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CYTOTOXICITY AS AN INDICATOR FOR TRANSPORT MECHANISM

EVIDENCE THAT MELPHALAN IS TRANSPORTED BY TWO LEUCINE-PREFERRING CARRIER SYSTEMS IN THE L1210 MURINE LEUKEMIA CELL

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Summary

Melphalan, L-phenylalanine mustard, is transported by the L1210 cell through carriers of the leucine (L) type. Its initial rate of transport is inhibited by both L-leucine, a naturally occurring L system amino acid and 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH), a synthetic amino acid which is transported by the L system in the Ehrlich ascites tumor cell. Both amino acids inhibited melphalan transport comparably in sodium-free medium. However, BCH, in medium containing sodium, was unable to reduce a component of melphalan transport which was readily inhibited by leucine but not by α-aminoisobutyric acid. Inhibition analysis indicated that leucine competes with BCH for transport but that a portion of leucine transport is not readily inhibited by BCH. These results suggest that in the L1210 cell melphalan is transported equally by a BCH-sensitive, sodium-independent L system and a BCH-insensitive, sodium-dependent L system.

Introduction

Cultured L1210 murine leukemia cells are refractory to the antitumor agent melphalan in growth medium containing amino acids [1]. Investigation of the effects of single amino acids upon melphalan cytotoxicity indicated that the L-isomers of leucine and glutamine were primarily responsible for reduced cytotoxicity [1], and that reduction in cytotoxicity is correlated with a reduc-

Abbreviations: BCH, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

tion in drug uptake [2,3]. Kinetic analysis indicated that at cytotoxic concentrations melphalan is transported by a high-affinity amino acid transport system of the leucine (L) type and that the protective nature of amino acids is related to their affinities for the leucine carrier sites as compared to melphalan [3]. Additional studies indicated that melphalan transport is mediated through a similar system by cells maintained in vivo and that administration of leucine with melphalan negated the latter's therapeutic efficacy [4,5].

During the course of this work it became apparent that 2-aminobicyclo-[2,2,1]heptane-2-carboxylic acid (BCH), a substrate specific for the leucine-preferring transport system in the Ehrlich ascites tumor cell [6–8], was considerably less effective than leucine in reducing melphalan transport and cytotoxicity in medium containing sodium. As a result of this observation a study was undertaken to examine the relationship between the transport of melphalan, BCH and leucine.

Materials and Methods

Bovine serum albumin was obtained as serum fraction V from Miles Laboratories, Inc., Elkhart, Indiana. Fetal calf serum was purchased from Flow Laboratories, Rockville, Maryland and RPMI 1630 medium and Dulbecco's phosphate-buffered saline were supplied by the NIH Media Unit. Gentamicin (Schering, 50 mg/ml) and Fungizone (250 μg/ml) were purchased from Microbiological Associates, Bethesda, Maryland, and Grand Island Biological Co., Grand Island, New York, respectively. Unlabeled melphalan was obtained from Burroughs Wellcome Co., Research Triangle Park, North Carolina. DL-2-Aminobicyclo[2,2,1]heptane-2-carboxylic acid was purchased from New England Nuclear, Boston, Massachusetts and α-aminoisobutyric acid was obtained from the Sigma Chemical Co., St. Louis, Missouri. All other unlabeled amino acids were obtained from Calbiochem, San Diego, California. Choline Chloride and (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (HEPES) were also purchased from Calbiochem. The silicone oil, Versilube F-50 (specific gravity 1.045 at 25°C; viscosity 70 centistokes at 25°C was obtained from Harwick Chemical Corp., Cambridge, Massachusetts.

L-[1-^{14}C]Leucine (56.9 mCi/mmol), DL-[*carboxyl*-^{14}C]BCH (4.78 mCi/mmol), [*carboxyl*-^{14}C]inulin (1.55 mCi/g) and α-[3-^{14}C]aminoisobutyric acid (19.9 mCi/mmol) were purchased from New England Nuclear, Boston, Massachusetts. [^{14}C]Leucine and α-[^{14}C]aminoisobutyric acid were diluted with the respective unlabeled amino acids and used at the concentrations indicated in the text. Analyses [8] performed by the supplier revealed that the labeled BCH was composed of 3.5% DL-α-2-aminobicyclo[2,2,1]heptane-2-carboxylic acid and 96.5% DL-β-2-aminobicyclo[2,2,1]heptane-2-carboxylic acid.

Melphalan (6.4 mCi/mmol), labeled in the chloroethyl groups with ^{14}C, was synthesized by Mr. Morris Leaffer under contract with the Stanford Research Institute, Menlo Park, California. Radiochemical purity was 97%, as determined by thin layer chromatography on Silica gel in *n*-butanol/acetic acid/water (7 : 2 : 1, v/v). Unlabeled and labeled melphalan solutions were prepared daily in 75% ethyl alcohol containing an equimolar concentration of hydrochloric

acid. Further dilutions were made in aqueous medium immediately prior to use in order to minimize hydrolysis. Dilutions of labeled melphalan with unlabeled material resulted in a corresponding decrease in uptake of radioactivity, suggesting that the labeled and unlabeled material exhibited similar transport characteristics. Also, the cytotoxic potency of [^{14}C]melphalan to L1210 cells as determined by clonal growth [1,9] corresponded to that of the unlabeled material. Intracellular radioactivity was identified as unhydrolyzed melphalan by its R_F value of approximately 0.5 following thin layer chromatography on Silica gel 60 in *n*-butanol/acetic acid/water (4 : 1 : 1, v/v) [10].

Cytotoxicity assays. The conditions for maintenance of cell cultures and for exposure of the cells to melphalan have been described [1]. Modifications included the use of cells in the logarithmic growth phase ($5 \cdot 10^5$—$10 \cdot 10^5$ cells/ml), incorporation of fungizone (0.25 μg/ml) in the maintenance medium and 0.25% glucose in the standard incubation medium of phosphate-buffered saline and 0.1 mM bovine serum albumin.

Cells were harvested after a 35 min exposure to melphalan or the solvent, ethanol, washed twice in RPMI 1630 medium supplemented with 20% fetal calf serum, and suspended in the same medium at $1.0 \cdot 10^5$—$1.2 \cdot 10^5$ cells/ml. The cytotoxicity of melphalan was assessed by clonal growth of surviving cells according to the procedure of Chu and Fischer [9] with minor modifications [1].

A minimal LD_{100} concentration of melphalan was used for appropriate studies described in the text. This concentration is defined [1] as the lowest concentration of melphalan which consistently results in 100% mortality after incubation with cells for 35 min in phosphate buffered saline containing 0.1 mM bovine serum albumin and 0.25% glucose, and was used to minimize experimental variation due to the sharp dose response curve of the drug. This drug dose is 6.5 μM for a cell concentration of 10^5 cells/ml and 7.0 μM for a cell concentration of 10^6 cells/ml.

Transport of melphalan and amino acids by L1210 cells. Logarithmic phase L1210 cells ($5 \cdot 10^5$—$10 \cdot 10^5$ cells/ml) were harvested by centrifugation at $300 \times g$ for 5 min and washed three times in transport medium, composed of Dulbecco's phosphate-buffered saline containing 0.1 mM bovine serum albumin and 0.1% glucose. They were then added at a concentration of $2.0 \cdot 10^6$ cells/ml to the appropriate volume of medium with or without amino acid and the uptake was initiated by addition of labeled material, as indicated in the individual experiments. Transport of melphalan was estimated after a 3 min incubation while that of L-leucine, BCH and α-aminoisobutyric acid was estimated after 24 s. Aliquots of the incubation mixture were layered on Versilube F-50 silicone oil in a microcentrifuge tube and transport was terminated by centrifugation of the cells through the oil at $12\,000 \times g$ for 1 min in an Eppendorf microcentrifuge. Individual uptake estimates were performed in duplicate and cell recovery was found to be greater than 99%. Tips containing the cell pellet were cut off, the cell pellets were solubilized in 0.2 M NaOH, liquid scintillation fluor was added and samples were counted on a Beckman liquid scintillation counter. An estimation of extracellular drug in the cell pellet was made with inulin as a marker. Approximately 0.04 pmol melphalan/10^5 cells resided in the extracellular space following exposure of cells to 6.5 μM

melphalan. Data are corrected for this small amount of extracellular drug.

Cells were maintained at 37°C with gentle agitation during all phases of the transport study and experiments were completed within 1 h of their removal from growth medium. Control populations after the transport study were found to be 90—100% viable as determined by clonal growth.

Sodium-free transport medium was prepared by substituting 137 mM choline chloride for sodium chloride and was buffered with 25 mM HEPES (pH 7.4). The composition in g/l was: $CaCl_2$ (0.1)/KCl (0.2)/KH_2PO_4 (0.2)/$MgCl_2 \cdot 6H_2O$ (0.1)/choline chloride (19.1)/HEPES (5.96). The concentration of choline chloride was determined by titration of chloride according to the method of Mohr.

Results

Effect of sodium on the initial rate of transport of leucine, BCH, α-aminoisobutyric acid and melphalan. The initial rate of transport of leucine and BCH by the L1210 cell exhibited minimal dependence upon sodium whereas the transport of α-aminoisobutyric acid and melphalan was substantially decreased in the absence of sodium (Table I). As can be seen in Fig. 1, the initial rate of melphalan transport was reduced comparably by BCH and leucine in sodium-free medium, but in medium containing sodium, BCH inhibited only 50% of melphalan transport whereas leucine effectively reduced nearly all drug uptake. α-Aminoisobutyric acid, an alanine (A) system model amino acid in the Ehrlich ascites cell [11], was without effect on the BCH-insensitive component of melphalan transport.

Effect of BCH and leucine on melphalan uptake and cytotoxicity in medium containing sodium. The failure of BCH to reduce the initial rate of melphalan transport by more than 50% in medium containing sodium (Fig. 1) prompted investigation of its effect on melphalan cytotoxicity. As can be seen in Fig. 2, leucine reduced net melphalan uptake to one-sixth of control and was accompanied by a corresponding increase in the surviving fraction of cells. This was in contrast to only a 50% reduction in melphalan uptake by 0.2 mM BCH and the

TABLE I

EFFECT OF SODIUM ON THE INITIAL RATE OF TRANSPORT OF LEUCINE, BCH, α-AMINOISOBUTYRIC ACID AND MELPHALAN

Cells, prepared as described in Materials and Methods, were incubated with 0.1 mM leucine, 0.1 mM α-aminoisobutyric acid, 0.2 mM BCH or 6.5 μM melphalan in transport medium containing 137 mM sodium chloride or choline chloride. The transport of amino acids, except melphalan, was terminated after 24 s by centrifugation of the cells through silicone oil. Melphalan transport was terminated at 3 min.

	+Na	—Na	Percent dependence upon sodium
	(pmol/min per 10^5 cells)		
Leucine	39.2	34.2	12.8
BCH	96.5	80.7	16.4
α-Aminosisobutyric acid	26.7	7.3	72.7
Melphalan	2.0	1.1	45.0

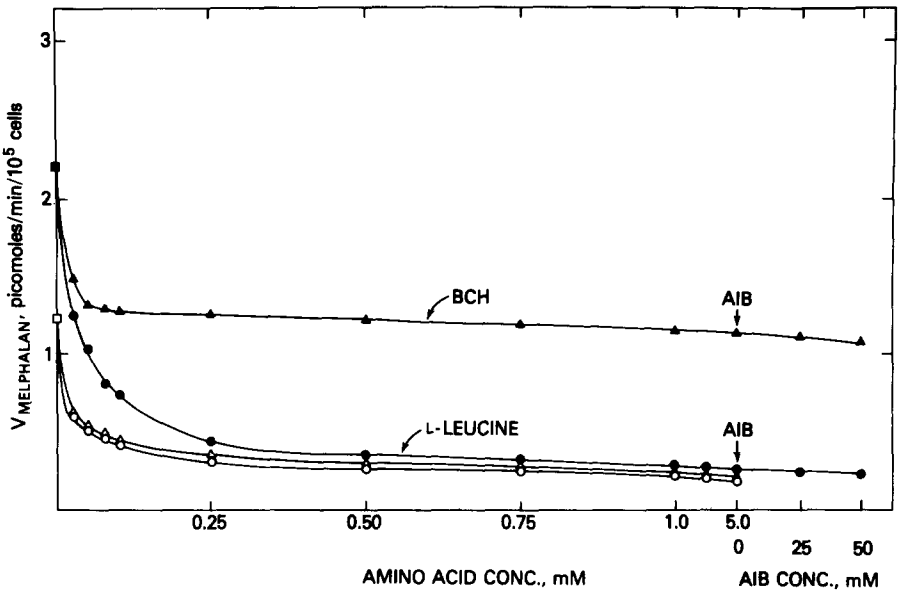

Fig. 1. Effect of leucine and BCH upon the initial rate of melphalan transport in the presence and absence of sodium. Cells ($2.0 \cdot 10^6$ cells/ml) in 300 μl transport medium with (■) or without (□) sodium were incubated with 300 μl of the respective medium containing 6.5 μM [^{14}C]melphalan and no amino acids (■, □), the indicated concentration of L-leucine (●, ○) or BCH (▲, △) either with or without α-amino-isobutyric acid (AIB). Actual BCH concentrations were twice that indicated on the abscissa. Duplicate 200 μl aliquots were removed and melphalan transport was terminated at 3.0 min by centrifugation of the cells through silicone oil at 12 000 × g.

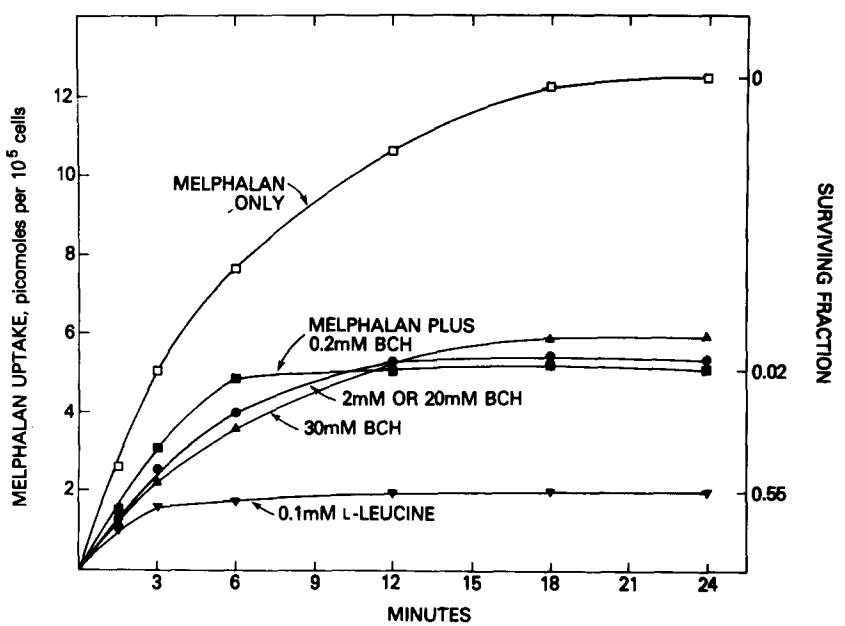

Fig. 2. Effect of leucine and BCH upon melphalan uptake and cytotoxicity in medium containing sodium. Cells ($1.33 \cdot 10^6$ cells/ml) were incubated for 15 min in transport medium under one of the following experimental conditions: no amino acid supplementation (□), 0.1 mM L-leucine (▼), 0.2 mM BCH (■), 2 mM or 20 mM BCH (●) and 30 mM BCH (▲). [^{14}C]Melphalan was added to yield a cell density of $1.0 \cdot 10^6$ cells/ml and a drug concentration of 6.5 μM. At appropriate intervals, duplicate 200 μl aliquots were removed and melphalan transport was terminated as described in the legend to Fig. 1. Melphalan uptake at the indicated time points is shown on the left ordinate and cytotoxicity following a 35 min exposure is indicated on the right ordinate. Sodium concentration, 137 mM.

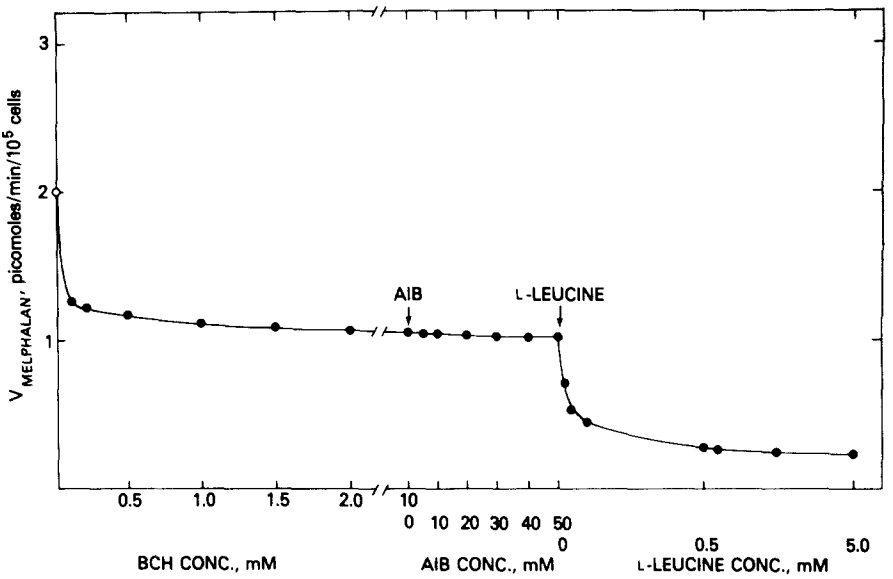

Fig. 3. Effect of leucine and α-aminoisobutyric acid (AIB) on the BCH insensitive component of melphalan transport. Cells, prepared as described in Materials and Methods, were incubated with transport medium containing no amino acids (○), with the indicated concentration of either BCH alone, with 10 mM BCH and the indicated concentration of α-aminoisobutyric acid or with 10 mM BCH, 50 mM α-aminoisobutyric acid and the indicated concentrations of L-leucine. Melphalan transport was terminated as described in the legend to Fig. 1. Sodium concentration, 137 mM.

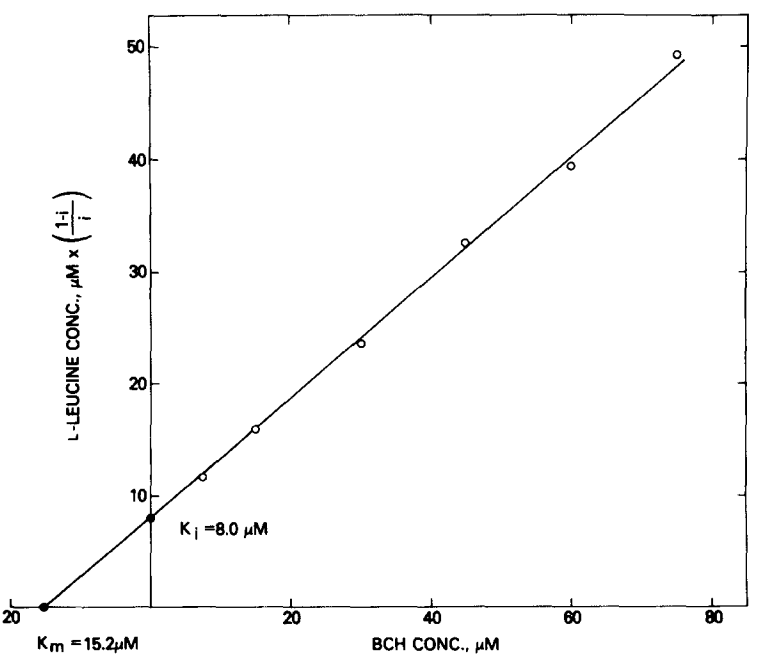

Fig. 4. Inhibition analysis of the rate of BCH transport by leucine. Cells, prepared as described in Materials and Methods, were incubated with the appropriate concentration of [^{14}C]BCH alone or [^{14}C]-BCH and L-leucine. Transport was terminated after 24 s as described in the legend to Fig. 1. Sodium concentration, 137 mM. *i* is the fractional inhibition of BCH transport occurring in the presence of leucine.

failure of increasing concentrations of BCH to reduce net drug uptake or cytotoxicity.

Effect of leucine and α-aminoisobutyric acid on the BCH-insensitive component of melphalan transport. Since a small portion of leucine transport has been shown to occur via the alanine (A) amino acid transport system [12] the effect of α-aminoisobutyric acid on the BCH-insensitive component of melphalan transport was investigated. α-Aminoisobutyric acid was without effect on this component of transport, whereas leucine, at concentrations of 0.1 mM and below, effectively reduced it (Fig. 3).

Effect of BCH and leucine on each other's transport. Inhibition analysis of the initial rate of transport was employed to determine the relationship between the leucine and BCH transport systems. A non-inverted kinetic plot [13] originally described by Hunter and Downs [14] was selected and such a plot for leucine inhibition of the rate of BCH transport (Fig. 4) indicated a competitive mechanism. A K_m for BCH transport of 15.2 μM and a leucine K_i of 8.0 μM were obtained. The reciprocal experiment, the effect of BCH on leucine transport (Fig. 5) indicated that a portion of leucine transport occurred which was not readily inhibited by BCH. In addition, the K_i for BCH inhibition of leucine transport was approximately 5 times higher than the leucine K_m. These data, as well as those of Figs. 2 and 3, suggest that melphalan is transported by a leucine system which is not readily inhibited by BCH.

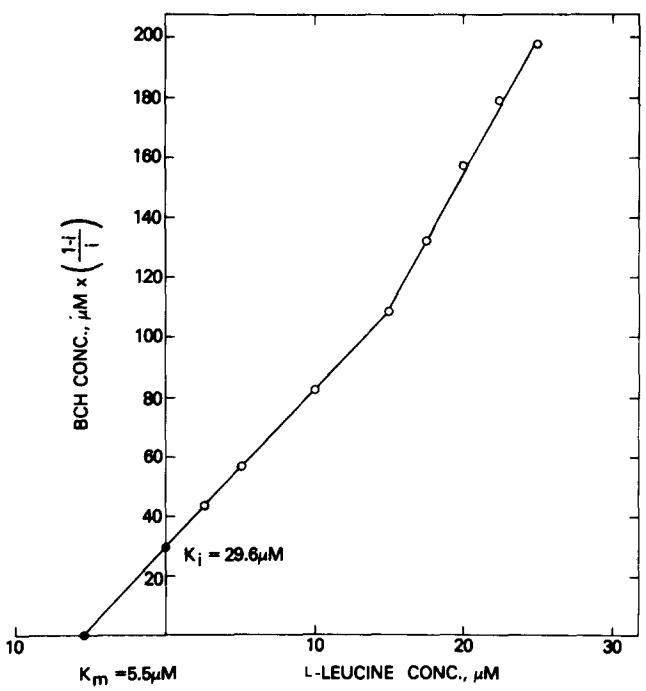

Fig. 5. Inhibition analysis of the rate of leucine transport by BCH. Cells, prepared as described in Materials and Methods, were incubated with the appropriate concentration of [^{14}C]leucine alone or [^{14}C]leucine and BCH. Transport was terminated after 24 s as described in the legend to Fig. 1. Sodium concentration, 137 mM. *i* is the fractional inhibition of leucine transport occurring in the presence of BCH.

Discussion

The results described in the present communication suggest that two high-affinity leucine transport systems exist in the L1210 murine leukemia cell and that melphalan, a cytotoxic agent, is transported equally by both systems. BCH, the model L system amino acid in the Ehrlich ascites cell, appears to be reactive with only one system, and consequently is ineffective in reducing melphalan transport to levels which affect the surviving fraction of cells. This conclusion is supported by three observations: (1) BCH, at high concentrations, is unable to reduce a component of melphalan transport (Figs. 1 and 3); (2) BCH, at high concentrations, is unable to reduce cytotoxicity (Fig. 2) and (3) BCH is unable to affect a component of leucine transport (Fig. 5). BCH is reactive with the L system responsible for the sodium-insensitive component of melphalan transport whereas leucine is able to reduce both the sodium sensitive and insensitive components of transport.

These results appear to be related to those recently described by Oxender et al. [12] for leucine uptake by 3T3 mouse cells. Kinetic plots for uptake were biphasic yielding K_m values of 10—20 μM and 90—100 μM. It has been assumed that biphasic plots represent uptake by two saturable mechanisms [15] although definitive experimental evidence is lacking.

Certain dissimilarities in the transport of melphalan and classical L system substrates such as leucine and BCH are apparent. Melphalan transport is highly dependent upon sodium, reminiscent of the alanine (A) transport system in the Ehrlich ascites tumor cell [6]. However, the inhibition kinetics resemble those of system L (ref. 3 and present study). Several lines of evidence indicate that BCH may not be reactive with all leucine transport systems. Christensen et al. [8] found that the K_i for BCH inhibition of leucine uptake by *Escherichia coli* was 10 times higher than the leucine K_i while the leucine K_i for inhibition of BCH uptake was 1 μM, a value identical with the leucine K_m. Indeed, these authors state that their results indicated that leucine and BCH transport may not be identical.

The results described here indicate that melphalan may be an important agent in discerning complex interactions in the transport of amino acids. Its potent cytotoxicity provides a sensitive means for measuring interference with transport by amino acids.

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