Melphalan Penetration of the Blood-Brain Barrier via the Neutral Amino Acid Transporter in Tumor-bearing Brain

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ABSTRACT

Melphalan, a nitrogen mustard derivative of the neutral amino acid L-phenylalanine, was transported across the rat blood-brain barrier by the large (L-system) neutral amino acid transporter in tumor-bearing brain, but no evidence for blood-brain barrier transport by the alanine-serine-cysteine system carrier was obtained in the present study. The ability of melphalan to inhibit phenylalanine uptake was compared in rats implanted with two experimental CNS tumors: the C-6 glioma (a model of primary brain tumors) and Walker carcinoma (a model of metastatic brain tumors). The melphalan concentration which caused 50% inhibition of blood-brain barrier (BBB) phenylalanine uptake (K_i) was 0.49 ± 0.18 mM in the Walker tumor, compared with 0.46 ± 0.19 mM in the contralateral control brain. In the ipsilateral hemisphere (K_i = 0.59 ± 0.25 mM) and contralateral hemisphere (K_i = 0.45 ± 0.19 mM), drug entry was also via the neutral amino acid transporter. In C-6 gliomas (K_i = 0.77 ± 0.20 mM) and contralateral control brain (K_i = 0.84 ± 0.29 mM), melphalan also inhibited BBB phenylalanine transport. A major finding was that, at melphalan concentrations greater than 1.0 mM, BBB permeability of radiolabeled indium (chelated to EDTA) increased in proportion to melphalan concentration. In the contralateral hemisphere of rats implanted with C-6 gliomas, brain extractions of indium-EDTA measured 3 to 4% in the absence of drug, 5 to 6% at 2.5 mM melphalan, and 9 to 10% at 5 mM melphalan. A similar phenomenon was observed in the nontumoral brain regions of rats implanted with Walker carcinoma cells. In normal (nonimplanted) rats, melphalan’s inhibition (K_i = 0.29 mM) of phenylalanine and tryptophan (K_i = 0.20 mM) uptake was confirmed, and brain extraction of sucrose (a nonspecific marker which does not penetrate the intact BBB) was observed to increase in proportion to melphalan concentration. We conclude that melphalan not only enters the brain via the neutral amino acid transporter, but at higher concentrations (>1 mM) may open the blood-brain barrier in a nonspecific manner.

INTRODUCTION

Melphalan is an antitumor drug used in treatment of carcinoma of the breast (1) and ovary (2) and in the treatment of multiple myeloma (3). This drug is a nitrogen mustard derivative of the neutral amino acid L-phenylalanine [see Greig et al. (4) for the structures]. Studies of tumor cells in vitro suggest that this drug is taken up by the sodium-independent L-system (5, 6) which transports large neutral amino acids (e.g., valine, leucine, tyrosine, isoleucine, phenylalanine, and tryptophan). In LPC-1 plasmacytoma cells, melphalan transport is reduced by the amino acids alanine, serine, and cysteine, suggesting that a second carrier, the ASC system, is also capable of transporting melphalan (7). In the rat BBB, melphalan was shown to be transported by the large neutral amino acid carrier, the L-system (4).

In metastatic brain tumors, fenestrated capillaries characteristic of the tissue of origin have been observed in contrast to the typical BBB endothelia (8). In primary brain tumors, abnormalities of interendothelial tight junctions, fenestrations, and vesicular profiles have been reported in several studies (9-11), indicating that mechanical features of the BBB are compromised. A question arises then as to whether nutrient transporter systems (which regulate blood-to-brain transit of glucose, essential amino acids, nucleic acid precursors, and choline) are quantitatively or qualitatively altered in brain tumors. Since melphalan enters brain tissue via the large neutral amino acid transporter, any alteration in transport of large neutral amino acids such as phenylalanine at the blood-tumor barrier would profoundly affect drug distribution to the target site. Pollay (12) has developed a method for the regional study of phenylalanine transport in both right and left forebrain hemispheres, which is particularly applicable to the comparative study of implanted tumor and contralateral nontumor, forebrain.

Brain capillary transporters can be characterized like enzymes by their kinetic constants K_m and V_max; the latter term describes the maximal rate at which substrate is transported, while the affinity constant (K_m) identifies that substrate concentration at which 50% of the transporters are occupied. The affinity of blood-brain barrier transporters for amino acids is unusually high compared with other organs (13), and this high affinity is characterized by low half-saturation constants (K_m) which are in the 25 to 100 μM range (14). These BBB K_m values approximate normal plasma concentrations of amino acids, but are 1 to 2 log orders less than the K_m values of cell membrane transporters mediating amino acid influx in non-brain tissues (13, 15). Because of these great differences in amino acid transport between CNS (μM K_m) and peripheral tissues (mM K_m) (15), the present study compares melphalan’s ability to inhibit phenylalanine uptake in two experimental CNS malignancies, both primary (C6 glioma) and metastatic (Walker 256 carcinoma) brain tumor models.

MATERIALS AND METHODS

Radiochemicals. The tin-indium TFC3 generator was obtained from Amersham International, Buckinghamshire, England. The other isotopes ([1H]phenylalanine and [14C]diazepam) were obtained from Amersham Australasia, Auckland, New Zealand. Radiochemical purity of the isotopes was confirmed by thin-layer chromatography on glass-backed silica gel plates. Isotopic scanning was performed on a Tracemaster Model LB285 linear analyzer (Berthold Analytical Instruments Corporation, Nashua, NH).

Injection Solutions. A mixture was prepared containing about 0.5 μCi of [14C]diazepam, 5 μCi of [1H]phenylalanine, and approximately 50 μCi of [113mIn] per 0.1 ml of buffered saline. The indium was eluted
from the generator in dilute HCl and chelated to EDTA as described previously (16). Each 1.0 ml of eluate was mixed with 10 μl of EDTA (150 mg/ml) and neutralized with 1 N NaOH. The saline was buffered with N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid to a final concentration of 10 mM (pH 7.55) and contained 0 to 20 μM melphan (phenylalanine mustard; Sigma Chemical Company, St. Louis, MO).

Experimental Tumor Models. Walker 256 tumor cells and C-6 glioma cells were grown in α-minimal essential medium supplemented with fetal bovine serum (10%, v/v), penicillin (100 units/ml), and streptomycin (100 μg/ml). To prepare the cells for cerebral implantation, C-6 glioma cells were trypsinized, washed, and resuspended in phosphate-buffered saline at a concentration of 5 × 10^6 cells per 10 μl. Walker 256 cells were maintained in medium but adjusted to a concentration of 1 × 10^6 cells per 10 μl for implantation.

Prior approvals from the respective institutional animal use committees were obtained. Female Wistar rats (150 to 200 g) were anesthetized with halothane and placed in a stereotactic frame for tumor implantation. A midline incision 1 to 2 cm in length was made, and a high-speed drill was utilized to make a small burr hole in the left parietal skull, 2 mm caudal of the coronal suture and 4 mm lateral of the sagittal suture. The medium containing tumor cells was drawn into a 50-μl graduated syringe (Hamilton Company, Reno, NV) fitted with a 30-gauge needle and mounted on one arm of the stereotactic frame. The needle was positioned vertically above the burr hole, inserted 5 mm deep into the cerebral tissue, and withdrawn 1 mm to a final depth of 4 mm. The tumor cells (10 μl) were injected into the cerebral tissue, and the burr hole was sealed with cyanoacrylate. The scalp incision was closed, and the animals were allowed to recover for subsequent use.

Brain uptake measurements were performed either 7 to 9 days postinoculation (Walker 256 carcinoma) or 14 to 21 days postinoculation (C-6 gliomas). They were maintained in a ventilated, temperature-controlled (21 ± 1°C) environment on a bed of wood shavings, with access to food and water ad libitum and a 12-h light-dark cycle.

**Drug Penetration into the Brain.** Rats were weighed and anesthetized by i.p. injection of sodium pentobarbital (Nembutal; Bomac Labs., New South Wales, Australia; 50 to 60 mg/kg), or in the case of nonimplanted rats, i.m. ketamine (230 mg/kg) and xylazine (2.3 mg/kg). The anesthetized rat, it was determined that the E3H@ = 0.61 ± 0.07 and that E3H@ = 0.77 (21). Thus, extractions (E%) can be derived from BUI measurements determined with either water or butanol as the reference.

Indium quantitation in a beta counter is described in detail elsewhere (22). The 113In, chelated to EDTA, does not cross intact cell membranes. Thus, in the above ratio the minuend indicates that proportion of test isotope (e.g., phenylalanine) remaining in the brain vasculature. In studies of tryptophan and phenylalanine entry into normal brain, this is a very small (<2%) fraction; BUIs were determined without indium, and the (uncorrected) BUI is simply defined as the subtraction (E3H@/E3H@) of the above ratio (18, 19). In pathophysiological states such as tumors, the intravascular quantity of test isotope may be quite variable, and indium-corrected measurements provide a more precise estimate of brain extractions by measuring changes in the vascular compartment.

Cerebral Blood Flow Rates. Cerebral blood flow rates in the tumor-containing hemisphere and contralateral control sides were compared using the artificial organ method of van Uitert and Levy (23). Under barbiturate anesthesia, an arterial cannula was connected to a peristaltic pump calibrated to withdraw at the rate of 0.50 ml/min. At time zero, a mixture (containing 1.5 μCi of [14C]butanol in 100 μl of buffered saline) was injected i.v., and the withdrawal pump was activated. Exactly 20 s later the animal was decapitated, and arterial withdrawal was abruptly halted. The brain regions of interest were dissected out and placed in tared scintillation vials, and cerebral blood flow rates were determined from the ratio of radioactivity in the tissue and artificial organ (23).

Fifty % Inhibition Constants. Estimates of transport parameters were derived as described by Partridge and Mietus (24) using Program BMDP3R (25). Unidirectional extraction (E) is a function of the PS and cerebral blood flow (F), i.e., $E = 1 - e^{-F/PS}$, and

$$PS = \frac{V_{\text{max}} K_m + C}{K_m + C}$$

where C is the substrate concentration injected, $V_{\text{max}}$ is the maximal velocity, $K_m$ is the half-saturation constant, and $K_d$ is the diffusion component. In the present analyses of unlabeled melphan (substrate) inhibition of neutral amino acid transport, the name of the half-saturation constant is changed to $K_i$ (50% inhibition constant) and is the only parameter reported. Data are presented in the form of mean ± SD (n = 3 to 6) unless otherwise indicated. The Student t test was used to compare control and treatment groups.

**RESULTS**

Brain extraction of a tracer concentration (≤0.01 mM) of radiolabeled phenylalanine, measured in rats 7 days after implantation of Walker 256 carcinoma cells, was inhibited in the...
MELPHALAN-INDUCED OPENING OF THE BBB

Table 1  Half-inhibition constant (K_i) of L-phenylalanine mustard (melphalan) competition for blood-brain barrier neutral amino acid (phenylalanine) transport in rat brains implanted with C-6 gliomas or Walker carcinomas.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Anesthetic</th>
<th>Cerebral blood flow rate (ml/min/g)</th>
<th>Melphalan K_i (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-6 glioma</td>
<td>PB*</td>
<td>0.51 ± 0.10*</td>
<td>0.77 ± 0.20*</td>
</tr>
<tr>
<td>Contralateral control</td>
<td>PB</td>
<td>0.56 ± 0.08</td>
<td>0.84 ± 0.29</td>
</tr>
<tr>
<td>Ipsilateral hemisphere</td>
<td>PB</td>
<td>0.60 ± 0.10</td>
<td>0.78 ± 0.52</td>
</tr>
<tr>
<td>Contralateral hemisphere</td>
<td>PB</td>
<td>0.56 ± 0.09</td>
<td>0.78 ± 0.43*</td>
</tr>
<tr>
<td>Walker carcinoma</td>
<td>PB</td>
<td>0.38 ± 0.12</td>
<td>0.49 ± 0.18</td>
</tr>
<tr>
<td>Contralateral control</td>
<td>PB</td>
<td>0.43 ± 0.09</td>
<td>0.46 ± 0.19</td>
</tr>
<tr>
<td>Ipsilateral hemisphere</td>
<td>PB</td>
<td>0.42 ± 0.08</td>
<td>0.57 ± 0.25</td>
</tr>
<tr>
<td>Contralateral hemisphere</td>
<td>PB</td>
<td>0.45 ± 0.10</td>
<td>0.45 ± 0.19</td>
</tr>
</tbody>
</table>

* PB, pentobarbital (60 mg/kg, i.p.).

Cerebral blood flow rates were measured in a separate group of animals using the artificial organ technique. In 5 barbiturate-anesthetized rats bearing C-6 glioma implants, cerebral blood flow rates averaged about 0.6 ml/min/g (Table 1). In contrast, in 5 rats bearing Walker carcinomas, cerebral blood flow rates were lower, about 0.4 ml/min/g. The life-span of the latter rats was only 8 to 10 days postimplantation, they lost 10 to 23% of their body weight, and their reduced cerebral blood flow rates correlated with observed increases in susceptibility to the barbiturate anesthetic.] Within each tumor group, no significant regional differences in blood flow rate were apparent in the tumor or ipsilateral or contralateral brain. Autoradiographic methods (which more precisely discriminate tumor tissue) have established that significantly lower flow rates occur in rat tumors, but flow rates derived in these studies (27-30) are comparable to the present estimates (Table 1). The half-maximal inhibition constant of phenylalanine uptake by melphalan was determined (K_i about 0.8 mM in glioma-bearing, and K_i about 0.5 mM in Walker carcinoma-implanted rats) for each of the brain regions and tumor conditions studied (Table 1).

Studies with cultured breast cancer cells and lymphocytes (31) suggest that melphalan is transported via an ASC system in addition to the large neutral L-system amino acid transporter. The suggestion of amino acid transport across the BBB via an ASC system has been raised by Tovar et al. (32). To test for the possibility of melphalan entry into experimental brain tumors in vivo via an ASC system, brain extraction of radiolabeled serine was measured in the presence and absence of melphalan in both glioma-implanted and carcinoma-implanted (Table 2) rat brains. As indicated, melphalan did not appear to inhibit brain serine uptake in either tumor or contralateral brain (Table 2; the listed brain extractions were not significantly different in any of the regions or treatment groups analyzed), and no evidence for melphalan entry via a serine locus (ASC system) was apparent.

Because intracranial hemorrhage was observed in Walker carcinoma-implanted rats, the possibility that alterations in the size of the vascular compartment (especially in the tumor and peritumoral brain) was considered. Examination of brain extractions of the 113mIn-EDTA chelate, a marker which behaves in vivo similarly to sucrose, confirmed this expectation but suggested unexpected effects of melphalan on the BBB. Brain extraction of 113mIn-EDTA, when measured in the absence of melphalan, was observed to be significantly higher in the C-6 glioma than in the other brain regions (Fig. 3), consistent with the concept that some loss of mechanical barrier function occurs.

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Fig. 1. Increasing concentrations of unlabeled phenylalanine mustard (melphalan) inhibit brain extraction of tracer concentrations (0.007 mM) of phenylalanine. Transport measurements were performed 7 days after implantation of tumor cells. Note that, at each of the drug concentrations tested, indium-corrected phenylalanine extraction is inhibited to a similar degree in both Walker carcinoma tissue and the contralateral control brain (bottom), as well as in the tumoral (ipsilateral) hemisphere and contralateral brain (top). Points, mean; bars, SD.

Fig. 2. Increasing concentrations of melphalan inhibit indium-corrected blood-brain barrier extraction of radiolabeled phenylalanine (0.008 mM). Transport measurements were performed 14 days after tumor cell implantation. The ability of melphalan to saturate phenylalanine extraction indicates that this drug is carried on the neutral amino acid transporter in C-6 glioma and control tissues, as well as ipsilateral and contralateral brain. Columns, mean; bars, SD.

Contralateral hemisphere PB 0.56 ± 0.08 0.78 ± 0.52
Ipsilateral hemisphere PB 0.56 ± 0.09 0.78 ± 0.43
Walker carcinoma PB 0.38 ± 0.12 0.49 ± 0.18
Contralateral control PB 0.43 ± 0.09 0.46 ± 0.19
Ipsilateral hemisphere PB 0.42 ± 0.08 0.57 ± 0.25
Contralateral hemisphere PB 0.45 ± 0.10 0.45 ± 0.19
in tumor and peritumoral brain. The unexpected finding was that at concentrations of melphalan > 1 mM, nonspecific uptake of $^{113m}$In-EDTA across the BBB more than doubled, and both BBB and blood-tumor barriers were compromised by high concentrations of melphalan (Fig. 3). Similarly, in Walker carcinoma-implanted rats, increased brain extraction of indium-EDTA was also seen; melphalan (>1 mM) compromised BBB function in a concentration-dependent manner (Fig. 4).

Additional brain uptake studies (without indium correction) were performed in normal (nonimplanted) rats. The inhibition of brain phenylalanine extraction by melphalan was confirmed (Fig. 5). At melphalan concentrations < 1 mM, inhibition of phenylalanine uptake was apparent; at > 1 mM (uncorrected), brain phenylalanine extraction increased. Brain extraction of sucrose, a compound which does not penetrate the intact BBB, also increases at melphalan concentrations above 1 mM (Fig. 5). Thus, it appears that high concentrations of melphalan compromise the BBB of normal rats, similar to the situation observed in tumor-bearing animals (Figs. 3 and 4).

Tryptophan is another amino acid which shares the BBB large neutral amino acid transporter. Brain tryptophan uptake in normal rats is also inhibited by increasing concentrations of melphalan (Fig. 6). The half-maximal constants of melphalan's inhibition of brain phenylalanine ($K_i = 0.29 \pm 0.10$ mM) and tryptophan ($K_i = 0.20 \pm 0.16$ mM) (extraction in the normal rat brain) did not differ significantly. Other studies indicated that melphalan has no affinity for either the BBB hexose transporter or the basic amino acid transporter. Brain glucose

$E = 9.8 \pm 0.4$% in the absence of melphalan, and $E = 9.9 \pm 1.0$% with the addition of 1.0 mM unlabeled melphalan. Similarly brain arginine $E = 6.6 \pm 0.5$% versus $E = 6.4 \pm 1.1$% in the presence of 1.0 mM melphalan in ketamine-anesthetized, normal rats.

**DISCUSSION**

If the tumor retains transporter properties comparable to its tissue of origin, then great differences in the metastatic (Walker carcinoma) versus the primary CNS (C-6 glioma) tumor would be anticipated. In the present study we found no evidence for such a phenomenon; forebrain implants of both tumor types appeared to exhibit BBB-like inhibition of the $L$-system neutral amino acid transporter, and melphalan inhibition of a possible ASC locus (see Refs. 32 and 33) could not be demonstrated. It should also be noted that the "tumor" tissues sampled in the present study invariably also contained some tumor-adjacent brain tissue. Therefore the possibility exists that small quantitative changes in blood-tumor barrier melphalan entry may have gone undetected. The observation that the half-saturation

**Table 2 Brain extraction of serine in rats implanted with C-6 glioma or Walker carcinoma cells**

<table>
<thead>
<tr>
<th>Brain region</th>
<th>C-6 glioma implants in the presence of</th>
<th>Walker carcinoma-implanted brain with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Phenylalanine (0.1 mM)</td>
</tr>
<tr>
<td>Tumor and peritumor</td>
<td>8.0 ± 0.7</td>
<td>5.2 ± 0.9</td>
</tr>
<tr>
<td>Contralateral control</td>
<td>5.5 ± 1.9</td>
<td>3.3 ± 1.4</td>
</tr>
<tr>
<td>Ipsilateral hemisphere</td>
<td>8.0 ± 2.5</td>
<td>5.2 ± 0.9</td>
</tr>
<tr>
<td>Contralateral hemisphere</td>
<td>7.1 ± 1.0</td>
<td>5.3 ± 1.2</td>
</tr>
</tbody>
</table>

* Mean ± SD.
drug concentrations (>1 mM) melphalan), brain extractions of both phenylalanine and sucrose are increased. Sucrose, like indium-EDTA, does not penetrate intact alanine uptake by melphalan was derived from these data. Note that, at higher rats. The half-maximal constant for inhibition ($K_i = 0.29 \pm 0.10 \text{ mM}$) of phenylalanine uptake by melphalan was derived from these data. Note that, at higher drug concentrations (>1 mM melphalan), brain extractions of both phenylalanine and sucrose are increased. Sucrose, like indium-EDTA, does not penetrate intact BBB endothelia, and the increased uptake of this marker indicates that the BBB is compromised by high melphalan levels in the normal rat brain. Points, mean; bars, SE.

constant for melphalan-inhibited neutral amino acid uptake was greater in glioma-bearing than Walker carcinoma-implanted brain (Table 1) is possibly related to the recent observation that factors secreted by C-6 glioma cells (in vitro) have been shown to increase brain capillary permeability in vivo (34).

This study establishes that, at >1 mM concentrations, melphalan is able to open the BBB in both normal and tumor-implanted brain. Therapeutic melphalan concentrations range from 0.1 to 9.0 $\mu\text{M}$ (4). This suggests that, in addition to competing for the large neutral amino acid locus in CNS and peripheral tissues, clinicians using this drug should be aware of its potential to open the BBB and thus produce CNS side effects. Patients receiving other drugs, such as cisplatin (35), in combination with melphalan may be at a greater risk for such side effects, with neurotoxic sequelae.

Intracarotid administration of chemotherapeutic agents, although still investigational, is sometimes used in the treatment of gliomas; the fact that some patients who fail systemic chemotherapy have responded to similar or lower doses of the same drugs is an often-cited argument in favor of this treatment method (36). For drugs which do not readily penetrate the BBB, Neuweit and coworkers (37, 38) have demonstrated that intraarterial delivery of hyperosmotic agents (such as mannitol or arabinose) transiently opens the BBB to deliver antineoplastic agents to the brain. Although controversial (see Ref. 27), this technique has shown promise in augmenting delivery of non-neurotoxic drugs (such as methotrexate, cyclophosphamide, and procarbazine) to the central nervous system (37, 38).

The present demonstration that melphalan, an antitumor agent, is also capable of opening the BBB after intracarotid administration suggests the possible role of phenylalanine mustard drugs in enhancing brain drug delivery. For example, if intracarotid delivery of melphalan is tested, side effects such as narrow toxicity might be reduced through this drug delivery route. Furthermore, although melphalan and cyclophosphamide are both alkylating agents, the other two drugs which have shown promise in intraarterial delivery studies, methotrexate (an antimitabolite agent) and procarbazine (a monoamine oxidase inhibitor) (37, 38), have different modes of pharmacological action, which might be augmented by concomitant melphalan administration.

The observation that high concentrations of melphalan cause a nonspecific increase in BBB permeability also serves to emphasize the need for assaying the blood vascular space or simultaneously measuring nonspecific permeation in studies of BBB transport. In the study of Greig et al. (4), PS area products on the order of 5.7 to 4.4 (ml/s/g X 10³) for melphalan were reported at 8 and 16 mM, concentrations of 100- and 50-fold greater than their estimated half-saturation constant. In contrast, leucine PS in the presence of 20 mM unlabeled amino acid was 0.6 to 1.1 ml/s/g X 10³ (39, 40), and the isoleucine PS in the presence of 10 mM unlabeled isoleucine is similarly low [0.8 to 1.3 ml/s/g X 10³ (41)]. At saturating concentrations of substrate, neutral amino acid PS values on the order of 1 ml/s/g X 10³ are seen (42), and the 5-fold greater value observed by Greig et al. (4) in the presence of saturating concentrations of melphalan could be attributed to the ability of melphalan to compromise BBB integrity.

Our estimates of the $K_i$ for melphalan’s inhibition of phenylalanine uptake in tumor-bearing ($K_i = 0.5$ to 0.8 mM; Table 1) and normal ($K_i = 0.2$ to 0.3 mM; Figs. 5 and 6) rats are comparable to the half-saturation constant for melphalan uptake in normal rat brain [$K_m = 0.15 \pm 0.06 \text{ mM}$; (4)]. The failure to detect melphalan-induced increases in nonspecific BBB penetration may also have contributed to a slight underestimation of the half-saturation constant by Greig et al. (4).

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