Facilitated Transport of Melphalan at the Rat Blood-Brain Barrier by the Large Neutral Amino Acid Carrier System

Nigel H. Greig, Seiji Momma, Daniel J. Sweeney, Quentin R. Smith, and Stanley I. Rapoport

ABSTRACT

Melphalan has been reported to be actively transported into tumor cells by two amino acid carrier systems. As amino acids are transported across cerebral capillaries by a facilitated mechanism, studies were undertaken to assess whether or not melphalan was transported similarly, and additionally to determine melphalan's plasma and brain pharmacokinetics. The brain uptake of [14C]melphalan was measured by an in situ brain perfusion technique in the anesthetized rat utilizing [14C]-melphalan. The cerebrovascular permeability-surface area product of [14C]melphalan was calculated at cold melphalan concentrations from 0 to 16.3 μmol/ml. The permeability-surface area product was concentration-dependent and decreased from 10.8 ± 0.6 (±SE) x 10^-5 s^-1 at 0.02 μmol/ml melphalan to 5.4 ± 0.3 x 10^-5 s^-1 at 16.3 μmol/ml. The system became saturated at a concentration in excess of 0.1 μmol/ml. The Michaelis-Menten parameters V_max and K_m determined by nonlinear regression analysis of the permeability-surface area product data, equaled 0.9 ± 0.3 x 10^-4 μmol/g and 0.15 ± 0.06 μmol/ml, respectively, for the saturable component of melphalan's brain uptake. The K_m of the nonsaturable component was 5.3 ± 0.3 x 10^-3 s^-1. Addition of the amino acid L-phenylalanine to the brain perfusate inhibited the saturable component of melphalan's brain uptake. The analysis of the plasma and brain concentrations of melphalan by high-performance liquid chromatography, following i.v. melphalan administration, demonstrated that approximately 15% of the drug that was present in plasma entered the brain. These data suggest that the brain uptake of melphalan is facilitated, demonstrating concentration-dependent uptake, saturation, and inhibition, and that melphalan shares the large neutral amino acid carrier system at the blood-brain barrier.

INTRODUCTION

Antineoplastic drugs have been responsible for considerable improvements in the treatment of a variety of extracerebral malignancies. However, the mainstays for the treatment of brain tumors remain surgery and radiation therapy (1). The presence of a blood-brain barrier, although of variable integrity in brain tumors (1, 2), restricts the brain uptake of water soluble compounds (3). While stereospecific, saturable carrier-mediated transport systems exist at the level of the cerebral capillary endothelium to regulate and facilitate the brain uptake of essential water soluble compounds (D-glucose, L-amino acids, and certain nucleic acid precursors and ions) (3, 4), no exogenous drugs have yet been demonstrated to be similarly transported (5).

Melphalan is an anticancer alkylating agent proven to be effective in the treatment of multiple myeloma (6) and of carcinoma of the breast and ovary (7, 8). It is a nitrogen mustard derivative of the large neutral amino acid L-phenylalanine (Fig. 1), and has been demonstrated to be transported into tumor cells via two amino acid carrier systems (9-18). Experiments were undertaken to assess whether melphalan was similarly transported across the blood-brain barrier. A brain perfusion technique was utilized to measure the brain uptake of melphalan independent of the uptake of the endogenous amino acids, which compete for the large neutral amino acid carrier system (19). In addition, as the brain pharmacokinetics of melphalan have not previously been reported, plasma and brain concentrations of drug were measured up to 4 h following the i.v. administration of melphalan to rats. Part of this work has been published as an abstract (20).

MATERIALS AND METHODS

Pharmacokinetic Study

Adult male rats (Fischer 344 strain), weighing 200-250 g, were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The left saphenous vein was exposed and melphalan (Sigma Chemical Co., St. Louis, MO), 10 mg/kg, was injected i.v. (1 ml/kg). At intervals from 15 min to 4 h following melphalan administration, blood was collected by cardiac puncture and the brain was removed and placed on 0.9% NaCl, ice-chilled filter paper. A minimum of five animals were killed per time point. The blood was centrifuged (7000 x g, 1 min), and the plasma removed and stored immediately at -70°C. Plasma and brain samples were analyzed for melphalan by high-performance liquid chromatography, as described by Sweeney and colleagues (21).

Brain Perfusion Study

Surgical Procedure. Adult male rats (Osborne-Mendel strain), weighing 250-350 g, were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The right external carotid artery was cannulated with a polyethylene catheter for retrograde infusion. In addition, the right pterygopatine artery was ligated, the right occipital and thyroid arteries were coagulated and cut, and the right common carotid artery was encircled with silk thread (Fig. 2). Blood flow through the right common carotid artery was never interrupted during the surgical procedure. Following the surgery, a heat lamp connected to a feedback device (YSI Indicating Controller, Yellow Springs, OH) maintained rectal temperature at 37°C.

Brain Perfusion. The cannula to the right external carotid artery was connected to a syringe containing 0.3 μCi/ml [14C]melphalan, 1.0 μCi/ml [3H]ljinulin, 0-16.3 μmol/ml of unlabeled melphalan (Sigma Chemical Co., St. Louis, MO), and 0-100 μmol/ml of L-phenylalanine (Sigma) dissolved in an HCO3-buffered physiological saline (in μmol/ml, 142 NaCl, 28 NaHCO3, 6.0 dextrose, 4.2 KH2PO4, 1.7 CaSO4, and 1.0 MgSO4). This perfusion fluid was prepared just prior to use to minimize any hydrolysis of the melphalan. The radiochemical purities (>98%) of the [14C] and [3H]tracers were confirmed by thin-layer chromatography and gel chromatography (22, 23), respectively. The perfusion fluid was filtered through a 10-μm polypropylene filter (Gelman Sciences, Ann Arbor, MI), oxygenated with 95% O2:5% CO2 and warmed to 37°C. The pH and CO2 tension of the perfusion fluid were 7.40 and 32 mm Hg, respectively. One second before perfusion, the right common carotid artery was ligated. Then perfusion fluid was infused retrograde into the external carotid artery at a constant rate of 8.3 x 10^-7 ml/s with an infusion pump (no. 944; Harvard Apparatus, South Natick, MA). At this infusion rate, the measured carotid artery pressure was between 130 and 140 mm Hg (below 160-190 mm Hg, which has been reported to damage the blood-brain barrier (3)), and circulating blood contributed less than 5% of the net flow to the right cerebral hemisphere (19). After 60-65 s brain perfusion, the rat was decapitated. A 100-μl aliquot of perfusion fluid was transferred to a vial and stored at -70°C for radiochemical analysis by thin-layer chromatography, to determine whether any hydrolysis of melphalan

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1 To whom requests for reprints should be addressed, at Laboratory of Neurosciences, National Institute on Aging, NIH, Bethesda, Maryland 20892.

1571
FACILITATED TRANSPORT OF MELPHALAN INTO THE BRAIN

Fig. 1. Structural formulas of the large neutral amino acid phenylalanine, and the alkylating agent melphalan.

Fig. 2. Diagram of the technique for perfusing the right cerebral hemisphere of a rat. ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery. With permission from Takasato et al. (19).

had occurred during the procedure. In addition, a 20-μl aliquot of perfusion fluid was transferred to a scintillation vial for the determination of radioactivities. The brain was removed form the skull and placed on 0.9% NaCl moistened, ice-chilled filter paper. The arachnoid membrane and meningeal blood vessels were removed, and six brain regions (frontal, parietal, and occipital cerebral cortex; hippocampus; caudate nucleus; and thalamus/hypothalamus) were dissected from the right cerebral hemisphere. They were placed in tared vials, reweighed, and digested overnight at 50°C in 1 ml of protosol (New England Nuclear, Boston, MA). Ten ml of Ready Solv MP (Beckman, Fullerton, CA) were added to the brain samples and to the 20-μl sample of perfusion fluid, following which their [14C] and [3H] radioactivities were measured by high-performance liquid chromatography, by subtracting the intravascular concentration.

Melphalan (>98%) was confirmed by thin-layer chromatography on silica gel 60 F-254 with n-butanol, acetic acid, and distilled water (7:2:1) as the eluting solvent. There was no detectable hydrolysis of melphalan (>98%) was confirmed by thin-layer chromatography on silica gel 60 F-254 with n-butanol, acetic acid, and distilled water (7:2:1) as the eluting solvent. There was no detectable hydrolysis of melphalan.

Calculations

Pharmacokinetic Study. The brain concentration of melphalan was calculated from the net regional brain concentration, as measured by high-performance liquid chromatography, by subtracting the intravascular volume at the time of death (T). The intravascular concentration equaled the plasma concentration of melphalan (nmol/ml) at time T, multiplied by the regional blood volume (ml/g of brain).

Regional cerebral blood volume was measured by injecting four adult male rats, 200–250 g weight, i.v. with [125I]human serum albumin (100 μCi/kg, Amersham Corp.). The [131I]human serum albumin was determined to be 99.9% pure by gas-liquid chromatography. Blood and brain samples were collected at 2 min as described for melphalan, and samples of plasma, cerebellum, and cerebral cortex were assayed for [125I] activity (Gamma 4000; Beckman Instruments, Palo Alto, CA). [131I]Human serum albumin was restricted to the cerebral vasculature during the 2-min experiment. Regional blood volumes of the cerebellum and cerebral cortex were calculated by dividing the [131I] activity of the brain samples by that of the plasma (dpm g⁻¹/dpm ml⁻¹), and were equal to 2.4 and 1.7%, respectively.

The melphalan concentration versus time data were fitted by nonlinear regression analysis to the biexponential equation:

\[ C = Ae^{-at} + Be^{-bt} \] (A)

The brain data, the peak concentration, and drug levels terminal to it, were fitted to a single exponential equation.

In Equation A, C is the concentration of melphalan (nmol/ml or nmol/g) at time t (min). A and B are defined as the theoretical 0 time concentrations in a central and peripheral compartment, respectively, and a and b are apparent first-order elimination rate constants (min⁻¹). Plasma half-lives were calculated from the parameters by the general formula:

\[ \text{Half-life} = \frac{0.693}{x} \] (B)

where x = a or b. Areas under the concentration-time profiles were calculated by the trapezoidal rule.

Brain Perfusion Study. The brain uptake of a test tracer, such as [14C]melphalan, during perfusion is given by a two-compartment model:

\[ dC_z/dt = k_aC_a - k_mC_z \] (C)

where \( C_z \) represents the concentration of [14C]melphalan in dpm/g in the brain parenchyma, \( C_a \) (dpm/ml) is the concentration of [14C] melphalan in the perfusion fluid, \( k_a \) and \( k_m \) are the transfer coefficients for influx and reflux, respectively, and t (s) represents the net perfusion time. In each experiment, the perfusion time was limited so that only a small quantity of [14C]melphalan accumulated in the brain, \( k_mC_z \approx k_aC_a \), and thus back diffusion from the brain is negligible and can be neglected. Under such circumstances brain uptake follows unidirectional kinetics and can be described as:

\[ dC_z/dt = k_aC_a \] (D)

In Equations C and D \( k_a \) is defined as

\[ k_a = F(1 - e^{-Ft}) \] (E)

where F is the regional cerebral blood flow (ml/s/g), and \( PA^2 \) is the capillary permeability-surface area product.

\( F \) was determined in a separate series of experiments from the unidirectional uptake of [14C]diazepam during a 10-s perfusion (19). To solve for \( PA^2 \), Equation D can be integrated from the time that perfusion fluid entered the cerebral capillaries (t = 0) to the time of decapitation (T).

\[ PA^2 = -F \ln \left[ 1 - \frac{C_z(T)}{FTC_a} \right] \] (F)

The cerebrovascular \( PA \) of [14C]melphalan can be calculated from Equation F. In this, \( C_z(T) \) is obtained by subtracting the intravascular [14C]melphalan, equal to the product of \( C_a \) and the regional intravascular volume, from the measured brain concentration of [14C]melphalan. [3H]Inulin, a polysaccharide that does not measurably enter the blood brain barrier, was utilized to determine the intravascular volume. In all experiments, 5 s were subtracted from the total
perfusion time, 65 s, to obtain \( T \), the time that the perfusate was actually within the cerebral capillaries (19).

The unidirectional influx (\( J_{\text{un}} \), \( \mu \text{mol/s/g} \)) of melphalan into the brain is defined as the product of cerebrovascular \( PA \) and the mean capillary concentration (\( C_{\text{cap}} \), \( \mu \text{mol/ml} \)) (24). \( F \) was considerably greater than the highest measured \( PA \), and as a consequence \( C_{\text{cap}} = \frac{k C_r}{PA} = C_r^* \) (24). Thus, for the present study, the expression of \( J_{\text{un}} \) can be simplified to

\[
J_{\text{un}} = PA C_r^*
\]

(G)

To examine the transport of melphalan across the cerebral capillaries the concentration dependence of melphalan influx was described by a Michaelis-Menten equation which contained a nonsaturable component (25). In this, the simple passive diffusion of the drug across the blood-brain barrier was represented as \( K_a \), a constant of nonsaturable diffusion, and that due to carrier mediated transport was represented by the saturable Michaelis-Menten component.

\[
J_{\text{un}} = \frac{V_{\text{max}} C_r^*}{K_m + C_r^*} + K_a
\]

(H)

where \( V_{\text{max}} \) is the maximal influx rate and \( K_m \) is the half-saturation concentration of melphalan. Because, from Equation \( G \), \( PA = J_{\text{un}}/C_r^* \), dividing Equation \( H \) by \( C_r^* \) gives

\[
PA = \frac{V_{\text{max}}}{K_m + C_r^*} + K_a
\]

(I)

Best-fit values for \( V_{\text{max}} \), \( K_m \), and \( K_a \) were obtained by fitting Equation \( I \) to the \( PA \) data with weighted nonlinear least squares (26). Weighting factors were calculated as the inverse square of the melphalan \( PA \). The nonlinear least squares analyses were performed on a DEC 10 computer using the MLAB program (27), which is based on the Marquardt-Levenberg curve-fitting algorithm (28).

Statistical Analysis

A two-tailed Student’s \( t \) test was performed for the comparison of two means. When more than two means were compared, one-way analysis of variance and the Bonferroni multiple-test were utilized to compare the individual means (29). Statistical significance for all tests was taken as \( P < 0.05 \). Means \( \pm SE \) are given routinely, unless otherwise stated.

RESULTS

Fig. 3 shows the plasma and brain concentrations of melphalan following its i.v. administration (10 mg/kg). Melphalan demonstrated a biphasic elimination from plasma, with an initial half-life of 1.9 min, and a secondary half-life of 78 min. Peak concentrations of 230 nmol/ml were achieved at 30 s, which fell to 8.9 nmol/ml by 4 h. Melphalan slowly entered the brain and reached approximately plateau concentrations between 30 and 120 min. In the cerebral cortex, a peak concentration of 2.3 nmol/g was achieved at 30 min, after which melphalan’s disappearance was monophasic with a half-life of 128 min. In the cerebellum, a peak melphalan concentration of 4.4 nmol/g was achieved at 60 min. Drug disappearance from the cerebellum was similarly monophasic, with a half-life of 174 min.

The areas under the concentration versus time curves for melphalan in the plasma, cerebral cortex, and cerebellum, from 15 min to 4 h, were 4151 nmol min/ml, 391 nmol min/g, and 861 nmol min/g, respectively. Calculated from these, the cerebral cortex/plasma concentration ratio for melphalan was 0.094 and the cerebellum/plasma ratio was 0.20, giving a mean brain/plasma ratio of 0.15.

The transport of melphalan across the blood-brain barrier was measured by an isolated, in situ brain perfusion technique. Table 1 presents the mean \( PA \) values (calculated from Equation \( F \)) for \([^{14}C]\)melphalan in six brain regions which were perfused with physiological saline containing \([^{14}C]\)melphalan, 0.02 \( \mu \text{mol/ml} \), and 0–16.3 \( \mu \text{mol/ml} \) of unlabeled melphalan. In each region, the \( PA \) of melphalan decreased as the concentration of unlabeled melphalan in the perfusion fluid was increased. This is consistent with the saturation of a transport site. The mean regional \( PA \) of the nonsaturable component of melphalan transport, equivalent to its passive diffusion across the blood-brain barrier, varied from \( 5.4 \times 10^{-5} \text{s}^{-1} \) in the frontal and parietal cortex to \( 4.3 \times 10^{-5} \text{s}^{-1} \) in the caudate nucleus. The \( PA \) values of melphalan in each brain area were not significantly different when measured following the addition of 8.0 and 16.3 \( \mu \text{mol/ml} \) of melphalan to the perfusion fluid. However, the \( PA \) values of melphalan, measured following the brain perfusion of \([^{14}C]\)melphalan alone (0.02 \( \mu \text{mol/ml} \)), were significantly greater in all brain areas compared to those measured following the addition of unlabeled melphalan, 16.3 \( \mu \text{mol/ml} \), to the perfusion fluid. The \( PA \) values of \([^{14}C]\)melphalan alone, 0.02 \( \mu \text{mol/ml} \), varied from \( 10.8 \times 10^{-5} \text{s}^{-1} \) in the parietal cortex to \( 7.6 \times 10^{-5} \text{s}^{-1} \) in the hippocampus. With the exception of the caudate nucleus and the combined thalamic/hypothalamic area, each of the \( PA \) values of \([^{14}C]\)melphalan alone was also significantly greater than those measured after 8.0 \( \mu \text{mol/ml} \) of melphalan was added to the brain perfusion fluid. The \( PA \) values of melphalan in cortical areas, following the brain perfusion of...
The half-lives of melphalan in cerebral cortex and cerebellum and 4.4 nmol/g at 30 and 60 min, respectively) represented cerebral cortex and cerebellum melphalan concentrations (2.3 nmol/g at 30 and 60 min, respectively) for brain perfusion fluid. The concentration of melphalan was achieved in the brain following phasic with a half-life longer than that of the drug in plasma. The half-time profiles of melphalan in the brain were approximately favorably with previous measurements (31, 32). Although peak plasma concentrations in the former were only 15% of that under the plasma concentration-time profile. This value is calculated from data that span three plasma half-lives of the i.v. administration of the drug. Melphalan disappeared from the brain, which has not previously been described, was mono-phasic with a half-life longer than that of the drug in plasma. The half-lives of melphalan in cerebral cortex and cerebellum were 128 and 174 min, respectively.

The concentration-dependent and saturable transport of melphalan into the brain, demonstrated by the isolated brain perfusion technique, suggests that, in addition to simple diffusion, melphalan was transported across the cerebrovascular endothelium and into the brain by a facilitated mechanism. The additional demonstration that this transport was completely inhibited by l-phenylalanine indicates that melphalan utilized the large neutral amino acid carrier system that is located at the blood-brain barrier (33, 34).

There have previously been several suggestions that exogenous agents, in particular amphetamine (35), penicillin (36), and a-methyl dihydroxyphenylalanine (Aldomet) (37) are transported into the brain by a transport system at the blood-brain barrier. However, Fenstermacher and colleagues (5), having reviewed the literature, concluded that the apparent demonstrations were neither particularly convincing nor physiological. The present study is the first to unequivocally demonstrate, as evidenced by self inhibition (i.e., concentration-dependent transport), saturation and competitive inhibition, that an exogenous agent, melphalan, which is structurally related to an amino acid, l-phenylalanine, can be transported by the large neutral amino acid carrier system into the brain. In a similar manner, the endogenous amino acid l-dihydroxyphenylalanine is transported into the brain by the large neutral amino acid facilitated transport system (3, 37), and, because it is a precursor of the neurotransmitter dopamine, is administered clinically for the alleviation of Parkinson’s disease.

In addition to that present at the blood-brain barrier (3, 38), amino acid transport systems have been demonstrated to exist in other tissues, particularly at the level of the gastrointestinal tract and on the cell membrane of tumor cells. The field has been extensively reviewed by Oxender and Christensen (33), Johnstone and Scholefield (39), Neame (40), and Christensen (41). Interestingly, melphalan has been reported to be transported across the gastrointestinal tract after oral administration, as well as into tumor cells via two amino acid transport systems.

Adair and McElnay (42) have demonstrated that the gastrointestinal absorption of melphalan is reduced by metabolic inhibitors, indicating that, in addition to simple diffusion across the gut wall, melphalan utilizes an active transport system at the level of the gastrointestinal epithelium. Further, Bosanquet and Gilbey (43) have demonstrated that, following similar administrations of melphalan to fed and fasted patients with multiple myeloma, peak plasma concentrations in the former were only one third of the value in the latter, and drug absorption was 49 versus 93%, respectively. Competitive inhibition of the transport site as well as an increased gut motility in the fed patients probably accounted for this.

In vitro drug uptake studies of melphalan into tumor cells have provided perhaps the clearest evidence of melphalan’s ability to utilize transport systems for essential endogenous substrates. Vistica and colleagues (17, 18) have demonstrated that melphalan entry into murine L1210 leukemia cells was an active, concentrative process, as evidenced by intracellular to extracellular distribution ratios of approximately 10, when transport was measured in an environment free of amino acids. Ratios of up to 35 have been reported (44). In addition, melphalan uptake into tumor cells has been shown to be temperature sensitive (12), and to be reduced by metabolic inhibitors and inhibitors of oxidative phosphorylation (11), indicating that the transport was an energy-requiring process. The ability of L-leucine to reduce melphalan transport into tumor cells suggested that the drug gained entrance via an amino acid transport system, specifically the sodium-independent L system (14, 15), for large neutral amino acids (33). As this transport was only partially inhibited by 2-amino-bicyclo[2,2,1]heptane-2-carboxy-
Facilitated Transport of Melphalan into the Brain

Cytosol cells by alanine, serine, and cysteine led Goldenberg additionally proposed to be utilized by melphalan (9, 12). The partial reduction of melphalan transport into LPC-1 plasmacytoma cells by alanine, serine, and cysteine led Goldenberg and colleagues (9–12) to suggest that the second carrier was the ASC system, which is responsible for the transport of specific neutral amino acids (46). Whether or not the second carrier is indeed the ASC system or an as yet unclassified system remains controversial (10, 11, 13, 14). Vistica and colleagues (16, 18) measured the Km value of melphalan transport at a single system in murine L1210 leukemia cells and reported a value of 0.019 µmol/ml. Begleiter and colleagues (10) reported the Km of melphalan in L5178Y lymphoblasts to be 0.08 µmol/ml and 0.01 µmol/ml, respectively, for the L and ASC systems. In the former study the Km of phenylalanine transport into L1210 cells was additionally measured, and was 0.01 to 0.015 µmol/ml (16), which closely matched that of melphalan.

The carrier system for amino acids at the blood-brain barrier is slightly different from those present elsewhere. Christensen (4, 41) and Wade and Katzman (37) have reported cogent evidence that the A system, for the transport of small neutral amino acids, does not function at the blood-brain barrier. Indeed, it is probable that blood-brain barrier amino acid transport is mediated solely by the L system, which is sodium independent and, unlike that present on tumor cells, is equilibrium. The lack of concentrative amino acid transport across the blood-brain barrier explains why brain melphalan concentrations were substantially lower than those in the plasma, and additionally, why cerebrospinal fluid amino acid concentrations are maintained at a low level (47). The Km value of melphalan, for the L system at the blood-brain barrier, was 0.15 µmol/ml. This was 15-fold greater than that of L-phenylalanine, 0.01 µmol/ml, for the same system measured by the same technique (38). The Km values for melphalan and L-phenylalanine transport into tumor cells are more closely matched and similar to those of L-phenylalanine transport at the blood-brain barrier. The affinity of L-phenylalanine for the L system at the blood-brain barrier (equal to the reciprocal of the Km value) is considerably greater than that of melphalan. As a consequence, the cerebrovascular permeability of L-phenylalanine is considerably larger than that of melphalan, the PA values are 790 × 10⁻⁴ s⁻¹ (38) and 10.8 × 10⁻⁴ s⁻¹, respectively. The endogenous amino acids in plasma therefore affect the transport of melphalan at the blood-brain barrier to a much greater degree than they do into tumor cells. Vistica and colleagues (13, 18) have reported that the minimal cytotoxic concentration of melphalan is approximately 6.5 nmol/ml. Although concentrations approaching this were achieved in the brains of rats, the amount of drug initially administered, 10 mg/kg, was substantially larger than that routinely given to humans. Thus, despite melphalan’s facilitated transport at the blood-brain barrier, due to its low affinity for the carrier system and its competition with endogenous large neutral amino acids, it is unlikely that therapeutic brain concentrations are achieved clinically following standard-dose therapy during which plasma melphalan concentrations range from 0.1 to 9.0 nmol/ml (31, 32). Gouyette and colleagues (48), however, have recently reported measurable cerebrospinal fluid levels of melphalan in children administered high-dose melphalan therapy, during which significantly higher plasma concentrations of drug are achieved. Studies are presently continuing in the design and testing of anticancer agents that compete for the blood-brain barrier, L system amino acid carrier with a higher affinity than melphalan.

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References
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