Experimental Chemotherapy of Human Medulloblastoma Cell Lines and Transplantable Xenografts with Bifunctional Alkylation Agents

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INTRODUCTION

Medulloblastoma, the most common malignant tumor of the central nervous system in childhood, continues to represent a frustrating therapeutic challenge (1, 2). Despite treatment with increasingly sophisticated neurosurgical and radiotherapeutic intervention, the majority of children with medulloblastoma will eventually die of progressive disease. Chemotherapy has produced responses in recurrent disease, but has only provided minimal increases in disease-free survival for patients with advanced disease (3).

Our laboratory has established and characterized a panel of human medulloblastoma models allowing the design of chemotherapeutic intervention for this tumor (4–6). Preliminary studies with TE-671, a human medulloblastoma continuous cell line and transplantable xenograft, suggested the antitumor activity of a series of classical alkylating agents, particularly melphalan, cyclophosphamide, and thiopeta. In vivo responses in patients with recurrent medulloblastoma (10, 11), supported a role for cyclophosphamide in producing objective therapeutic trials for patients with medulloblastoma.

MATERIALS AND METHODS

Cell Lines. The human medulloblastoma-derived cell lines TE-671, Daoy, and D283 Med were used in these studies. Their establishment, characterization, and tissue culture techniques have been previously described (4, 5, 20, 21). Chromosomal analysis of D283 Med revealed a stemline of 46,XY,i(17q),der(8)(q13,q1)q32,der(20)(p5;20)(q1;ql1)q13 (5). TE-671 and Daoy contained near-tetraploid stemlines with distinctive marker chromosomes identical to those described originally (20, 21). Phenotypic analysis revealed that TE-671 and Daoy demonstrated a glial-like profile, with reactivity with monoclonal antibodies directed against epidermal growth factor receptor, tenascin, HLA-A,B epitopes, β2 microglobulin, and 5 of 8 of glioma-associated antigens, but not with an antineurofilament protein antibody. D283 Med demonstrated a neuronal-like profile, with expression of neurofilament protein, but without reactivity with monoclonal antibodies against tenascin, epidermal growth factor receptor, HLA-A,B epitopes, β2 microglobulin, or 6 of 8 of glioma-associated antigens (22).

Cytogenetic Analysis. All drug assays were performed by using a 1-h drug incubation with a final cell concentration of 2.5 × 10^6 cells/dish for TE-671 and 1.0 × 10^6 cells/dish for Daoy and D283 Med, as previously described (9). Response was assessed by the ratio of treated versus control colony formation. All drugs and solutions were provided and prepared as previously described (9).

Animals. Male or female athymic BALB/c mice (nu/nu genotype, 6 weeks or older) were used for all in vivo studies and were maintained as previously described (23).
Xenograft Transplantation. TE-671 and Daoy grown as s.c. and intracranial xenografts were used for in vivo studies. Lack of homoge-
neous growth and survival curves with D283 Med prevented in vivo use of
of this xenograft. Xenograft transplantation (s.c.) was performed as
previously described with inoculation volumes of 30 μl of TE-671 and
100 μl for Daoy (8). Intracranial xenograft transplantation of TE-671
was performed as previously described (8). Daoy cells for intracranial
inoculation were harvested in vitro at 90% confluence, centrifuged at
1000 rpm for 5 min, and resuspended in 1.0 ml minimum essential
medium to a concentration of 4.0 × 10^5-6.75 × 10^6 cells/ml. Injections
(30 μl) of the homogeneous suspension were given into the right cerebral
hemisphere with a 500-μl Hamilton syringe through a 27-gauge needle
equipped with a sleeve allowing 4.5-mm penetration.

Tumor (s.c.) Measurements. The s.c. tumors were measured as pre-
viously described (8).

Drug Toxicity. The lethal toxicity of individual drugs was assessed
by probit analysis (24). A minimum of 4 dose levels with 10 animals/
dose was used to calculate the 10% lethal dose of each drug. The
regimen used in each experiment was 100% of the calculated 10%
lethal dose administered as a single i.p. injection in a volume of 90 ml/m^2.
The doses used were: melphalan, 71.3 mg/m^2; cyclophosphamide,
1391 mg/m^2; phenylketocyclophosphamide, 279.9 mg/m^2; iophos-
phamide, 2014.9 mg/m^2; thiopeta, 61.8 mg/m^2; BCNU, 100 mg/m^2;
and busulfan, 60.3 mg/m^2. Melphalan was administered in 16.7% DMSO,
phenylketocyclophosphamide in 33% DMSO, busulfan in 10% DMSO,
and BCNU in 3.3% ethanol. Cyclophosphamide, iophosphamide, and
thiopeta were administered in 0.9% NaCl solution.

Tumor Therapy. Therapy experiments for s.c. and intracranial xen-
ografts were performed as previously described (8). Xenografts (s.c.)
were treated when the median tumor volume exceeded 200 mm^3 (TE-
671) and 350 mm^3 (Daoy); intracranial xenografts were treated on day
12 (TE-671) and day 21 (Daoy) after implantation.

Response of s.c. xenografts was assessed by mean treated versus
control tumor volume (T/C), the difference in days between the median
of individual treated animals' and individual control animals' tumors
to reach a volume of 5 times the treatment volume (T - C), and treated
versus control tumor regressions. Response of intracranial xenografts
was assessed by comparing median survival time and long term survi-
vors (>90 days) between treated and control groups.

Statistical analyses were performed as previously described (7).

RESULTS

Clonogenic Assay. The in vitro dose response curves for
melphalan, 4-hydroperoxycyclophosphamide, thiopeta, 4-hy-
droperoxyiposphamide, and phenylketocyclophosphamide
against each cell line are presented in Figs. 1–5. The curves for
each drug tested were similar in slope and demonstrated de-
creasing survival with increasing dose. The calculated in vitro
drug dose at which there is a 90% reduction in the number of
colonies in comparison to controls for the 5 drugs tested in

![Fig. 1. In vitro dose-response curves showing the chemosensitivity of TE-671, Daoy, and
D283 Med to melphalan; 95% confidence limits.](image)

vitro are presented in Table 1. Within each line, the relative
and absolute activity of each drug was similar, with melphalan
and phenylketocyclophosphamide being most active on a molar
basis and thiopeta and 4-hydroperoxyiposphamide least ac-

Drug Toxicity. Twelve deaths in 220 and twenty-two deaths
in 217 tumor-bearing animals for TE-671 and Daoy, respec-
tively, were attributed to drug toxicity with the following dis-
tribution: melphalan, 1 of 40 and 5 of 50; cyclophosphamide,
1 of 40 and 1 of 39; phenylketocyclophosphamide, 0 of 40
and 0 of 29; thiopeta, 0 of 40 and 0 of 39; iophosphamide, 0 of 40
and 0 of 40; busulfan, 0 of 10 and 0 of 10; and BCNU, 0 of 10
and 0 of 10 for TE-671 and Daoy, respectively. Mean nadir
weight loss was: melphalan, 15.9 and 20.9%; cyclo-
phosphamide, 17.6 and 22.0%; phenylketocyclophosphamide, 17.7
and 12.8%; thiopeta, 17.0 and 10.9%; iophosphamide, 12.6 and
14.7%; busulfan, 3 and 1.1%; and BCNU, 2 and 0% for TE-
671 and Daoy, respectively.

Tumor Therapy (s.c.). The response to chemotherapy for each
line is summarized in Table 2. Growth curves illustrating the
response to melphalan, cyclophosphamide, thiopeta, iophos-
phamide, phenylketocyclophosphamide, BCNU, and busulfan
for Daoy are presented in Figs. 6 and 7 (growth curves for TE-
671 to melphalan, cyclophosphamide, thiopeta, iophosphamide,
and phenylketocyclophosphamide have been previously pub-
lished (9)).

All agents except BCNU and busulfan produced statistically
significant (P < 0.01) growth delays in TE-671 and Daoy.
Melphalan and cyclophosphamide were the only agents that
produced a statistically significant (P < 0.01) number of regres-
sions in TE-671 with neither agent producing cures (defined as
complete regression without regrowth). All agents with the
exception of phenylketocyclophosphamide which produced
growth delays in Daoy also demonstrated consistent tumor
regressions. There were no spontaneous regressions in control
animals for either TE-671 or Daoy.

Intracranial Tumor Therapy. The five drugs which were active
against the s.c. xenografts were also tested against intracranial
xenografts (Table 3). Mortality distribution for animals bearing
Daoy xenografts treated with melphalan, cyclophosphamide, thiopeta,
iophosphamide, and phenylketocyclophosphamide are
presented in Figs. 8 and 9 (mortality distribution curves for TE-
671 to melphalan, cyclophosphamide, thiopeta, iophosphamide,
and phenylketocyclophosphamide have been previously pub-
lished (9)). All agents tested produced statistically significant
(P < 0.01) increases in median survival in both lines with the
exception of phenylketocyclophosphamide against intracranial
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Fig. 2. *In vitro* dose-response curves showing the chemosensitivity of TE-671, Daoy, and D283 Med to 4-hydroperoxycyclophosphamide. Data from TE-671 are reproduced as published in Ref. 9, Fig. 1, p. 2828. It is reshown for comparative purposes; 95% confidence limits.

Fig. 3. *In vitro* dose-response curves showing the chemosensitivity of TE-671, Daoy, and D283 Med to thiotepa. Data from TE-671 are reproduced as published in Ref. 9, Fig. 2, p. 2828. It is reshown for comparative purposes; 95% confidence limits.

Fig. 4. *In vitro* dose-response curves showing the chemosensitivity of TE-671, Daoy, and D283 Med to phenylketocyclophosphamide. Data from TE-671 are reproduced as published in Ref. 9, Fig. 1, p. 2828. It is reshown for comparative purposes; 95% confidence limits.

Fig. 5. *In vitro* dose-response curves showing the chemosensitivity of TE-671, Daoy, and D283 Med to 4-hydroxyphosphamide. Data from TE-671 are reproduced as published in Ref. 9, Fig. 1, p. 2828. It is reshown for comparative purposes; 95% confidence limits.

Daoy. Melphalan was the most active drug against intracranial TE-671 with percentage of increased median survival of 42 and 64% in duplicate trials; thiotepa was the most active drug against intracranial Daoy with percentage of increased median survival of 61 and 36% in duplicate trials. No agent produced statistically significant numbers of long term survivors.
Table 1 In vitro cytotoxicity

<table>
<thead>
<tr>
<th>Drug</th>
<th>ID50 (μM)</th>
<th>TE-671*</th>
<th>D283 Med</th>
<th>Daoy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melphalan</td>
<td>2.13</td>
<td>5.29</td>
<td>4.72</td>
<td></td>
</tr>
<tr>
<td>Phenylketocyclophosphamide</td>
<td>4.60</td>
<td>5.01</td>
<td>4.34</td>
<td></td>
</tr>
<tr>
<td>4-Hydroperoxycyclophosphamide</td>
<td>12.80</td>
<td>22.47</td>
<td>29.90</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxyphenylphosphamide</td>
<td>20.80</td>
<td>47.13</td>
<td>104.98</td>
<td></td>
</tr>
<tr>
<td>Thiopeta</td>
<td>94.6</td>
<td>136.4</td>
<td>76.30</td>
<td></td>
</tr>
</tbody>
</table>

* ID50 in vitro drug dose at which there is a 90% reduction in the number of colonies in comparison to controls.

DISCUSSION

The role of chemotherapeutic intervention for medulloblastoma remains poorly defined, limited by a lack of active agents which can be utilized in adjuvant therapy trials. Recent clinical and laboratory studies have suggested the potential role of the classical alkylating agent cyclophosphamide in the treatment of human medulloblastoma (7, 10). More recent studies in our laboratory have documented the activity of a series of classical bifunctional alkylators in the treatment of the human medulloblastoma continuous cell line and transplantable xenograft TE-671, and have directly led to a Phase II trial of melphalan for recurrent medulloblastoma (9). This trial, now in progress, supports the potential role of melphalan, with 2 responses (1 complete response and 1 partial response) in the first 7 patients treated.

Nevertheless, clinical translation of experimental therapeutic profiles for medulloblastoma based on studies limited to one cell line and xenograft ignore the known heterogeneity of medulloblastoma, and may well lead to inappropriate clinical trials. Studies demonstrating the wide spectrum of histological malignancy, tissue pattern, neuronal and/or glial differentia-
tion, and genotypes seen in medulloblastoma (12–19) support consideration of this heterogeneity in the selection of chemotherapeutic agents for clinical trials. The 3 human medulloblastoma cell lines utilized in our current alkylation studies reflect the wide spectrum of genotypic and phenotypic patterns seen in this tumor. TE-671 and Daoy, derived from cerebellar medulloblastomas, are adherence-dependent cell lines which demonstrate a glial-like profile with reactivity against epidermal growth factor receptor, tenascin, HLA-A,B epitopes, β2 microglobulin, and 5 of 8 of glioma-associated antigens, but not with an antineurofilament protein antibody. TE-671 and Daoy also demonstrate marked differences in the transport and glutathione-mediated detoxification of melphalan. Karyotypically, both cell lines contain near-tetraploid stemlines but each possesses distinctive marker chromosomes. D283 MED, derived from peritoneal medulloblastoma metastases, is an adherence-independent cell line which demonstrates a neuronal-like profile, with expression of neurofilament protein, but without reactivity with monoclonal antibodies against tenascin, epidermal growth factor receptor, HLA-A,B epitopes, β2 microglobulin, or 6 of 8 glioma-associated antigens. Chromosomal analysis reveals a pseudodiploid stemline karyotype with i(17q) and unbalanced translocations involving chromosomes 5, 8, and 20. The most common chromosomal abnormality seen in medulloblastoma biopsies is i(17q). This structural abnormality characterizes D-283 Med and TE-679 but is not present in Daoy. All three lines demonstrate rearrangements involving chromosomes 5 and 8, but the breakpoints are in different regions. Thus, although each cell line contains abnormalities in common with other medulloblastoma cell lines and medulloblastoma biopsies, each line maintains an individual and distinctive karyotypic pattern.

The current studies extend our previous work with classical alkylators, demonstrating the activity of nitrogen and phosphoramide mustard-based alkylators in the treatment of this panel of genotypically and phenotypically heterogeneous human medulloblastoma continuous cell lines and transplantable xenografts. The anti-neoplastic activity of melphalan, a water soluble (log P = −1.70) bifunctional alkylator transported via a

Table 2 Chemotherapeutic response of s.c. xenografts

<table>
<thead>
<tr>
<th>Drug</th>
<th>Daoy</th>
<th>TE-671*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melphalan</td>
<td>36.7</td>
<td>22.9</td>
</tr>
<tr>
<td>Phenylketocyclophosphamide</td>
<td>24.3</td>
<td>39.6</td>
</tr>
<tr>
<td>4-Hydroperoxycyclophosphamide</td>
<td>12.6</td>
<td>26.3</td>
</tr>
<tr>
<td>4-Hydroxyphenylphosphamide</td>
<td>20.80</td>
<td>37.2</td>
</tr>
<tr>
<td>Thiopeta</td>
<td>36.1</td>
<td>37.1</td>
</tr>
<tr>
<td>Phenylketocyclophosphamide</td>
<td>60.5</td>
<td>48.9 (NS)</td>
</tr>
<tr>
<td>Busulfan</td>
<td>98.7 (NS)</td>
<td>118.0 (NS)</td>
</tr>
<tr>
<td>BCNU</td>
<td>120 (NS)</td>
<td>83.8 (NS)</td>
</tr>
</tbody>
</table>

* Data from TE-671 to melphalan, cyclophosphamide, iphosphamide, thiopeta, and phenylketocyclophosphamide are reproduced exactly as published in Table 2, p. 2830, Ref. 9, reshowen for comparative purposes.

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Fig. 6. Groups of 10 randomly assigned mice bearing s.c. Daoy were treated with chemotherapeutic agents or with an equivalent volume of drug vehicle. Drugs were delivered by i.p. injection at 100% of the 10% lethal dose when the median tumor volume exceeded 350 mm³.

Daoy

carrier-mediated pathway(s), noted initially against TE-671, was also seen against Daoy and D283 Med. This agent consistently demonstrated the greatest activity of all agents tested against the 3 lines in vitro, and the 2 lines tested as s.c. xenografts. The results with melphalan against intracranial Daoy were less impressive than those with cyclophosphamide or thiotepa, but these may not be biologically relevant differences. These results, coupled with our current Phase II study with melphalan, indicate that the role of this agent in the treatment of patients with medulloblastoma deserves further study. Laboratory studies in progress are evaluating potential mechanisms modulating sensitivity and resistance to melphalan in human medulloblastoma, including transport, glutathione levels, and formation and repair of DNA interstrand cross-links.

Cyclophosphamide, previously shown to be active in clinical and experimental studies with medulloblastoma (7-10) was active against all 3 lines in vitro and both xenografts in vivo. (Tables 1-3). Iphosphamide (4-hydroperoxycyclophosphamide) was less active on a molar basis in vitro than cyclophosphamide (4-hydroperoxycyclophosphamide), but produced similar growth delays and increases in median survival against s.c. and intracranial xenografts, respectively. Phenylketocyclophosphamide was markedly more active than 4-hydroperoxycyclophosphamide against the 3 cell lines in vitro, but produced smaller growth delays and increases in median survival against s.c. and intracranial xenografts, respectively. This may be due to dose limitations secondary to increased hematological toxicity or more rapid plasma clearance (9). The stem cell protection from cyclophosphamide afforded by aldehyde dehydrogenase will not be operational with phenylketocyclophosphamide, which instead undergoes a simple elimination reaction to generate phosphoramide mustard (25). This agent may provide an approach to bypass aldehyde dehydrogenase-mediated resistance to cyclophosphamide, although myelosuppression may prove unacceptably severe.

Thiotepa is a phosphoramide mustard analogue that has been minimally active in the intrathecal treatment of advanced medulloblastoma, quite possibly due to the low dosages used (26, 27). Although it was less effective in vitro, it produced similar growth delays (s.c. xenografts) and increases in median survival (intracranial xenografts) compared to the other agents tested. The disparity between in vitro, s.c. xenograft, and intracranial xenograft results demonstrates the complex interactions of cellular sensitivity and drug delivery operational in each system, and supports inclusion of data from all three systems in determining selection of drugs for entry into clinical trials. On the basis of our results thiotepa may be considered for new Phase II trials of recurrent medulloblastoma.

Neither busulfan, a methane sulfonate derivative, nor BCNU, a highly lipophilic (log P = 1.53) nitrosourea demonstrated activity against s.c. TE-671 or Daoy. Busulfan has not been utilized in the treatment of patients with medulloblastoma, and our studies do not support clinical investigations with this agent. BCNU has demonstrated moderate efficacy in the treatment of adults with glioma (28), but is not active in the treatment of children with medulloblastoma (29). Recent laboratory studies suggest that the presence of high levels of O⁶-alkylguanine-DNA alkyltransferase in the medulloblastoma...
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Table 3 Chemotherapeutic response of intracranial xenografts

<table>
<thead>
<tr>
<th>Drug</th>
<th>% IMS*</th>
<th>Median survival (range)**</th>
<th>LTS*</th>
<th>% IMS</th>
<th>Median survival (range)**</th>
<th>LTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melphalan</td>
<td>33</td>
<td>64 (52–126+)</td>
<td>2 (NS)</td>
<td>64</td>
<td>59 (24–75+)</td>
<td>2 (NS)</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>14</td>
<td>64.5 (59–91+)</td>
<td>1 (NS)</td>
<td>42</td>
<td>54 (38–72+)</td>
<td>1 (NS)</td>
</tr>
<tr>
<td>Thiotepa</td>
<td>46</td>
<td>70 (52–100)</td>
<td>1 (NS)</td>
<td>30</td>
<td>35 (39–38)</td>
<td>0 (NS)</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>73 (53–81)</td>
<td>0 (NS)</td>
<td>44</td>
<td>49 (45–54)</td>
<td>0 (NS)</td>
</tr>
<tr>
<td>Iphosphamide</td>
<td>61</td>
<td>88 (31–117)</td>
<td>0 (NS)</td>
<td>42</td>
<td>49 (33–54)</td>
<td>0 (NS)</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>68 (55–94)</td>
<td>0 (NS)</td>
<td>42</td>
<td>49 (31–54)</td>
<td>0 (NS)</td>
</tr>
<tr>
<td>Phenylketocyclophosphamide</td>
<td>14</td>
<td>54.5 (40–73)</td>
<td>0 (NS)</td>
<td>26</td>
<td>38 (32–42)</td>
<td>0 (NS)</td>
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<tr>
<td></td>
<td>34</td>
<td>67 (54–86)</td>
<td>0 (NS)</td>
<td>26</td>
<td>38 (31–48)</td>
<td>0 (NS)</td>
</tr>
</tbody>
</table>

* Data from TE-671 are reproduced as published in Ref. 9, Table 3, p. 2831, resown for comparative purposes.
* % IMS, percentage of increased median survival of treated animals versus control animals. Median day of death for control animals ranged from 27–38 for TE-671 and 48–56.5 days for Daoy. Median survival in days of treated animals (range of deaths of treated animals excluding toxic deaths secondary to drug).
* LTS, long term survivors; ≥90 days. No long term survivors were seen in control groups.
* P values are ≤0.01, unless otherwise designated not significant (NS).

Fig. 8. Groups of 10 randomly assigned mice bearing intracranial Daoy were treated with chemotherapeutic agents or with an equivalent volume of drug vehicle by i.p. injection of 100% of the 10% lethal dose on day 21 after tumor implantation.

Fig. 9. Groups of 10 randomly assigned mice bearing intracranial Daoy were treated with chemotherapeutic agents or with an equivalent volume of drug vehicle by i.p. injection of 100% of the 10% lethal dose on day 21 after tumor implantation.

Our results demonstrate that specific bifunctional alkylators, notably melphalan, cyclophosphamide, thiotepa, and i phosphamide are active in all cell lines and xenografts tested, thus confirming our previous studies with TE-671 and suggesting that a homogeneous response profile of this tumor to classical alkylators, paralleling its homogeneous radiosensitivity (1), may well exist, similar to that seen in rhabdomyosarcoma or neuroblastoma. Clinical trials using alkylating agent therapy for newly diagnosed patients prior to the use of radiotherapy will be needed to determine if this alkylator response rivals that seen to radiation therapy. Nevertheless, clinical trials in patients with recurrent medulloblastoma have demonstrated high response rates, albeit in limited numbers of patients (10). Continued studies both in the laboratory with our newer medulloblastoma cell lines and xenografts (31), and clinically with larger numbers of patients with medulloblastoma treated with melphalan or other bifunctional alkylators (in Phase II and Phase III trials), will be needed to determine if the observed alkylator response of the three cell lines and xenografts tested is indeed representative of the response of medulloblastoma to specific bifunctional alkylating agents.

Future studies in our laboratory will continue to define the therapeutic profile of medulloblastoma, to determine if synergistic interactions between specific alkylating agents are present, and to quantitate cross-resistance and collateral sensitivity. Analysis of the antigenic profile and the biochemical differences in drug transport, drug metabolism, glutathione detoxification, and formation/repair of DNA interstrand cross-links seen in medulloblastoma may allow better drug design and selection for clinical trials based on tumor profiles obtained at initial neurosurgical intervention. Clinical protocols will continue to attempt translation of these laboratory studies into effective Phase II and III trials.
REFERENCES

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