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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ABSTRACT
Earlier studies from this laboratory have shown that the uricosuric agent probenecid (PBCD) will inhibit the extrusion of folate analogues from tumor cells mediated by a plasma membrane ATPase resembling the canicular multispecific organic anion transporter/multidrug resistance-related protein (MRP) family of ATP binding cassette transporters. This inhibition of this outwardly directed membrane ATPase has been shown to have a favorable impact upon the cellular pharmacokinetics, cytotoxicity, and efficacy of methotrexate in vivo. In an extension of these earlier studies, which had focused only on murine ascites tumors, we now report that parental co-administration of PBCD will also enhance net intracellular accumulation in vitro and intracellular persistence in vivo of a new folate analogue, 10-propargyl-10-deazaaminopterin (PDX) in tumor cells. This resulted in marked enhancement of the efficacy of PDX against murine and human lung neoplasms and human prostate and mammary neoplasms growing as solid tumors in mice. As possible ATPases targeted by PBCD, all of these tumors expressed MRP-1, -4, and -7 genes, with expression of MRP-4 being greatest in each case. Four other MRP genes were expressed to a variable extent in some tumors but not others. The therapeutic enhancement of PDX by PBCD was manifested as tumor regression, where PDX alone was only growth inhibitory (A549 NSCL tumor), or as a substantial increase (3–4-fold) in overall regression and/or number of complete regressions (Lewis and LX-1 lung, PC-3 and TSU-PRI prostate, and MX-1 mammary tumors) compared to PDX alone. Also, only in the case of PDX with PBCD, a significant number of mice transplanted with LX-1 or MX-1 tumors that experienced complete regression did not have regrowth of their tumor. In view of these results, clinical trials of this therapeutic modality appear to be warranted, especially in the case of new more efficacious folate analogues that are also permissive for this canicular multispecific organic anion transporter/MRP-like plasma membrane ATPase.

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.

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3 The abbreviations used are: ABC, ATP binding cassette; cMOAT, canicular multispecific organic anion transporter; PBCD, probenecid; MTD, maximum tolerated dose; MTX, methotrexate; EDX, 10-ethyl-10-deazaaminopterin cedatrexate; PDX, 10-propargyl-10-deazaaminopterin; MRP, multidrug resistance-related protein; NSCL, non-small cell lung.
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/MRP 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 complete medium with 10% FCS. For A549 and the prostate tumors, an equal volume of Matrigel (Becton Dickinson, Franklin Lakes, NJ) was used to suspend the cell pellet. Aliquots of tumor cell suspension were implanted s.c. at the suprascapular region in nude mice. Working solutions of PBCD, MTX, and PDX are provided in earlier reports (29, 30) of similar studies from this laboratory. Working solutions of PBCD, MTX, and PDX were prepared in PBS (0.14 m NaCl plus 0.01 m sodium phosphate, pH 7) by adjusting to pH 7 with 1 N NaOH. These solutions were held frozen at −20°C for no longer than 2 weeks. These studies were performed in accordance with the NIH Principles of Laboratory Animal Care (34).

PDX was synthesized at the Memorial Sloan-Kettering Cancer Center. Its purity was established (35) as greater than 97% by high performance liquid chromatography. PBCD was purchased from Sigma Chemical Co. BD2F1 and NCR-nu (AT) mice were purchased from Sprague Dawley (Madison, WI). PDX was formulated as a sodium salt in 0.9% NaCl and 0.9% benzyl alcohol (pH 7). PBCD was formulated in distilled H2O as a sodium salt (pH 7.8).

Methodologies used for carrying out experiments measuring the effect of PBCD on the cellular pharmacokinetics of PDX in vitro and the pharmacokinetics of PDX in tumor and plasma in vivo have been described in considerable detail in an earlier (32) report. The analysis of tissue and plasma content of PDX and its polyglutamates with time after the administration 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 µl of each primer (Life Technologies, Inc.). The following primers were used. MRP-1: sense, 5'-GAC TTC ACC AAG TGC TTT CAG AAC-3'; antisense, 5'-GTA GAA GTA 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 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 AT-3'; MRP-4: sense, 5'-GAA GAC CCG CTC ACA GCA CCT TG-3', antisense, 5'-CTG ACA CCC TCT CCT CAA TGG CTG A-3'; MRP-5: sense, 5'-AAG TGT AGG GGA AAC CAG C-3', antisense, 5'-CTC GCC CCA TTT TTT GAA CAC TCT G-3'; MRP-6: sense, 5'-GGT GTG GTC GGA TCA ATG TCC TC-3', antisense, 5'-GAG GAA GAG TGC GTG GGA GCA-3'; MRP-7: sense, 5'-GGG CAC CTA CAG GTT TGA GGA G-3', antisense, 5'-GAC AAC TCT GCA GGA TGT GGA TT-3'. (GenBank accession numbers were as follows: MRP-1, NM_004996; MRP-2, NM_00392; MRP-3, NM_003786; MRP-4, NM_005845; MRP-5, NM_005688; MRP-6, NM_001171; MRP-7, U66684). Cycling conditions included an initial 3-min denaturation at 94°C, followed by 40 cycles of 30 s at 94°C, 45 s at 55°C, and 1 min at 72°C. A final
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.

In the context of the current studies, we have shown (Table 1) that PDX is markedly more cytotoxic than MTX and significantly more cytotoxic than EDX during a 3-h pulse exposure against all four human tumors (A549, NSCL, and TSU-PR1, and PC-3 prostate) studied in cell culture. In the presence of 1 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 the 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 studies by 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 pteridinol 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 A549, 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.

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 and 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 Table 1 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.

### Table 1

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>MTX</th>
<th>EDX</th>
<th>PDX</th>
<th>PDX + PBCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>6.1 ± 1</td>
<td>0.75 ± 0.13</td>
<td>0.24 ± 0.03</td>
<td>0.028 ± 0.004</td>
</tr>
<tr>
<td>TSU-PR1</td>
<td>13.2 ± 2</td>
<td>4.1 ± 0.6</td>
<td>0.75 ± 0.09</td>
<td>0.093 ± 0.02</td>
</tr>
<tr>
<td>PC-3</td>
<td>9.2 ± 1</td>
<td>1.4 ± 0.2</td>
<td>0.48 ± 0.02</td>
<td>0.065 ± 0.01</td>
</tr>
</tbody>
</table>

Note: 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.
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 several fold 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,
the co-administration of PBCD with PDX resulted in nearly complete regression of the tumor, and 75% (P < 0.01) of the mice compared to 25% with PDX alone were tumor-free after treatment. Moreover, in 5 of 12 of the PDX plus PBCD-treated mice, there was no regrowth of the tumor.

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 modulation therapy as ascites tumors (29) growing i.p. in mice. Thus, the potential for PDX with PBCD in the common clinical setting focusing on metastatic solid tumors would appear to be high. Given that PBCD is already commercially available and used by patients with a variety of medical needs, the clinical development of this regimen of therapy for patients with neoplastic disease should be relatively facile and straightforward. However, in view of the potential for PBCD to alter plasma concentration of PDX as well as other classical folate analogues (39, 40) by inhibiting multispecific anion transport systems in the liver and kidney, caution will be needed during the planning and conduct of Phase I trials of this agent, which should also include a pharmacokinetic component. Although similar concerns did emerge during the initial (29) in vivo studies with MTX, they proved to be unwarranted, at least in the context of these animal model systems. In that case and in the present study, PBCD actually brought about (29) more rapid clearance of MTX and PDX from plasma probably by preferentially inhibiting reabsorption in the kidney.

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) 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 cMOAT/MRP family of ATPases in mediating outward transport of folate analogues

The co-administration of PBCD with PDX resulted in nearly complete regression of the tumor, and 75% (P < 0.01) of the mice compared to 25% with PDX alone were tumor-free after treatment. Moreover, in 5 of 12 of the PDX plus PBCD-treated mice, there was no regrowth of the tumor. In that case and in the present study, PBCD actually brought about (29) more rapid clearance of MTX and PDX from plasma probably by preferentially inhibiting reabsorption in the kidney.

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The role of specific members of the cMOAT/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.

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