacturer’s instructions (Life Technologies, Inc.). PCR was done using 2 µl of cDNA in a 100-µl reaction containing 2.5 units of Taq polymerase, 10 µl of 10× PCR buffer without MgCl2, 0.2 mM each dNTP, 1.5 mM MgCl2, and 0.5 µM of each primer (Life Technologies, Inc.). The following primers were used. MRP-1: sense, 5′-GAC TTC ACC AAG TGC TTT CAT AAC-3′, antisense, 5′-GTA GAA GTC GCC CTG CCA GTC T-3′; MRP-2: sense, 5′-CAT CTG CCA TTC GAC ATG ACT GC-3′, antisense, 5′-CAC ATT CCG AGT TTT CAT CAA GGA GT-3′; MRP-3: sense, 5′-CCA AGG CAG AGG GTG AGA TCT C-3′, antisense, 5′-GCT TGA TGC GCG AGT CCT TCA-3′; MRP-4: sense, 5′-GAA GAC CCG CTC ACA AGC TG-3′, antisense, 5′-CTG ACA CCC TCT CAA TGG CTG A-3′; MRP-5: sense, 5′-AAG TGT GAG GGA GAG AAC CAG C-3′, antisense, 5′-CTG GCG CCA TTT TTT CTT GAA CCT TCT G-3′; MRP-6: sense, 5′-GGT GTC GAA GTC TAC ATG TCC TC-3′, antisense, 5′-GAG GAA GAG TGC AGT GAG GCA G-3′; MRP-7: sense, 5′-GGC CAC CTA CAG GGT TGA GGA G-3′, antisense, 5′-GAG AAC TCT GCA GGA GGT TGA TT-3′. (GenBank accession numbers were as follows: MRP-1, NM_004996; MRP-2, NM_000392; MRP-3, NM_003786; MRP-4, NM_005845; MRP-5, NM_005688; MRP-6, NM_001171; MRP-7, U66684).
extension was carried out at 72°C for 10 min. In a parallel reaction, β-actin was used as a standard (sense primer, 5'-CAT GGG TCA GAA GGA TTC CTA TG-3'; antisense primer, 5'-GTT GAA GGT CTC AAA CAT GAT CTG-3'). The linear range of the reaction was determined in experiments using different cDNA concentrations and cycle numbers. To determine any contamination by genomic DNA, the cDNA reaction mixture without reverse transcriptase was included in the PCR (data not shown).

RESULTS AND DISCUSSION

Preliminary Considerations. Other earlier studies from our laboratory have shown (35–37) that most analogues among a wide variety of pteridinyl, quinazolinyl, and pyridopyrimidinyl folate analogues are similarly effective as permeants for extrusion by a cMOAT/MRP-like ATPase in tumor cells. Among the 4-amino folate analogues, the 10-deazaaminopterins were equivalent (36–38) to aminopterin and MTX as permeants. Among the 5-dinyl folate analogues are similarly effective as permeants for extrusion by a cMOAT/MRP-like ATPase in tumor cells. Although some inhibition of internalization of PDX was also observed (Fig. 1), these results reflect a substantially greater inhibitory effect of PBCD on the extrusion of this and other folate analogues (12, 28) compared to its effect on mediated influx. Overall, these results are consistent with the results of the cytotoxicity studies given in Table 1.

In related in vivo experiments, we examined the pharmacokinetics of PDX in plasma and in the PC-3 tumor after the administration of 60 mg/kg of the antifolate with and without 125 mg/kg PBCD. This dose of PBCD was shown (Ref. 29 and below) to be the maximum dose of PBCD that could be given without toxicity to mice with an MTD of PDX on a schedule of once every 3–4 days for a total of 4 doses. Also, after the administration of this dose, a maximum plasma concentration of >0.5 mM of PBCD was achieved (data not shown) within the first 10 min. The data in Fig. 2 show that co-administration of PBCD resulted in a somewhat lower rate of plasma clearance of PDX within the first 2–3 h, but within the next 5–6 h, the rate of clearance was increased over that found in mice receiving PDX alone. These results are virtually the same as that obtained with MTX with and without PBCD in our earlier (29) studies. Related data on the pharmacokinetics of PDX in the PC-3 tumor are given in Fig. 3. These data show that the persistence of freely exchangeable PDX (both parent and polyglutamated forms) in intracellular water of tumor was substantially greater in mice treated with PDX and PBCD than those treated with PDX alone. In this case, the 2-fold greater level of intracellular PDX seen initially in the PBCD-treated mice was increased to greater than 5-fold within 3–7 h and remained, albeit at a somewhat lower level, elevated compared to mice treated with PDX alone for at least 24 h. These data and the related data on the cellular pharmacokinetics of PDX with and without PBCD (Fig. 1) clearly show that the enhancement in net accumulation of PDX observed in this tumor in the PBCD-treated animals occurred as a result of its effects at the cellular level. It is reasonable to assume that similar results would be obtained with other tumors used in the current studies.

Relative Expression Levels of cMOAT/MRP Genes among the Test Tumors. Because cMOAT/MRP ATPase are likely targets of PBCD in tumor, it was of interest to determine the extent, if any, to which identified cMOAT/MRP genes (14–27) were actually expressed in the tumors to be used for co-administration of PBCD results from a favorable pharmacokinetics effect in the target tumors that is achieved by inhibition of a resident cMOAT/MRP ATPase. This appears to be a reasonable assumption because the 10-deazaaminopterins, like all pteridine folate analogues (38), including MTX, are effective permeants for ATPase-mediated efflux from tumor cells. To provide direct support for this assumption in the context of the present studies, we carried out the following experiments. In Fig. 1, we show the effect of PBCD on the net accumulation of PDX at steady-state in AS49, PC-3, and TSU-PR1 cells. These cells lines were chosen for these experiments because they are among the tumors used in the current studies that will grow in culture. The time course data in Fig. 1 document a potent concentration-dependent effect of PBCD in increasing net accumulation of PDX in each case. These results with these solid human lung and prostate tumors are similar to that which we obtained (28) earlier using MTX and PBCD in murine tumor cells. Because cMOAT/MRP ATPase are like all pteridine folate analogues (38), including MTX, are effective permeants for ATPase-mediated efflux from tumor cells. To provide direct support for this assumption in the context of the present studies, we carried out the following experiments. In Fig. 1, we show the effect of PBCD on the net accumulation of PDX at steady-state in AS49, PC-3, and TSU-PR1 cells. These cells lines were chosen for these experiments because they are among the tumors used in the current studies that will grow in culture. The time course data in Fig. 1 document a potent concentration-dependent effect of PBCD in increasing net accumulation of PDX in each case. These results with these solid human lung and prostate tumors are similar to that which we obtained (28) earlier using MTX and PBCD in murine tumor cells. Although some inhibition of internalization of PDX was also observed (Fig. 1), these results reflect a substantially greater inhibitory effect of PBCD on the extrusion of this and other folate analogues (12, 28) compared to its effect on mediated influx. Overall, these results are consistent with the results of the cytotoxicity studies given in Table 1.

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Table 1 The effect of PBCD on the cytotoxicity of PDX in cell culture

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Growth inhibition (IC50, M, ±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS49</td>
<td>MTX: 6.1 ± 1; EDX: 0.75 ± 0.13; PDX: 0.24 ± 0.03; PDX + PBCD: 0.028 ± 0.004</td>
</tr>
<tr>
<td>TSU-PR1</td>
<td>13.2 ± 2; 4.1 ± 0.6; 0.75 ± 0.09; 0.093 ± 0.02</td>
</tr>
<tr>
<td>PC-3</td>
<td>9.2 ± 1; 1.4 ± 0.2; 0.48 ± 0.02; 0.065 ± 0.01</td>
</tr>
</tbody>
</table>

Growth inhibition (IC50; M, ±SE)
in our *in vivo* studies. Determinations of relative expression levels for a total of seven genes in this family were carried out by semiquantitative RT-PCR. These included MRP-1, -2 (cMOAT), -3 (MOAT-D or cMOAT-2), -4 (MOAT-B), -5 (MOAT-C or SMRP), -6 (MOAT-E), and -7. The sources of total RNA for these PCRs were human lung (LX-1 and A549), prostate (PC-3 and TSU-PR1), and breast (MX-1) tumors xenografted to nude mice. The results of these studies are given in Fig. 4 and show a markedly different pattern of relative expression for these genes among the tumors examined. Only MRP-1, -4, and -7 were expressed at detectable levels in all five tumors. Among these three genes, MRP-4 was consistently expressed at the highest level. In addition, MRP-2 was highly expressed in both lung tumors (LX-1 = A549) and to a lesser extent in the prostate tumors (PC-3 > TSU-PR1). There was some expression of MRP-3 in A549 and to a lesser extent in TSU-PR1.

Some expression of MRP-5 occurred in PC-3, and MRP-5 was expressed to a lesser extent in A549 and TSU-PR1. Finally, low levels of expression of MRP-6 were seen in A549 and TSU-PR1.

**In Vivo Antitumor Studies.** Experiments were first carried out in non-tumor-bearing mice to determine the maximum dose of PBCD that could be given i.p. with PDX at its MTD on the schedule of administration that was to be used in these studies, once every 3–4 days for a total of 4 doses. The data showed (Fig. 5) that at doses of PBCD of 125 mg/kg or below, there was no increase in toxicity in either BD2F1 or NCR-nu mice. In addition, the tumors were excised, blotted to remove excess moisture, and weighed. The tumors were disrupted and homogenized with cold (0–4°C) 0.14 M NaCl plus 0.01 M potassium phosphate (pH 7) to allow for removal of PDX from the extracellular space. The cells were washed once with the same solution by centrifugation and resuspension. The tumor cells were resuspended to a final volume of 2 ml and heated in a boiling water bath in a sealed tube for 10 min. After being cooled to ice bath temperature, the solids were centrifuged at 10,000 × g, and the supernatant was collected and recentrifuged at 12,000 × g in a microcentrifuge. The clarified supernatant was analyzed for PDX content by high performance liquid chromatography (20).

**Fig. 1** Time courses for the net intracellular accumulation of PDX at steady state in the presence and absence of PBCD in A549, PC-3, and TSU-PR1 cells. Cells were incubated with 2 µM PDX in transport buffer (pH 7.5) at 37°C with and without 0.1 or 0.5 mM PBCD. Aliquots of cells were removed for processing at the times indicated. Additional experimental details are given in the text. The data are an average of three experiments, with a SE of <14%.

**Fig. 2** Plasma pharmacokinetics for PDX in mice receiving PDX with or without PBCD. Athymic mice bearing the PC-3 tumor were given 60 mg/kg PDX i.p. with or without 125 mg/kg PBCD, and blood plasma was collected at varying times thereafter. The data shown are from a representative experiment using two mice per data point.

**Fig. 3** Pharmacokinetics for PDX in the PC-3 tumor implanted in athymic nude mice. Tumor-bearing mice were given 60 mg/kg PDX i.p. with or without 125 mg/kg of PBCD i.p., and after collection of plasma (see legend of Fig. 2), the tumors were excised, blotted to remove excess moisture, and weighed. The tumors were disrupted and held for 15 min in cold (0–4°C) 0.14 M NaCl plus 0.01 M potassium phosphate (pH 7) to allow for removal of PDX from the extracellular space. The cells were washed once with the same solution by centrifugation and resuspension. The tumor cells were resuspended to a final volume of 2 ml and heated in a boiling water bath in a sealed tube for 10 min. After being cooled to ice bath temperature, the solids were centrifuged at 10,000 × g, and the supernatant was collected and recentrifuged at 12,000 × g in a microcentrifuge. The clarified supernatant was analyzed for PDX content by high performance liquid chromatography (20).
mice compared to that seen with PDX alone. Higher doses of PBCD with PDX resulted in a significant increase ($P < 0.01$) in toxic deaths. These results were similar to that obtained earlier (29) with MTX, when PDX was given with and without PBCD to BD2F1 mice on a different schedule (once every 2 days for a total of 5 doses) of administration.

Using the dosages and schedule of administration found above to be well tolerated, a large series of experiments was carried out in mice bearing either a murine tumor or one of five human tumors. In the case of the murine Lewis lung tumor, experiments were carried out in BD2F1 mice comparing MTX and PDX given i.p. either alone or with PBCD i.p. at the MTD for each agent or the combination. The data showed (Table 2) that PBCD was essentially without effect on the growth of this relatively chemoresistant tumor. They also show that whereas the effect of MTX was minimal on tumor growth, PDX was substantially more active, inhibiting tumor growth by more than 90%. Despite this extreme difference in relative antitumor properties of these folate analogues, co-administration of PBCD with these analogues appreciably improved their efficacy against this tumor. MTX with PBCD was 5-fold more effective than MTX alone ($P < 0.01$) in inhibiting tumor growth, and PDX with PBCD was at least severalfold more effective than PDX alone ($P < 0.005$), causing major regression of the tumor; 4 of 12 tumor-free mice were observed only with the combination.

Extremely favorable results were also obtained (Table 3) with PDX and PBCD compared to PDX alone when given i.p. during the treatment of human lung, prostate, and mammary tumors in NCR-nu mice. In these experiments, PBCD alone was without effect (data not shown). Although the relative efficacy of PDX against these tumors varied substantially, co-administration of PBCD with this new folate analogue consistently increased its efficacy to a significant extent. Treatment of mice bearing the LX-1 tumor resulted in more regression ($P = 0.05$) and a 3-fold increase ($P < 0.01$) in the number of complete regressions compared to that seen with PDX alone. Also, 4 of 12 of these animals showed no regrowth of tumor. A similar increase in efficacy was obtained (Table 3) against the A549 NSCL tumor. Although less responsive to PDX than the LX-1 tumor, co-administration of PBCD with this folate analogue resulted in some tumor regression, whereas PDX alone was only growth inhibitory. Data obtained with the human prostate tumors (Table 3) were also consistent with these results. Whereas PDX alone brought about some regression of the PC-3 and TSU-PR1 tumors, PDX with PBCD resulted in substantially more regression and tumor free-mice. A striking result was obtained with the MX-1 mammary carcinoma. This tumor was extremely responsive to PDX, which induced major tumor regression and a substantial number of tumor-free mice. However,
Table 2  Treatment of the Lewis lung tumor with folate analogues with or without simultaneous PBCD
Treatment initiated i.p. 4 days postimplantation using 1 dose given every 3–4 days for a total of 4 doses. Additional details were provided in the text. Shown are the results of three experiments with four mice per group done on separate days.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MTD (mg/kg)</th>
<th>Average weight change(^a) (%)</th>
<th>Average tumor diameter(^b) (mm, ±SE)</th>
<th>Change in average tumor mass (mg)</th>
<th>No. of tumor-free mice/total no. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>+1</td>
<td>16.2 ± 0.8</td>
<td>+2232</td>
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<tr>
<td>PBCD</td>
<td>125</td>
<td>+2</td>
<td>16.4 ± 0.9</td>
<td>+2315</td>
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<tr>
<td>MTX</td>
<td>35</td>
<td>−4</td>
<td>14.8 ± 1.1</td>
<td>+1719</td>
<td>0/12</td>
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<tr>
<td>PDX</td>
<td>60</td>
<td>−3</td>
<td>7.9 ± 1.4</td>
<td>207</td>
<td>0/12</td>
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<tr>
<td>MTX + PBCD</td>
<td>35 + 125</td>
<td>−5</td>
<td>8.4 ± 1.2</td>
<td>329</td>
<td>0/12</td>
</tr>
<tr>
<td>PDX + PBCD</td>
<td>60 + 125</td>
<td>−3</td>
<td>2.4 ± 1.3</td>
<td>−32</td>
<td>4/12</td>
</tr>
</tbody>
</table>

\(^a\) Initial weight, 26 ± 1 g.
\(^b\) Initial tumor volume, 74 mg (5.2 ± 0.3 mm).

Table 3  Treatment of human lung, prostate, and mammary tumors with PDX with or without simultaneous PBCD
Treatment was initiated on a schedule of one dose every 3–4 days for a total of 4 doses i.p. 4 days posttransplantation. Tumor size measurement 2–4 days posttreatment. Shown are the results of two or three experiments with three or four mice per group. See text for additional details.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Prescription compound</th>
<th>Prescription dose (mg/kg)</th>
<th>Average tumor diameter (mm, ±SE)</th>
<th>Change in average tumor mass (mg)</th>
<th>No. of tumor-free mice/total no. of mice</th>
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<td>LX-1</td>
<td>Control</td>
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<td>17.8 ± 4</td>
<td>+2952</td>
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<tr>
<td></td>
<td>PDX</td>
<td>60</td>
<td>2.6 ± 1</td>
<td>−39</td>
<td>2/12</td>
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<td></td>
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<td>&lt;1</td>
<td>−88</td>
<td>8/12(^a)</td>
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<td></td>
<td>A549 Control</td>
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<tr>
<td></td>
<td>A549 PDX</td>
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<td>5.6 ± 1</td>
<td>+46</td>
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<td></td>
<td>A549 PDX + PBCD</td>
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<td>2.4 ± 1</td>
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<td></td>
<td>PC-3 Control</td>
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<td>PC-3 PDX</td>
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<td>PC-3 PDX + PBCD</td>
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<td>TSU-PR1 Control</td>
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<td>14.3 ± 2</td>
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<td>TSU-PR1 PDX</td>
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<td>4.8 ± 1</td>
<td>−25</td>
<td>0/12</td>
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<tr>
<td></td>
<td>TSU-PR1 PDX + PBCD</td>
<td>60 + 125</td>
<td>3.9 ± 1</td>
<td>−57</td>
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<tr>
<td></td>
<td>MX-1 Control</td>
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<td>11.2 ± 2</td>
<td>+690</td>
<td>0/12</td>
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<td></td>
<td>MX-1 PDX</td>
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<td>2.8 ± 0.5</td>
<td>−38</td>
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<td></td>
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<td>60 + 125</td>
<td>&lt;1</td>
<td>−81</td>
<td>9/12(^a)</td>
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\(^a\) 4/9 (LX-1) and 5/12 (MX-1) mice exhibited no regrowth of tumor in 28 days.

The results of these experiments substantially extend our prior (29) findings with MTX and PBCD. They show that the co-administration of PBCD will also improve the efficacy of a classical folate analogue with inherently greater antitumor potency (30) than MTX. They also show that established, s.c. implanted, solid tumors are equally responsive to this form of therapy. Moreover, in 5 of 12 of the PDX plus PBCD-treated mice, there was no regrowth of the tumor.

The current studies extend our earlier (29) work, which was the first known example in animal model systems of the successful pharmacological modulation of a cytotoxic agent as an approach to the improvement of therapy of neoplastic disease. It emerged from basic studies conducted in this laboratory (9, 10) and subsequently elsewhere (11) that identified a role for an ATP-dependent efflux process in limiting net intracellular accumulation of folate analogues in tumor cells. Subsequent (13) studies from our laboratory identified this process as mediated by an outwardly directed ATPase and further confirmed the potential merit of seeking agents that inhibit this process in tumor cells. To this end, the studies described here are mechanism-based and would appear to provide a sound rationale for clinical trials testing this therapeutic modality in patients.

The role of specific members of the eMOAT/MRP family of ATPases in mediating outward transport of folate analogues...
from tumor cells is controversial. Of the seven family members of this ABC cassette of transporters identified (14–27), overexpression of MRP-1, -2, -3, and -4 after transfection has been associated (21–23, 27) with enhanced efflux and acquired resistance of tumor cells to MTX. However, the identity of the resident ATPase(s) that extrude MTX or other folate analogues in untransfected, drug-naive tumor cells has yet to be directly documented. Based upon the overlapping specificity of these ATPases reported (14–27), it is likely that all identified members of this family, once overexpressed, are able to mediate efflux of folate analogues. This notion would appear to be consistent with the findings of our own studies, described herein, which documented consistent expression of some of these ABC cassette family members but not others among five human tumors. It seems probable that the effects of PBCD on the efficacy of PDX documented in our studies relates to the action of this agent (17, 23) on one or more of these cMOAT/MRP ATPases. Based upon our own analysis of their gene expression (Fig. 4) in the test tumors, the ATPase encoded by MRP-4 would be a likely target with MRP-1 and -7 ATPases as additional probable targets. However, further work on molecular and cellular pharmacokinetic aspects will be required to shed additional light on this question.

REFERENCES


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