Melphalan Transport, Glutathione Levels, and Glutathione-S-transferase Activity in Human Medulloblastoma


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ABSTRACT

Melphalan transport, glutathione levels, and glutathione-S-transferase activity were measured in two continuous human medulloblastoma cell lines established in athymic nude mice, TE-671 and Daoy. In vitro mean glutathione levels were 10.06 nmol/10^6 cells in TE-671 and 2.96 nmol/10^6 cells in Daoy. In vitro mean glutathione-S-transferase activities were 91.52 nmol/min/mg protein in TE-671 and 50.31 nmol/min/mg protein in Daoy. Transport studies revealed kinetic parameters related to the sensitivities of the two tumors to melphalan. Our studies revealed transport parameters confirming facilitated transport of melphalan in human medulloblastoma, a mean murine melphalan plasma concentration above the in vitro drug dose at which there is a 90% reduction in the number of colonies in comparison to controls for TE-671 and Daoy for 2 h, and glutathione and glutathione-S-transferase levels in the same range previously reported in other melphalan-sensitive and melphalan-resistant human tumors.

INTRODUCTION

Medulloblastoma, the most common malignant brain tumor of childhood, represents a therapeutic challenge with the majority of patients ultimately failing conventional treatment with surgical and radiotherapeutic intervention (1). The role of adjuvant chemotherapy is poorly defined, with recent clinical studies suggesting minimal benefit for this modality (2). Nevertheless, review of Phase II trials for patients with recurrent medulloblastoma suggests the antitumor activity of several chemotherapeutic agents, primarily alkylating agents, in the treatment of this tumor (3, 4). Newer approaches, exploiting models of human medulloblastoma in the design of chemotherapy (5-7), were prepared in methanol containing 0.1 N hydrochloric acid. Standards were prepared in minimal essential media containing melphalan throughout the course of the assay procedure. The unknown melphalan concentrations were determined from the linear regression analysis of the standards, using integrated peak areas.

Transport Studies

*[^1]^Melphalan (specific activity, 150 mCi/mmol, radiochemical purity ≥95%) was obtained from the Amersham Corp. (Arlington Heights, IL).

Choline chloride was obtained from Sigma Chemical Co. (St. Louis, IL).
MO). BCH\(^3\) was obtained from Calbiochem (Behring Diagnostics, San Diego, CA). Cell size was determined with a Coulter electronic particle analyzer equipped with a channel analyzer (Models ZB and C1000; Coulter Electronics, Hialeah, FL).

Transport studies for TE-671 and Daoy were carried out by incubating 1-3 \times 10^6 cells in 60-mm Petri dishes (90% confluent) with varying concentrations of \(\left[^{3}H\right]\)melphalan (1-100 \mu M, 32 \mu Ci/\mu mol) in a volume of 2.5 ml at 37 \(^\circ\)C. The radiolabeled drug supplied as a powder was dissolved in ethanol: 1 \text{ml HCl} (5:1, v/v) and was diluted to the desired concentration in Dulbecco’s PBS with glucose (1 g/l). The cells were incubated for 0.5-20 min, the medium was suctioned off, and the cells were washed 5 times with cold (0 \(^\circ\)C) PBS-phosphate buffer. The cells were covered with 2 ml cold (0 \(^\circ\)C) trichloroacetic acid (10\%) and then scraped with a rubber policeman. The trichloroacetic acid-cell solution (1.5 ml) was placed in 1.8-ml microcentrifuge tubes and centrifuged at 12,000 \(\times g\) for 1 min in a Beckman Microfuge 12 (Beckman Instruments, Inc., Palo Alto, CA) at 0 \(^\circ\)C. One ml of supernatant was placed in a scintillation vial with 8 ml of Aquasol-2 (NEN Research Products, Boston, MA) and 200 \mu l acetic acid, and the radioactivity was measured in a Packard Tri-Carb 4640 scintillation counter (Packard Instrument Co., Downers Grove, IL). Binding of \(\left[^{3}H\right]\)melphalan to the cell surface was measured by incubating the cells at 0 \(^\circ\)C for 15 s with varying concentrations of the drug.

Initial uptake velocity was calculated from the linear portion of the curve drawn through the uptake points and the origin.

Inhibition studies were similarly performed as above in the presence of 5 mM BCH or sodium-depleted media (Dulbecco’s PBS with Na\(^+\) replaced by choline, Na\(_2\)HPO\(_4\) replaced by K\(_2\)HPO\(_4\)). Drug influx in cells incubated in PBS served as control.

The drug uptake data were processed by linear regression analysis, and the reciprocal initial velocities were plotted against reciprocal melphalan concentrations. The resulting Lineweaver-Burk plots were analyzed by linear regression, allowing calculation of the apparent \(K_m\) and \(V_{max}\).

GSH Measurements

GSH measurements were performed by using the assay of Habig et al. (15). All reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Cells were washed in Dulbecco’s PBS centrifuged at 1000 rpm for 5 min, and were resuspended in 200 \mu l of 0.1 mM potassium phosphate buffer/1 mM EDTA, pH 7.4 (all procedures were performed at 4 \(^\circ\)C). Following freezing/thawing twice in methanol in dry ice, the suspension was centrifuged at 20,000 \(\times g\) for 30 min at 4 \(^\circ\)C. The assay was performed by placing NADPH (700 \mu M), Ellman’s reagent (100 \mu M!), stock buffer (170 \mu M), and supernatant or stock (100 \mu l) consecutively into a cuvet. Glutathione reductase (10 \mu l) was added to the mixture at room temperature and the cuvet was scanned on a Gilford Response spectrophotometer with recorder at 412 nm for 3 min; 5 \mu l of reduced glutathione (0.5 nmol) were added to the sample cuvet and it was rescanned. Sample glutathione levels were determined by comparison of the rate of 5,5’-dithiobis(2-nitrobenzoic acid) reduction to the internal glutathione standard (0.5 nmol). Glutathione levels were expressed per 10\(^6\) cells, per mg protein, and per g wet weight (xenografts).

Animals

Male or female athymic BALB/c-nu/nu mice, 6 weeks or older were used for all in vivo studies. Animals were maintained as described previously (19).

RESULTS

Tumor Lines

The human medulloblastoma cell lines TE-671 and Daoy were grown s.c. and intracranially in athymic nude mice as described previously (5, 11). Animals were inoculated s.c. with 30 \mu l (TE-671) or 100 \mu l (Daoy) due to differences in tumor latency.

Tumor Transplantation

Tumor transplantation (s.c.) was performed as described previously (5).

Tumor (s.c.) Measurement

Tumors s.c. were measured every 3 to 4 days with vernier calipers (Scientific Products, McGraw Park, IL) until the volume exceeded 2500 mm\(^3\). Width and length in mm\(^2\) were measured and volume was calculated by the formula \((\text{W} \times \text{L})^2/2\), where \(W\) is width and \(L\) is length (19).

Melphalan Pharmacokinetics (in Vivo)

Non-Tumor-bearing Animals. Sixty non-tumor-bearing athymic nude mice were given injections i.p. of melphalan at the 10\% lethal dose (71.3 mg/m\(^2\)). Groups of 5-6 mice were anesthetized with halothane and blood was obtained by cardiac puncture at 1, 10, 15, 30, 45, 60, 90, 120, 150, and 180 min postinjection. Plasma obtained from these samples was frozen (−70 \(^\circ\)C) for subsequent measurement of melphalan levels.

Tumor-bearing Animals. Two groups of athymic nude mice bearing s.c. TE-671 or Daoy xenografts, respectively, were given injections i.p. of melphalan at the 10\% lethal dose when the median tumor volumes exceeded 200 mm\(^3\) (TE-671) or 350 mm\(^3\) (Daoy). Groups of 3 mice in each group were anesthetized with halothane and blood was obtained by cardiac puncture, and the xenografts were surgically resected at 15, 60, 120, and 180 min following injection of drug. Plasma obtained from the blood samples was frozen (−70 \(^\circ\)C) for subsequent measurement of melphalan levels.

Transport Studies

Time Course of \(\left[^{3}H\right]\)Melphalan Uptake. The uptake of \(\left[^{3}H\right]\)melphalan by TE-671 and Daoy cells was linear with time over the first 5 min with subsequent nonlinear drug accumulation. Representative data are shown in Fig. 1, demonstrating that drug accumulation at 20 min was markedly greater in TE-671 cells. The cellular ratio of \(\left[^{3}H\right]\)melphalan, corrected

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\(^3\) The abbreviations used are: BCH, dl-\(\alpha\)-2-aminobicyclo[2.2.1]heptane-2-carboxylic acid; PBS, phosphate-buffered saline; GSH, glutathione; GST, glutathione-S-transferase; \(\left[^{3}H\right]\)melphalan, L-\(\alpha\)-dl-2-chloroethylamino)-(\(\alpha\)-methyl)phenylalanine.
for the surface binding of \(^{3}H\) melphalan, is plotted in Fig. 2 as a function of the concentration of free drug. Cell volumes for TE-671 and Daoy were measured as 23.0 \(\times 10^{-13}\) liter/cell and 36.0 \(\times 10^{-13}\) liter/cell, respectively. Uptake of \(^{3}H\) melphalan was strongly dependent upon temperature. At 37°C, cell:medium ratios in excess of unity were observed for both TE-671 and Daoy, but at 0°C no uptake could be demonstrated. Rapid surface binding of \(^{3}H\) melphalan to TE-671 and Daoy at 0°C was subtracted from each time recording of \(^{3}H\) uptake at 37°C (this represented 5% of the total radioactivity at 5 min).

Lineweaver-Burk plots of \(^{3}H\) melphalan uptake by TE-671 and Daoy revealed linear relationships (correlation coefficients of 0.975 and 0.965, respectively) allowing derivation of apparent kinetic parameters in the concentration range of 3.3 to 100 \(\mu M\) (Table 1). Comparison of the respective \(K_m\) values (\(P = 0.8273\)) and \(V_{max}\) values (\(P = 0.0495\)) show significant differences only for the \(V_{max}\) values.

Inhibition by BCH or Absence of Sodium. The effects of BCH (5 mM) or sodium absence upon \(^{3}H\) melphalan uptake at 1 min are shown in Table 2. Although biphasic Lineweaver-Burk plots were not seen between melphalan concentrations of 3.3–100 \(\mu M\), these inhibition studies support the presence of at least two carrier systems mediating drug uptake (similar if not identical to the BCH-sensitive Na\(^+\)-independent L system and the BCH insensitive Na\(^+\)-dependent ASC-like system) (20–22).

GST and GSH Measurements (in Vitro)

The two cell lines differed in their GST values (measured with 1-chloro-2,4-dinitrobenzene) (Table 1) with levels of (higher) 91.52 ± 13.50 (SD) in TE-671 and 50.31 ± 16.36 nmol/min/mg protein in Daoy.

The GSH levels for the two cell lines (Table 1) were likewise different, 10.06 ± 2.2 in TE-671 and 2.96 ± 2.11 nmol/10\(^6\) cells in Daoy.

Tumor (s.c.) GSH Measurements

The GSH values of the s.c. xenografts at the time of melphalan administration were TE-671, 1.02 ± 0.39, and Daoy, 2.01 ± 0.57 \(\mu M/g\) wet weight tumor (7.79 ± 1.61 and 13.68 ± 5.15 nmol/mg protein, respectively). This 2-fold difference in the GSH levels of the s.c. tumors was smaller than for the cell lines (Table 1).

Pharmacokinetics of Melphalan (in Vivo)

Analysis of plasma melphalan levels in non-tumor-bearing mice revealed a mean peak plasma concentration of 50.3 \(\mu M\) occurring at 10 min, a half-life of 29.9 min, and a concentration \(\times\) time value (0 to \(\infty\)) of 2862 \(\mu M\) \(\cdot\) min (Fig. 3). The pharmacokinetics of melphalan in plasma in mice bearing s.c. TE-671 or Daoy xenografts was similar to that in non-tumor-bearing animals. However, melphalan levels in the tumors were 2- to 3-fold higher in the TE-671 s.c. xenografts than in the Daoy xenografts (Fig. 4) (Table 3).

**DISCUSSION**

Laboratory and clinical studies to date suggest the antitumor activity of alkylating agents in the treatment of medulloblas-
The identification of melphalan in our previous laboratory work as the most active alkylating agent studied against TE-671, Daoy, and D283 MED (8, 9), coupled with the early results of our current Phase II trial of melphalan in the treatment of patients with recurrent medulloblastoma (1 complete response and 2 partial responses in the first 12 patients treated) prompted this current study of mechanisms potentially modulating cytotoxicity of this alkylator in human medulloblastoma. Mechanisms analyzed included melphalan transport, glutathione levels, and glutathione-S-transferase activity.

The role of altered transport in mediating changes in melphalan cytotoxicity has been previously described in non-human cell lines. The competitive effects of similarly transported large neutral amino acids (such as leucine or glutamine) in decreasing melphalan transport and cytotoxicity have been defined in murine L1210 leukemia cells (26). Intrinsic transport alterations resulting in decreased intracellular accumulation and cytotoxicity of melphalan have been demonstrated in murine L1210 leukemia cells (27) and Chinese hamster ovary cells (28, 29). Studies addressing melphalan transport in human cell lines or tumors are more limited. Begleiter et al. (30) defined the melphalan transport parameters in human lymphocytes and breast cancer cells, revealing the presence of both BCH-sensitive and Na⁺-sensitive amino acid carriers. Dufour et al. (31) defined melphalan transport parameters in a human adenocarcinoma of unknown etiology, revealing $K_m$, $37 \mu M$, and $V_{max}$, 45 pmol/intracellular $\mu l/min$. Our results revealed transport parameters confirming facilitated transport of melphalan in human medulloblastoma. The $K_m$ values in the medulloblastoma lines reported here (Table 1) were considerably higher than those reported for breast cancer cells, human adenocarcinoma, or human lymphocytes. The $V_{max}$ values for the two medulloblastoma lines were much higher than for human lymphocytes and similar (Daoy) to adenocarcinoma. There was greater intracellular drug accumulation in TE-671 compared to Daoy (presumably due to the higher $V_{max}$ in TE-671) (Figs. 1 and 2).

The differences in drug transport seen in vitro appear to be reflected in vivo as well, with higher intratumor and tumor/plasma melphalan levels in s.c. TE-671 following administration of the therapeutic dose (Fig. 4). In order to relate the in vivo concentrations of melphalan at therapeutic doses with those used in transport studies in vitro, the pharmacokinetics of melphalan was measured. The peak plasma concentration of melphalan, determined 10 min after an i.p. dose of 71.3 mg/m² (the 10% lethal dose) was 50.3 $\mu M$. With a half-life of 29.9 min, the plasma concentrations remained above the in vitro drug dose at which there is a 90% reduction in the number of colonies in comparison to controls for the TE-671 and Daoy cell lines, as previously determined by clonogenic assay (8, 9), for approximately 2 h (Fig. 3). The intracellular concentrations of melphalan in the s.c. TE-671 xenografts were 26.7 $\mu M$ and 10.4 $\mu M$ at 1 and 2 h, respectively, and 13.4 and 3.1 $\mu M$ for Daoy at the same time points.

The relationship between melphalan cytotoxicity and cellular transport and thiols in medulloblastoma. Previous studies in our laboratory have demonstrated the cytotoxicity of a series of classical alkylating agents to the human medulloblastoma cell lines and transplantable xenografts TE-671 and Daoy grown in vitro and in s.c. and intracranial sites in athymic nude mice (8, 9). The clinical studies of Allen et al. (23, 24) have confirmed cyclophosphamide as the most active single agent in the treatment of medulloblastoma. Combination of cyclophosphamide at a lower, less myelosuppressive dose (than used in Allen's studies) with vincristine yielded similarly impressive results (25).

![Graph](image-url)  
Fig. 3. Murine plasma levels of melphalan after i.p. administration of 100% of the 10% lethal dose. Points, mean values of 5-6 determinations.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Plasma (µM)</th>
<th>Tumor (nmol/g)</th>
<th>Tumor/plasma ratio</th>
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<tbody>
<tr>
<td>15</td>
<td>36.8 ± 5.9*</td>
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<td>0.46</td>
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<tr>
<td>60</td>
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<tr>
<td>120</td>
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<td>10.4 ± 3.1</td>
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<td>180</td>
<td>6.1 ± 4.3</td>
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<td>1.49</td>
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* Mean ± SD.

<table>
<thead>
<tr>
<th>Time (min)</th>
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<th>Tumor (nmol/g)</th>
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<tr>
<td>60</td>
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<tr>
<td>180</td>
<td>1.0 ± 0.5</td>
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![Graph](image-url)  
Fig. 4. Xenograft (s.c.) melphalan levels after i.p. administration of 100% of the 10% lethal dose. Points, mean values of 3 determinations.
glutathione levels has been described previously. Suzukake et al. (32, 33), using murine L1210 leukemia cells resistant to melphalan, have defined the role elevated glutathione plays in mediating this resistance. Recent studies have identified a spectrum of glutathione values in human cell lines (34–38). Furthermore, identification of melphalan-resistant ovarian carcinoma cell lines with glutathione values of 4.58 and 6.13 nmol/10^6 cells indicates that elevated GSH levels conferring drug resistance may be tumor cell line specific; values of GSH conferring alkylator resistance in one cell line may be associated with drug sensitivity in a different tumor cell line. Our results revealed a high GSH level (in comparison to other human tumor cell lines) of 10.06 ± 2.2 nmol/10^6 cells in TE-671 cells, which were nevertheless sensitive to melphalan. Definition of GSH levels in human tumors associated with drug resistance will require studies with the specific tumor target of interest, since these levels will clearly vary in different malignancies.

Glutathione levels have not been previously measured in human medulloblastoma with the exception of TE-671 s.c. xenografts (39), and further studies will be needed to define the range of values seen in this tumor. We have previously demonstrated enhancement of melphalan activity against s.c. TE-671 xenografts in athymic mice following buthionine sulfoximine-mediated glutathione depletion to 0.29 μmol/g (39), supporting this approach as both an enhancer of alkylator activity in drug-sensitive cells (as well as its potential value of reversing GSH-dependent alkylator resistance) in medulloblastoma.

The relationship between glutathione-S-transferase and resistance to chemotherapeutic agents is still being defined. Elevated levels of glutathione-S-transferase have been seen in a few drug-resistant nonhuman and human tumor cell lines. The initial observation of Wang and Tew (40) of increased glutathione-S-transferase in a Walker 256 carcinoma cell line resistant to bifunctional nitrogen mustards is provocative, particularly, since the sensitive and resistant cell lines demonstrated similar total glutathione levels. Batist et al. (41) have identified an increase of an anionic glutathione-S-transferase in multidrug-resistant human breast cancer cells, with values of 161 nmol/min/mg protein in the resistant line compared to 3.6 nmol/min/mg protein in the parent line. Teicher et al. (42) demonstrated glutathione-S-transferase activity of 1145 nmol/min/mg protein in a cis-diaminedichloroplatinum(II)-resistant human squamous carcinoma cell line (compared to a baseline value of 503 nmol/min/mg protein in the parent sensitive cell line). Recent studies (43) have defined a diverse spectrum of glutathione-S-transferase activity in human tumor biopsies. Our results with TE-671 (91 nmol/min/mg protein) and Daoy (50 nmol/min/mg protein) are the first measurements of glutathione-S-transferase in human medulloblastoma, and are in the same range as previously reported values in other human tumors. Nevertheless, identification of glutathione-S-transferase-mediated alkylator resistance in human medulloblastoma will require studies limited to this tumor, since, as with glutathione, glutathione-S-transferase levels associated with drug resistance will no doubt vary in different malignancies. Our investigations with TE-671 and Daoy are the first to measure melphalan transport, glutathione levels, and glutathione-S-transferase activity in human medulloblastoma and will allow further work defining their role in mediating resistance to melphalan in this tumor. Since TE-671 and Daoy are both sensitive to melphalan, analyses of differences in the cytotoxicity of this drug mediated by these mechanisms are of limited value, and further studies, in spontaneous and acquired melphalan-resistant human medulloblastoma cell lines and xenografts (which are not presently available) will be necessary. Nevertheless, the current results in TE-671 and Daoy highlight the complex interrelationships mediating melphalan cytotoxicity. As discussed above, transport differences facilitating greater intracellular melphalan accumulation in TE-671 compared to Daoy may be responsible for the greater sensitivity of TE-671 in vitro, and are consistent with the higher intratumor and tumor/plasma melphalan levels in TE-671 s.c. xenografts following administration of the therapeutic dose. In vivo therapeutic studies with TE-671 and Daoy have demonstrated responses of both xenografts at s.c. and intracranial sites. TE-671, despite greater in vitro melphalan cytotoxicity and 2-fold higher accumulation of melphalan in the s.c. xenografts, displayed growth delays smaller than those seen for s.c. Daoy xenografts. Growth delays following treatment with melphalan for s.c. TE-671 were 20.9 and 19.5 days (duplicate trials) and 90+ and 63.4 days (duplicate trials) for Daoy. Glutathione values in the s.c. xenografts did not explain these results, with higher levels in the Daoy xenografts. However, the apparent greater sensitivity of the s.c. Daoy xenografts was not evident at the intracranial site. Increases in median survival for animals bearing intracranial TE-671 and Daoy following treatment with melphalan were 64 and 42% (duplicate trials) and 33 and 14% for Daoy (8, 9). Although we have previously defined the blood flow and blood-to-tissue transport in TE-671 xenografts grown intracranially in nude athymic rats, demonstrating high rates of blood-to-tissue transport (44), similar data are not yet available for Daoy. More restricted delivery to the intracranial site of Daoy xenografts is a potential explanation for the dichotomy in the response of s.c. and intracranial Daoy xenografts.

Thus, precise analysis of the contribution of transport and glutathione alterations in modulating cytotoxicity in human medulloblastoma may prove difficult, due to the potential presence of multiple alterations (with opposing actions). Nevertheless, definition of transport parameters, glutathione levels, and glutathione-S-transferase activity in human medulloblastoma will facilitate identification of those mechanisms important in the generation of drug resistance. Studies in progress are evaluating mechanisms of resistance operational in TE-671 serially exposed to melphalan in vitro or in vivo with resultant decreased cytotoxicity to this drug, as well as in cell lines and xenografts derived from the tumors of patients which proved resistant (either at initial or subsequent chemotherapy intervention). This approach, in conjunction with similar studies with fresh medulloblastoma specimens obtained from patients failing treatment with our Phase II trial of melphalan for recurrent medulloblastoma, will provide an understanding of those mechanisms mediating clinical resistance to this agent and the utility of the cell lines and xenografts to serve as models allowing definition (and modulation) of this resistance.

REFERENCES
MELPHALAN TRANSPORT AND THIOLS IN MEDULLOBLASTOMA

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