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

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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 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 polyglutamylation 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
(PDX) was identified (8), which exhibited therapeutic activity superior to EDX. PDX was found (8) in companion pharmacological studies to be the most efficient permeant for reduced folate carrier-mediated internalization and substrate for folypolyglutamate synthetase in human tumors among all of the earlier 10-deazaaminopterin analogues 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 [3H]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. 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 × 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 × 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 mm3 (mm3 ± 3/4π r3).

Statistical analysis was carried out by the χ2 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 (Taquin; 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 (β-actin) were amplified separately with the Taqman using an oligonucleotide probe with a 5’ fluorescent reporter dye (6FAM) and a 3’ quencher dye (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’-CATATGGCAGCTCAATG-3’, reverse primer starting at nt 1698, 5’-CGATTGCTTTGTGCTTCTCATGTG-3’; probe starting at nt 1671, 6FAM-ACCTGATACGTCTTGGTCTC- TAMRA; MRP-2, forward primer starting at nt 4049, 5’-GCTGGAAAGTCATCCCTCACA-3’, reverse primer starting at nt 4148, 5’-GGACACATGGGAAAGGATAT-3’, probe starting at nt 4122, 6FAM-CCATCAATGATATCATCTGAC- TAMRA; MRP-3, forward primer starting at nt 1380, 5’-CTATCCTTGGGATCTACTTCTC-3’, reverse primer starting at nt 1470, 5’-CCGTTGAGTGGAATCAGCAAA-3’, probe starting at nt 1447, 6FAM-CATGAAAGCGACTCCAG-GACTATCTGGCCTGTGGTTT-3’; MRP-4, forward primer starting at nt 614, 5’-CGAAGGCACTCAGTGCGTGGCATG-3’, reverse primer starting at nt 700, 5’-GACTATCGTGCCCTTGTTGCTT-3’, probe starting at nt 686, 6FAM-CCATGAGCTGTATTTACAA-GACGAGGAAAGTCGCTTCT-3’, MRP-5, forward primer starting at nt 767, 5’-CCCTGAGTACAGCTTGGTGTAGTG-T-3’, reverse primer starting at nt 860, 5’-TCTGGTAAATCAAT-
Fixed threshold. The amount of target gene (\( C_\text{T} \)) normalized to an endogenous reference (\( C_\text{T ref} \)).

\[
\text{Efficiency} = \frac{1}{\text{Slope}} - 1
\]

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 \( r = 0.999 \).

**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 tissue bank 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 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 IC50 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 to of experiments using a 3-h pulse exposure to these folate analogues for 3 h then washed and resuspended in drug-free medium. Average of three determinations ± SE of the mean.

**Table 2 Relative growth inhibition of three platinum compounds against human mesothelioma cell lines**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>CDDP IC50 (μM)</th>
<th>CBEDA IC50 (μM)</th>
<th>OXCTA IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAMT-1</td>
<td>6.5 ± 1</td>
<td>116 ± 22</td>
<td>10.3 ± 2</td>
</tr>
<tr>
<td>JMN</td>
<td>7.0 ± 1</td>
<td>189 ± 26</td>
<td>10.8 ± 2</td>
</tr>
</tbody>
</table>

**Table 1 Relative growth inhibition by three folate analogues against human mesothelioma cells**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>MTX IC50 (μM)</th>
<th>EDX IC50 (μM)</th>
<th>PDX IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAMT-1</td>
<td>634 ± 86</td>
<td>77 ± 11</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>JMN</td>
<td>683 ± 65</td>
<td>98 ± 12</td>
<td>27 ± 4</td>
</tr>
</tbody>
</table>

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.
Tumor are given in Table 3. The data show that tolerances of the organoplatinum CBCDA was substantially less potent against these cell lines. By comparison, the cytotoxic CDDP was somewhat more cytotoxic than the organoplatinum human mesothelioma cell lines. The data in Table 2 show that platinum compounds in the same manner against these same 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 OXCDA against these cell lines. By comparison, the cytotoxic potency of the organoplatinum CBCDA was substantially less than the other two platinum compounds. Values for IC₅₀ obtained with CBCDA were 10–15- and 18–25-fold higher against both VAMT-1 and JMN.

Animal Studies. Because of the minimal growth potential as xenografts in nude mice of most human mesotheliomas available to us, we were only able to conduct efficacy studies in vivo with the JMN tumor. However, the results obtained with the folate analogues and platinum compounds with this tumor were for the most part impressive and consistent with results obtained (8, 10) with these agents against other human tumors. The results of experiments comparing the efficacy of MTX, EDX, and PDX on a schedule of q3–4 × 4 against the JMN tumor are given in Table 3. The data show that tolerances (MTD) of these mice to these agents differed by ≥50% with greatest tolerance seen in the case of PDX. The MTD for each antifolate was determined in extensive (6, 8) preliminary experiments relating dosage to weight loss and lethality of NCR-nu mice treated on the same schedule of administration. In these experiments, MTX was found to be highly inhibitory to tumor growth, suppressing growth to ~90%. EDX was found to be more inhibitory (P < 0.05) than MTX suppressing growth by 95%. In sharp contrast to these results, which documented no regression of tumor, PDX brought about 50% regression of the JMN tumor with some animals showing no palpable tumors shortly after cessation of treatment.

Also, shown in this table (Table 3) are the results of experiments in which PBCD was coadministered with PDX against the JMN tumor. In our other studies, this organic anion had been shown (10–12) to inhibit the extrusion of PDX and MTX from tumors ostensibly by an MRP-like ATPase, thus, increasing their net accumulation within tumor cells. In the case of other human tumors, the probable effect of PBCD on this ATPase(s) was shown (10, 16, 17, 19) to markedly retard extrusion of PDX from the tumor and increase its efficacy. In the new experiments, PDX also exhibited (data not shown) the same MTD (60 mg/kg) alone and when coadministered with 125 mg/kg PBCD on the same schedule of administration as in the earlier studies. The data given in Table 3 show that there was marked enhancement in the efficacy of PDX against the JMN tumor. This was seen as an increase in the degree of regression (P < 0.01) and in the number of mice (P < 0.01) showing no tumor after cessation of treatment. Moreover, some animals treated with PDX and PBCD did not have tumor regrowth after complete regression.

In view of the above results, 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-

<table>
<thead>
<tr>
<th>Rx</th>
<th>Dose (mg/kg)</th>
<th>Weight change (%)</th>
<th>Average tumor diameter (mm ± SE)</th>
<th>Change in tumor vol (mm³)</th>
<th>Average regression (%)</th>
<th>Complete regressions (no./total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>+0.3</td>
<td>8.3 ± 1</td>
<td>+1771</td>
<td>0/12</td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>40</td>
<td>—0.1</td>
<td>5.6 ± 1</td>
<td>+205</td>
<td>0/12</td>
<td></td>
</tr>
<tr>
<td>EDX</td>
<td>40</td>
<td>—0.2</td>
<td>4.8 ± 1</td>
<td>+92</td>
<td>0/9</td>
<td></td>
</tr>
<tr>
<td>PDX</td>
<td>60</td>
<td>—0.1</td>
<td>3.0 ± 1</td>
<td>—25</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>PDX + PBCD</td>
<td>60 + 125</td>
<td>—0.1</td>
<td>2.5 ± 1</td>
<td>—41</td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>

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

— Initial tumor volume = 4.5 ± 0.6 (48 mm³).

— Two of nine mice exhibited no regrowth of tumor 3 weeks after cessation of treatment.
Table 4 Relative expression of MRP genes in human mesothelioma

<table>
<thead>
<tr>
<th>MRP genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>Cell lines</td>
</tr>
<tr>
<td>JMN</td>
</tr>
<tr>
<td>VAMT1</td>
</tr>
<tr>
<td>Patient samples</td>
</tr>
<tr>
<td>1 mixed</td>
</tr>
<tr>
<td>2 mixed</td>
</tr>
<tr>
<td>3 (epith)</td>
</tr>
<tr>
<td>4 (epith)</td>
</tr>
<tr>
<td>5 mixed</td>
</tr>
<tr>
<td>6 (sarco)</td>
</tr>
<tr>
<td>7 (epith)</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.

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

<table>
<thead>
<tr>
<th>Rx</th>
<th>Dose (mg/kg)</th>
<th>Weight change (%)</th>
<th>Average tumor diameter (mm ± SE)*</th>
<th>Change in tumor vol (mm³)</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>CBDDCA</td>
<td>50</td>
<td>−3</td>
<td>4.3 ± 1</td>
<td>+53</td>
<td>9</td>
</tr>
<tr>
<td>OXDDCA</td>
<td>7</td>
<td>−5</td>
<td>7.8 ± 2</td>
<td>+950</td>
<td>9</td>
</tr>
</tbody>
</table>

* See text for experimental details. Average of three experiments of the 3 mice/group.

sion of MRP-1, -3, -4, -5, and -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, and -7 but less so for MRP-4 in several mesotheliomas derived directly from patients. Although there was considerable variability in the level of expression of these genes among these tumors, the overall pattern of expression was the same in both epithelioid and mixed tumor (epithelioid/sarcomatoid) samples. Interestingly, relative expression of these MRP genes was highest in one sample of a sarcomatoid tumor that also showed higher levels of expression of MRP-2 and -6 compared with the other samples.

Again, in preparation for combination therapy experiments, we also examined the relative effects in vivo of the three platinum compounds against the JMN tumor, which can be seen in Table 5. Tolerances of these mice to these agents on the schedule used varied substantially in the order of CBCDDCA >> OXDDCA > CDDP. Despite these differences in tolerance, CDDP and CBDDCA were found to strongly inhibit tumor growth to a similar extent (97%). This can be explained by the higher cytotoxic potency of CDDP (Table 2) compared with CBDDCA, which offsets the lower tolerance to CDDP. However, in comparison to this marked effect on tumor growth by CDDP and CBCDDCA, OXDDCA was only moderately inhibitory (P < 0.005) to the growth of this tumor (<50%).

In light of the above results, we compared only CDDP and CBDDCA in combination with PDX against the JMN tumor. 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 compared with MTX and EDX obtained against several other human tumor types, there is a reasonable basis for assuming that PDX could have significant impact on the treatment of this neoplastic disorder in patients. This potential is clearly manifested in the case 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-naive human NSCLC, prostate, and mammary neoplasms. Because drug-naive 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 mesothe-
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 of these tumors. 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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