Experimental Therapeutics with a New 10-Deazaaminopterin in Human Mesothelioma: Further Improving Efficacy through Structural Design, Pharmacologic Modulation at the Level of MRP ATPases, and Combined Therapy with Platinums

Nushmia Z. Khokhar, Yuhong She, Valerie W. Rusch, and F. M. Sirotnak

ABSTRACT

Studies described here sought to evaluate the therapeutic potential of a new 10-deazaaminopterin analogue, 10-propargyl-10-deazaaminopterin (PDX), alone and in combination with platinum compounds in the treatment of human pleural mesothelioma. In vitro studies documented 25–30-fold and 3-fold, respectively, greater cytotoxic potency of PDX compared with methotrexate and another 10-deazaaminopterin, edatrexate, against VAMT-1 and JMN cell lines derived from human mesothelioma. These tumor cell lines were also inhibited by platinum compounds. Cisplatin (CDDP) was somewhat more inhibitory than oxaloplatin and > 1 log order in magnitude more inhibitory than carboplatin (CBCDA). Against the JMN tumor xenografted in nude mice, whereas methotrexate and, more so, edatrexate, were potently growth inhibitory, only PDX brought about substantial regression. By comparison, CDDP and CBCDA, but not oxaloplatin were markedly growth inhibitory to this same tumor in vivo. This high level of therapeutic activity of PDX could be additionally enhanced by coadministration of probenecid, an inhibitor of canicular multispecific organic anion transporter/multidrug resistance-related protein (MRP)-like ATPases, which increased the number of complete regressions by > 3-fold. Canicular multispecific organic anion transporter/MRP genes, primarily 1, 3, 4, 5, and 7, were in fact expressed in these human mesothelioma cell lines as determined by real-time reverse transcription-PCR. These same MRP genes, including, to a lesser extent, MRP-4, were also expressed in pleural mesotheliomas derived from patients as shown by the same methodology. When combined with CDDP or CBCDA, PDX achieved 2-fold greater overall regression of the JMN tumor with a 3–4-fold increase in complete regressions, although some attenuation of dosages of each were required in the combination. These results strongly suggest that PDX has significant potential in the treatment of human pleural mesothelioma, particularly when coadministered with probenecid or combined with platinum compounds.

INTRODUCTION

Malignant pleural mesothelioma is a human neoplasm against which current therapeutic approaches have yielded little success (1, 2). Among cytotoxic agents evaluated (1, 2), modest activity has been observed with anthracyclines, platinums, alkylating agents, and folate antagonists. However, duration of response has been short, and there was only a minimal impact on survival. Combination therapy with these agents has also been disappointing (1, 2). More recently, a 10-deazaaminopterin analogue, EDX, has shown (3) promising activity against this neoplasm in the context of a group cooperative study.

The 10-deazaaminopterins were designed (4) as 4-amino folate analogues with more efficiency than MTX (1) for reduced folate carrier-mediated internalization and folylpolyglutamate synthetase-mediated polyglutamylvation in tumor cells. Both of these cellular properties are important determinants (5) of cytotoxicity and therapeutic response to the general class of classical folate antagonists. On the basis of its greater therapeutic activity compared with other 10-deazaaminopterins (6) against human tumor xenografts, EDX was selected for clinical development. This analogue was subsequently found (7) to have promising activity against human NSCLC and breast cancer and one form of soft tissue sarcoma in addition to mesothelioma as mentioned above (3).

After additional studies in experimental models of human cancer, a new analogue within the 10-deazaaminopterin series

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The abbreviations used are: EDX, edatrexate; MTX, methotrexate; TAMRA, 6-carboxytetramethylrhodamine; NSCLC, non-small cell lung cancer; PDX, 10-propargyl-10-deazaaminopterin; PBOD: probenecid; MRP, multidrug resistance-related protein; CDDP, cisplatin; CBCDA, carboplatin; OXCA, oxaloplatin; nt, nucleotide; MTD, maximum tolerated dose; CT, threshold cycle; MSKCC, Memorial Sloan-Kettering Cancer Center.
(PDX) was identified (8), which exhibited therapeutic activity superior to EDX. PDX was found (8) in companion pharmacological studies to be the most efficient perment for reduced folate carrier-mediated internalization and substrate for poly-ribozyme synthetase in human tumors among all of the earlier 10-deazaaminopterinomas examined. All told, the above studies characterized PDX as an excellent candidate for clinical development, which is now underway (9).

We now describe studies of this newest 10-deazaaminopterin analogue, which focus on models of human pleural mesothelioma. Our results document the significantly enhanced cytotoxicity of PDX compared with MTX and EDX against two mesothelioma cell lines of different histological subtypes. They also give evidence of the superiority of PDX compared with MTX and EDX against human mesothelioma xenografted in nude mice. We also markedly enhanced the therapeutic activity of PDX by coadministration of PBCD. This represents an extension of our earlier (10) studies in which we demonstrated that PBCD will selectively inhibit ATP-dependent efflux of \( ^{14} \text{H} \text{MTX} \), increase cytotoxicity of folate analogues, including PDX, against tumor cells in culture, and enhance the therapeutic activity of these analogues against tumors when coadministered to animals. This uricosuric agent is a selective inhibitor (11, 12) of canicular multispecific organic anion transporter/MRP class ATPases (13–22), which are known to extrude folate analogues from tumor cells and limit their effectiveness. Finally, we also demonstrate substantial improvement in the therapy of human experimental mesothelioma by combining PDX with either one of two platinums analogues, CDDP or CBCDA. Although CBCDA has a more acceptable toxicity profile, the relative efficacy of these two platinums has been controversial (23, 24). There seems to be equivalence in activity against some human neoplasms but documented superiority of CDDP against others. Moreover, the usefulness of CBCDA in mesothelioma has not been established (1, 2). The results of our studies are presented below.

MATeRIALS AND METHODS

Cell Culture Studies. The mesothelioma cell lines were cultured in RPMI 1640 in accordance with published (4, 8) procedures. The VAMT-1 (sarcomatoid) and JMN (mixed epithelioid/sarcomatoid) cell lines were generously provided by Drs. Brenda Gerwin and Curtis Harris at the National Cancer Institute, Bethesda, MD. Procedures used for the cytotoxicity determinations in cell culture were also described previously (4, 8).

In Vivo Studies. During in vivo studies the JMN tumor was maintained in athymic NCR-nu mice by s.c. transplantation at the flank. After tumor growth, a cell suspension in RPMI 1640 was prepared from the excised tumor, centrifuged for 5 min at 1000 \( \times g \) and the pellet resuspended in RPMI 1640 with 10% FCS for implantation with an 18-g needle. Later (3–4 days) when tumor diameters averaged 4–5 mm, the mice were randomized among control and treated groups. The MTDs of the various agents alone or in combination were determined in preliminary experiments comparing the effect of various doses. All of the agents were administered on a schedule of once every 3–4 days for a total of four injections (q3–4d \( \times 4 \)). This schedule of administration was chosen for convenience, to conserve drug supplies, and because it was highly efficacious when comparing these agents (6, 8, 10). The doses eventually selected as MTD resulted in <10% weight loss and no toxic deaths. These values were obtained in preliminary experiments relating dosage of individual agents or combinations to weight loss and lethality of treated mice. The average tumor diameter (two perpendicular axes of the tumor) was measured in control (diluent treated only) and treated groups by caliper at intervals during treatment and after treatment for 2–10 days or until the nadir in regression was attained. The data are expressed as the increase or decrease in tumor volume in mm\(^3\) (mm\(^3\) = 4/3\( \pi \)r\(^3\)).

Statistical analysis was carried out by the \( \chi^2 \) method (25). Working solutions of MTX, EDX, PDX, CDDP, CBDDA, and PBCD were prepared in 0.9% NaCl (pH 7.0). These studies were performed in accordance with the “Principles of Laboratory Animal Care” (NIH publication No. 85–23 revised 1985). NCR-nu mice were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN).

Quantitative Reverse Transcription-PCR Analysis. Total RNA was prepared with Trizol reagent (Life Technologies, Inc.) from all of the tumor cell lines. First strand cDNA was prepared using the Superscript System (Life Technologies, Inc.). Mesothelioma tumor samples were harvested from patients at the time of surgical resection and snap frozen in liquid nitrogen. mRNA was purified from these tissue samples using the Quick Prep mRNA Purification kit (Amersham Pharmacia) and then reverse transcribed using M-MLV reverse transcriptase and random hexamers (Life Technologies, Inc.). The quantitation of MRP gene expression was carried out with the aid of an ABI Prism 7700 Sequence Detection System (Taqman; PE Biosystems, Foster City, CA). A detailed description of this methodology has been provided previously (26). Specific cDNAs of interest (MRPs 1–7) and reference cDNA (\( \beta \)-actin) were amplified separately with the Taqman using an oligonucleotide probe with a 5'-fluorescent reporter dye (6FAM) and a 3' quencher dye (TAMRA) (TAMRA; Ref. 26). The original descriptions of the MRP genes have been presented in various publications (13–18, 20, 22, 27–29). The primer and probe sets were designed using Primer Express Software (Applied Biosystems). The sequences are as follows: MRP-1, forward primer starting at nt 1620, 5'-TCATGTGAGCCTAATG-3', reverse primer starting at nt 1698, 5'-CGATTGTCTTTGCTCTCATG-3', probe starting at nt 1671 6FAM-ACCTGATACGTCTTTGGTCT-3'; MRP-2, forward primer starting at nt 4049, 5'-GCTGAGAAGTCACTCCCTACA-3', reverse primer starting at nt 4148, 5'-GGGAGCTATGGGAGAAT-3', probe starting at nt 4122, 6FAM-CCATCAAATGTACATACG-3'; MRP-3, forward primer starting at nt 1380, 5'-TCATCTTGGCATATCATCCTT-3', reverse primer starting at nt 1470, 5'-CCGGTGAAGGATGCAAGC3-3', probe starting at nt 1447, 6FAM-CATGAAACGCACTGCAA-TGAG-3', MRP-4, forward primer starting at nt 614, 5'-CGAAGATCGCCATG-3', reverse primer starting at nt 700, 5'-GACTATTGCTTTGCTTTCTTCTT-3', probe starting at nt 686, 6FAM-CATGAAACGCACTGCAA-TGAG-3', MRP-5, forward primer starting at nt 767, 5'-CTTGCACTGATGCTTGTAGT-3', reverse primer starting at nt 860, 5'-TGGCGTTAATCATT-
fractional cycle number at which the amplified target reaches a

-G9252/H9252-actin.

The C T indicates the

4 Genebank accession numbers for the genes are 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, AL133613; and β-actin, XM_004814.

GCCAAGT-3′, probe starting at nt 828, 6FAM-ACCAAGACCCACGATTTCGTC-TAMRA; MRP-6, forward primer starting at nt 245, 5′-GCTTGAGTTCGCCCTCATAG-3′, reverse primer starting at nt 321, 5′-CAGGCCTCCTCTGTTGG-GAT-3′, probe starting at nt 299, 6FAM-CAAAAGAGCGA-GCTTGAG-3′, forward primer starting at nt 456, 5′-GGAAACCTCTACATCCAGTTGTC-3′, reverse primer starting at nt 545, 5′-GGTCGCATACAGGGTAGGTA-3′, probe starting at nt 498, 6FAM-CCTATCCACCGGAGTACT-3′, probe starting at nt 1053, 6FAM-TCAAGATCATTCTACT-3′, forward primer starting at nt 245, 5′-GCTTGAGTTCGCCCTCATAG-3′, reverse primer starting at nt 1099, 5′-GCCATCCACCGGAGTACT-3′, probe starting at nt 1053, 6FAM-TCAAGATCATTCTACT-3′, reverse primer starting at nt 245, 5′-GCTTGAGTTCGCCCTCATAG-3′, reverse primer starting at nt 1099, 5′-GCCATCCACCGGAGTACT-3′, probe starting at nt 1053, 6FAM-TCAAGATCATTCTACT-3′, reverse primer starting at nt 245, 5′-GCTTGAGTTCGCCCTCATAG-3′, reverse primer starting at nt 1099.

Relative quantitation was done using the comparative C T method. The C T indicates the fractional cycle number at which the amplified target reaches a fixed threshold. The amount of target gene (MRPs 1–7) normalized to an endogenous reference (β-actin) is given by 2 -ΔCT (30, 31) where ΔCT is C T (target gene) – C T (reference gene). However, this relation is based on the assumption that efficiency of the target and reference amplification be approximately equal. A validation experiment was done comparing serial dilutions of genes and β-actin. The absolute value of the slope of log input amount versus ΔC T should be <0.1, indicating that efficiencies of target and reference amplification are approximately equal (32). In our case it varied between 0.021 and 0.051. For comparison we also calculated efficiencies of different runs. Serial dilutions of a control cDNA were run in triplicate amplifying β-actin to generate a standard curve (Fig. 1). The slope of this curve is used for calculating the run efficiency. The relation is given by E = 10 -1/s – 1, where E is the efficiency, and S is the slope. In our hands the efficiencies varied from 0.966 to 0.986 with correlation coefficient ≥0.998.

Tissues and Clinical Database. Surgical specimens were obtained from consecutive patients undergoing thoracotomy and surgical resection. They were divided into small samples (3 × 3 × 3 mm), snap frozen, and stored at −140°C. Histological sections were reviewed by a pathologist to identify those tumor samples that had predominantly tumor cells among nucleated cells on the slide. Relevant clinical data were collected for all of the patients and linked to the specimens and the link-up to patient identifiers destroyed by the Tumor Bank before distribution to this project to protect patient confidentiality. Tissue collection and these experiments were performed in accordance with the guidelines of MSKCC.

Materials. Samples of MTX and EDX were obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, and Novartis, East Hanover, NJ, respectively. PDX was synthesized at MSKCC. PBCD was obtained from Sigma Chemical Co. (St. Louis, MO) and recrystallized at MSKCC. All of these agents were found to be >98% pure by high-performance liquid chromatography (33). CDDP and CB-CDA were obtained from the pharmacy at MSKCC. NCR-nu mice were purchased from Harlan Sprague Dawley.

RESULTS AND DISCUSSION

Cell Culture Studies. In vitro experiments were carried out with PDX in comparison with MTX and EDX to assess their relative cytotoxicity against two human mesothelioma cell lines. The results of experiments using a 3-h pulse exposure to these folate analogues are given in Table 1. Against both the VAMT-1 and JMN cell lines PDX was found to be 25–30-fold and 3-fold, respectively, more potent than MTX or EDX with values for IC 50 varying from low nm (PDX) to high nm (MTX) in concentration. It had already been established in our earlier (10) studies that PBCD will enhance the cytotoxicity of PDX in cell culture against a variety of tumor cell types. Because there is no reason to expect that similar effects of PBCD on PDX cytotoxicity would be any different for mesothelioma cells, we proceeded directly to in vivo studies combining PBCD with PDX.

In preparation for combination studies to be performed in

\begin{table}
\centering
\caption{Relative growth inhibition by three folate analogues against human mesothelioma cells\textsuperscript{a}}
\begin{tabular}{|l|l|l|l|}
\hline
Tumor & MTX IC\textsubscript{50} (nm) & EDX IC\textsubscript{50} (nm) & PDX IC\textsubscript{50} (nm) \\
\hline
VAMT-1 & 634 ± 86 & 77 ± 11 & 23 ± 4 \textit{n} = 3 \\
JMN & 683 ± 65 & 98 ± 12 & 27 ± 4 \textit{n} = 3 \\
\hline
\end{tabular}
\textsuperscript{a} The cells were exposed to various concentrations of each analogue for 3 h then washed and resuspended in drug-free medium. Average of three determinations ± SE of the mean.
\end{table}

\begin{table}
\centering
\caption{Relative growth inhibition of three platinum compounds against human mesothelioma cell lines\textsuperscript{a}}
\begin{tabular}{|l|l|l|l|}
\hline
Tumor & CDDP IC\textsubscript{50} (μM) & CB-CDA IC\textsubscript{50} (μM) & OX-CDA IC\textsubscript{50} (μM) \\
\hline
VAMT-1 & 6.5 ± 1 & 116 ± 22 & 10.3 ± 2 \textit{n} = 3 \\
JMN & 7.0 ± 1 & 189 ± 26 & 10.8 ± 2 \textit{n} = 3 \\
\hline
\end{tabular}
\textsuperscript{a} The cells were exposed to various concentrations of each agent for 3 h and then the agent was removed by washing the cells followed by resuspension in drug-free medium. Average of three experiments ± SE of the means.
\end{table}
Table 3 Treatment of the JMN human mesothelioma with classical folate analogues with and without probenecid

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (mg/kg)</th>
<th>Weight change (%)</th>
<th>Change in tumor vol (mm³)</th>
<th>Complete regressions (no./total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>+0.3</td>
<td>+1771</td>
<td>0/12</td>
</tr>
<tr>
<td>MTX</td>
<td>40</td>
<td>-0.1</td>
<td>5.6 ± 1</td>
<td>205/12</td>
</tr>
<tr>
<td>EDX</td>
<td>40</td>
<td>-0.2</td>
<td>4.8 ± 1</td>
<td>0/9</td>
</tr>
<tr>
<td>PDX</td>
<td>60</td>
<td>-0.1</td>
<td>3.0 ± 1</td>
<td>25/5</td>
</tr>
<tr>
<td>PDX + PBCD</td>
<td>60 + 125</td>
<td>-0.1</td>
<td>2.5 ± 1</td>
<td>86/5/9</td>
</tr>
</tbody>
</table>

*See text for experimental details. Three to four experiments using three to four mice/group.

1. Initial tumor volume = 4.5 ± 0.6 (48 mm³).
2. Two of nine mice exhibited no regrowth of tumor 3 weeks after cessation of treatment.

vivo (see below), we also compared the cytotoxicity of three platinum compounds in the same manner against these same human mesothelioma cell lines. The data in Table 2 show that CDDP was somewhat more cytotoxic than the organoplatinum OXCDMA against these cell lines. By comparison, the cytotoxic platinum compounds in the same manner against these same (see below), we also compared the cytotoxicity of three in vivo – available to us, we were only able to conduct efficacy studies against both VAMT-1 and JMN.

Animal Studies. Because of the minimal growth potential as xenografts in nude mice of most human mesotheliomas, it was of interest to examine these mesothelioma cell lines for the expression of MRP family genes. The expression of these genes in these tumors would be consistent with the notion that the enhanced therapeutic effect of PBCD with PDX was mediated through inhibition of one or more of these ATPases. Biochemical evidence of such an effect in other human tumor cells was actually provided (10–12, 19, 20) earlier by showing that PBCD was a strong inhibitor of ATP-dependent efflux of [³H]MTX. Our earlier studies (10) with human NSCLC, prostate, and mammary tumors have also documented consistent expression of MRP-1, -4, and -7 genes in these tumors. We used real-time PCR (Fig. 1) to determine the relative expression levels of MRP genes in human mesothelioma. We found (Fig. 2; Table 4) consistently prominent expres-
levels of expression of human mesothelioma cell lines. We also found (Table 4) similar expression of MRP-1, -3, -4, -5, -7 genes in the JMN and VAMT-1 human mesothelioma cell lines. We also found (Table 4) similar levels of expression of MRP-1, -3, -5, -7 genes in the JMN and VAMT-1 mixed tumor (epithelioid/sarcomatoid) samples. Interestingly, the pattern of expression was the same in both epithelioid and sarcomatoid tumors, but less so for MRP-4 in several mesotheliomas derived directly from patients. Although there was considerable variability in the level of MRP-4 in several mesotheliomas derived directly from patients, some reduction in dosage of both PDX and the platinums was required (Table 6) to obtain tolerances (MTD) that were equivalent to either PDX or the platinums alone. The MTD for each platinum alone and in combination with PDX were determined (data not shown) in extensive experiments relating dosage to weight loss and lethality of NCR-nu mice. However, despite this dosage attenuation, the combined effect of these agents was greater (Table 6) than either PDX or platinum alone. Compared with PDX both combinations brought about ∼2-fold more regression ($P < 0.05$) of the tumor and a 3–4-fold increase ($P < 0.01$) in mice showing no palpable tumors.

Our results clearly document the superiority of PDX compared with MTX and EDX against the JMN human mesothelioma xenografted in nude mice. Given the consistency of the results obtained in vivo with the companion in vitro data (Table 1) and the well-documented (8, 9) improvement in activity of PDX combined with platinum analogues, because members within both categories of agents have already been identified (1–3) as active in human mesothelioma.

Of interest as well were our results showing that PBCD markedly enhanced the activity of PDX against the JMN tumor. As in the case of this human mesothelioma, we have shown (10) similar enhancement in the efficacy of PDX by PBCD against drug-naïve human NSCLC, prostate, and mammary neoplasms. Because drug-naïve human mesothelioma, like these other tumors, expresses several members of the MRP ATPase family of genes, the likely targets of PBCD (16–21), this mechanism-based approach may clearly extend to human pleural mesothelioma.

### Table 4 Relative expression of MRP genes in human mesothelioma

<table>
<thead>
<tr>
<th>MRP genes</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JMN</td>
<td>4.8</td>
<td>0.11</td>
<td>2</td>
<td>2.4</td>
<td>1.8</td>
<td>0.008</td>
<td>1.3</td>
</tr>
<tr>
<td>VAMT1</td>
<td>9.5</td>
<td>0.074</td>
<td>0.9</td>
<td>6</td>
<td>0.9</td>
<td>0.05</td>
<td>0.85</td>
</tr>
<tr>
<td>Patient samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mixed</td>
<td>4.7</td>
<td>0.27</td>
<td>2.43</td>
<td>1</td>
<td>2.3</td>
<td>0.034</td>
<td>3.6</td>
</tr>
<tr>
<td>2 mixed</td>
<td>12.8</td>
<td>0.24</td>
<td>6.7</td>
<td>1.15</td>
<td>3.3</td>
<td>0.34</td>
<td>6.2</td>
</tr>
<tr>
<td>3 (epith)</td>
<td>3.8</td>
<td>0.04</td>
<td>1.7</td>
<td>0.35</td>
<td>1.1</td>
<td>0.05</td>
<td>2.3</td>
</tr>
<tr>
<td>4 (epith)</td>
<td>13</td>
<td>0.23</td>
<td>0.87</td>
<td>0.4</td>
<td>3.25</td>
<td>0.094</td>
<td>4.15</td>
</tr>
<tr>
<td>5 mixed</td>
<td>5.3</td>
<td>0.1</td>
<td>2.4</td>
<td>0.35</td>
<td>2.2</td>
<td>0.12</td>
<td>4.48</td>
</tr>
<tr>
<td>6 (sarco)</td>
<td>14.2</td>
<td>1.0</td>
<td>18.5</td>
<td>0.72</td>
<td>9.65</td>
<td>1.75</td>
<td>16.7</td>
</tr>
</tbody>
</table>

### Table 5 Treatment of the JMN human mesothelioma with various platinum compounds

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (mg/kg)</th>
<th>Weight change (%)</th>
<th>Average tumor diameter (mm ± SE)</th>
<th>Change in tumor vol (mm$^3$)</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDDP</td>
<td>2</td>
<td>−4</td>
<td>4.2 ± 1</td>
<td>+52</td>
<td>9</td>
</tr>
<tr>
<td>CBDDA</td>
<td>50</td>
<td>−3</td>
<td>4.3 ± 1</td>
<td>+53</td>
<td>9</td>
</tr>
<tr>
<td>OXDDA</td>
<td>7</td>
<td>−5</td>
<td>7.8 ± 2</td>
<td>+950</td>
<td>9</td>
</tr>
</tbody>
</table>

All values are averages of two determinations done in duplicate differing < ± 15% using human β actin as the internal reference gene. The values shown are normalized to the reference gene × 1000.

### Note

- See text for experimental details. Average of three experiments of the 3 mice/group.
- Initial tumor diameter was 4.3 ± 1 mm (43 mm$^3$).
lioma. Whereas it is not yet known which MRP-ATPase(s) is primarily responsible for extruding PDX from these tumors, the results obtained with PBCD provide proof-of-principle that these ATPases do impact on intrinsic sensitivity of these tumors by their role in determining net intracellular accumulation of PDX and that these ATPases can at least be partially suppressed by pharmacological means. It should also be mentioned that potential exists for the favorable modulation by PBCD of intrinsic resistance to the platinum compounds in the same context as that discussed above. However, the coadministration of 125mg/kg PBCD with either CDDP or CBDDCA had no beneficial effect compared with these platinum alone (data not shown) on the JMN tumor in mice. We attribute this negative result to the fact that MRP-2, which is associated with resistance to CDDP (13) and its encoded ATPase, is inhibited (19) by PBCD and is not expressed in this tumor. In contrast, 3 MRP genes appear to be relevant to the cytotoxic action of PDX. Clearly, additional work will be required addressing this issue of PBCD-mediated pharmacological modulation of intrinsic resistance to other agents, which may be permeants for MRP-2 or other MRP-encoded ATPases.

REFERENCES

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