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Carrier Mechanism and Specificity Accounting for the Increase in Intracellular Melphalan by the Basic Amino Acids

DAVID T. VISTICA AND BARBARA P. SCHUETTE

Laboratory of Medicinal Chemistry and Biology, National Cancer Institute, Bethesda, Maryland 20205

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SUMMARY

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The basic amino acids arginine and its lower homologue, α-amino-γ-guanidinobutyric acid, increased the intracellular levels of melphalan only in the presence of leucine and sodium ions. This increase occurred solely through the monovalent cation-dependent, high-affinity, leucine-preferring transport system, one of the two amino acid transport systems responsible for melphalan uptake. Acceleration of melphalan exodus by leucine is antagonized by α-amino-γ-guanidinobutyric acid, resulting in increased cellular retention of melphalan.

INTRODUCTION

Melphalan (L-phenylalanine mustard) cytotoxicity to murine L1210 leukemia cells in culture is reduced in growth medium containing amino acids (1). Leucine and glutamine were found to be the amino acids primarily responsible for this decrease in drug cytotoxicity. The decreased cytotoxicity is due to a corresponding reduction in melphalan transport, and the ability of leucine to protect L1210 cells from melphalan cytotoxicity is related to the higher affinity of leucine for the transport system (2). Further studies indicated that melphalan is transported by two separate amino acid transport systems (3–5). One system is presumed to be the leucine-preferring transport system designated System L by Christensen and his colleagues (6–8), since melphalan uptake is reduced, although incompletely, by BCH,[1] a substrate specific for that transport system. The second transport system is insensitive to both BCH and AIB, a substrate specific for System A (6). The transport system which is insensitive to BCH and AIB exhibits its highest affinity for the branch-chain amino acid leucine (9) and tolerates substitution of lithium or potassium ions for sodium ions (9), a criterion which was used to differentiate this transport system from System ASC (10).

During the course of the work with the murine L1210 leukemia cell system, it was discovered that protection of cells from melphalan cytotoxicity by leucine in complete culture medium containing amino acids is less than that obtained in a balanced salt solution containing albumin and glucose (11). An examination of the ability of amino acids to interfere with leucine protection of melphalan cytotoxicity indicated that the basic amino acids arginine and to a lesser extent lysine were primarily responsible for increasing melphalan cytotoxicity. The elevation in both intracellular drug concentration and cytotoxicity decreased with increasing carbon chain length within the arginine homologous series (α-amino-γ-guanidinobutyric acid > arginine > homoarginine). As a result of these observations the present study was undertaken to elucidate the mechanism by which α-amino-γ-guanidinobutyric acid raised the intracellular melphalan concentration and the participation of each of the two amino acid transport systems responsible for melphalan uptake.

MATERIALS AND METHODS

Materials. Bovine serum albumin (regular and salt-free) was obtained as serum Fraction V from Miles Laboratories, Inc., Elkhart, Ind.; fetal calf serum was purchased from Flow Laboratories, Rockville, Md. RPMI 1630 medium and Dulbecco's phosphate-buffered saline were supplied by the National Institutes of Health Media Unit. Unlabeled melphalan was obtained from Burroughs Wellcome Company, Research Triangle Park, N. C. BCH was purchased from New England Nuclear Corporation, Boston, Mass. AIB was obtained from the Sigma Chemical Company, St. Louis, Mo. All other unlabeled amino acids were obtained from Calbiochem, San Diego, Calif. Choline chloride and Hepes were also purchased from Calbiochem. The silicone oil, Versilube F-50 (specific gravity 1.045 at 25°; viscosity 70 centistokes at 25°) was

[1] The abbreviations used are: BCH, β(±)-2-aminobicyclo[2,2,1]heptane-2-carboxylic acid; AIB, α-aminoisobutyric acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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obtained from Harwick Chemical Corporation, Cambridge, Mass.

[*carboxy*-^{14}C]Inulin (1.55 mCi/g) was purchased from New England Nuclear. L-α-Amino-γ-guanidino[^{14}C]butyric acid (55 mCi/mmole) was purchased from ICN Pharmaceuticals Inc., Irvine, Calif. and was purified prior to use by thin-layer chromatography on Silica Gel 60 in a solvent system consisting of *n*-butyl alcohol-acetic acid-water (4:1:1, v/v). Labeled material was diluted with unlabeled α-amino-γ-guanidinobutyric acid and used at concentrations indicated in the text. Melphalan (11 mCi/mmole), labeled in the chloroethyl groups with ^{14}C, was synthesized by Mr. Morris Leaffer under contract with the Stanford Research Institute, Menlo Park, Calif. Radiochemical purity was 97% as determined by thin-layer chromatography on silica gel in *n*-butyl alcohol-acetic acid-water (7:2:1, v/v). 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) corresponded to that of the unlabeled material.

Uptake and exodus of melphalan by L1210 cells. Cells were maintained at 37° with gentle agitation during all phases of the uptake study, and experiments were completed within 1 hr of their removal from growth medium. Control populations after the uptake study were found to be 90%–100% viable as determined by clonal growth. The conditions for maintenance of cell cultures have been described (1, 2). Logarithmic phase L1210 cells (5×10^5–10×10^5 cells/ml) were harvested by centrifugation at $300 \times g$ for 5 min and washed three times in transport medium containing 0.1 mM bovine serum albumin and 0.1% glucose (pH 7.4). Sodium ion-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 use of this sodium ion-free transport medium allowed examination of the interaction of melphalan, BCH, and α-amino-γ-guanidinobutyric acid with the presumed System L, a leucine-preferring transport system which exhibits little or no dependence upon sodium ions for substrate uptake (12). The composition of the sodium ion-free transport medium in grams per liter was as follows: $CaCl_2$, 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 transport medium was supplemented with 0.1 mM salt-free bovine serum albumin and 0.1% glucose. Selected experiments, described in the appropriate figure legends, were performed in Dulbecco's phosphate-buffered saline supplemented with 0.1 mM bovine serum albumin and 0.1% glucose.

Cells, in the respective transport medium, were then added at a concentration of 2.0×10^6 cells/ml to the appropriate volume of medium with or without amino acid, and the uptake of melphalan was initiated by addition of the labeled material. Aliquots of the incubation mixture were layered on Versilube F-50 silicone oil in a microcentrifuge tube, and melphalan uptake was terminated at the indicated time points 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 triplicate, 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 at 37° for 3 hr, and neutralized with acetic acid; scintillation fluid was then added and the 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 and indicated 0.04 pmole of extracellular melphalan per 10^5 cells following exposure of the cells to 6.5 μM melphalan. Data are corrected for this small amount of extracellular drug. Melphalan exodus studies were performed by loading L1210 cells with melphalan for 15 min at 37° in transport medium composed of Dulbecco's phosphate-buffered saline containing 0.1 mM bovine serum albumin and 0.1% glucose. The cells were then pelleted by centrifugation at $300 \times g$ for 5 min. The cell pellets were then resuspended in transport medium alone or in transport medium containing amino acids as indicated in the appropriate figure legend. Aliquots (200 μl) (2×10^5 cells) were centrifuged at appropriate times through silicone oil as described above.

RESULTS

Sodium ion and leucine requirement for the L-α-amino-γ-guanidinobutyric acid-induced increase in intracellular melphalan. The reduction of melphalan up-

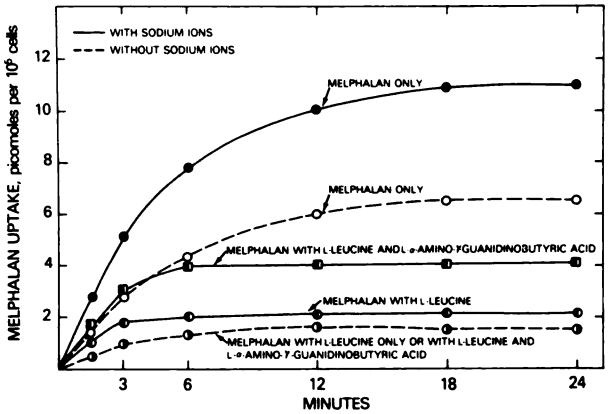

FIG. 1. *α-Amino-γ-guanidinobutyric acid increases intracellular melphalan only in the presence of leucine and sodium ions*

Murine L1210 leukemia cells were harvested from growth medium, washed twice in transport medium with (——) or without (- - -) sodium ions and suspended in the respective medium at a density of 2.0×10^6 cells/ml. [^{14}C]-Melphalan (22 cpm/pmole) was added alone (●, ○), with 100 μM L-leucine (◐, ◑) or with 100 μM each of L-leucine and α-amino-γ-guanidinobutyric acid (◧, ◑) to yield a drug concentration of 6.5 μM and a cell density of 1.0×10^6 cells/ml. Aliquots (200 μl) were removed, and melphalan uptake was terminated at the indicated time points by centrifugation of the cells through silicone oil at $12,000 \times g$ for 1 min. Tips containing the cell pellets were removed and processed as described under Materials and Methods.

take by leucine is partially negated by α-amino-γ-guanidinobutyric acid (Fig. 1). This interference with leucine inhibition did not occur when only the sodium-independent component of uptake, presumably System L, was operative.

Carrier specificity. The failure of L-α-amino-γ-guanidinobutyric acid to increase intracellular melphalan in the absence of sodium ions suggested that the observed increase was mediated by the leucine transport system distinct from System L in the L1210 leukemia cell (9). To examine this relationship further, experimental conditions were utilized which permitted independent examination of the two high-affinity leucine transport systems responsible for melphalan uptake.

The interaction of the arginine homologue with System L was examined both in medium containing sodium ions and in sodium ion-free medium using BCH, a substrate specific for that system (6–8). The interaction of the homologue with the sodium-dependent leucine transport system was examined in sodium ion-containing medium with excess BCH (2 mM) and AIB (50 mM) to block that part of melphalan uptake (50%) which occurs by System L (3) and amino acid uptake which occurs by Systems L and A as described by Christensen (6). A BCH concentration of 2 mM was chosen to ensure saturation of System L, which exhibits an apparent K_m for BCH transport of 15 μM (3). These experimental conditions allowed examination of the interaction of melphalan, leucine, and α-amino-γ-guanidinobutyric acid with the leucine transport system which exhibits dependence upon sodium ions and is insensitive to BCH (3, 9).

L-α-Amino-γ-guanidinobutyric acid did not increase intracellular melphalan in the presence of BCH when both transport systems were operative (Fig. 2) or when only System L was operative (Fig. 3). However, the interaction of the arginine homologue with the monovalent cation-dependent transport system (9) is a substantial one. Leucine reduced melphalan uptake by this system, and L-α-amino-γ-guanidinobutyric acid, in the presence of leucine, increased intracellular levels of melphalan by 2 pmoles/10^5 cells (Fig. 4), a value which corresponds exactly with that found under conditions when both amino acid carriers are operative (Fig. 1).

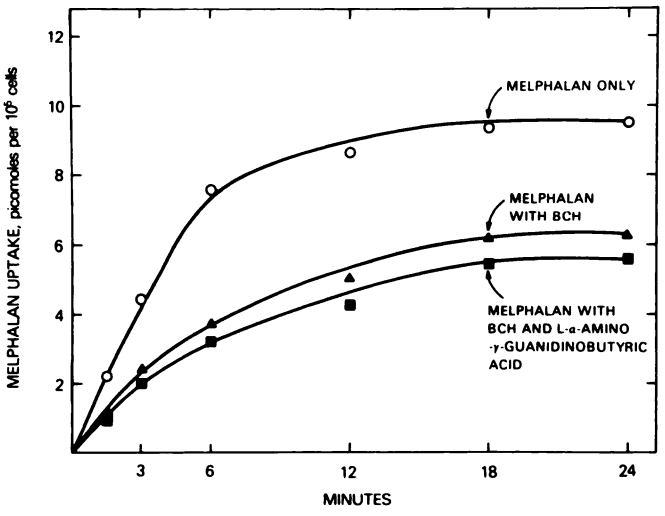

FIG. 2. *Failure of α-amino-γ-guanidinobutyric acid to increase intracellular melphalan in the presence of BCH under conditions in which both leucine transport systems are operative*

Murine L1210 leukemia cells, prepared in transport medium containing sodium ions as described in legend to Fig. 1, were exposed to 6.5 μM [^{14}C]-melphalan alone (○), to [^{14}C]-melphalan and 100 μM BCH (▲), or to [^{14}C]-melphalan and 100 μM each of BCH and α-amino-γ-guanidinobutyric acid (■). The experiment was continued as described in legend to Fig. 1.

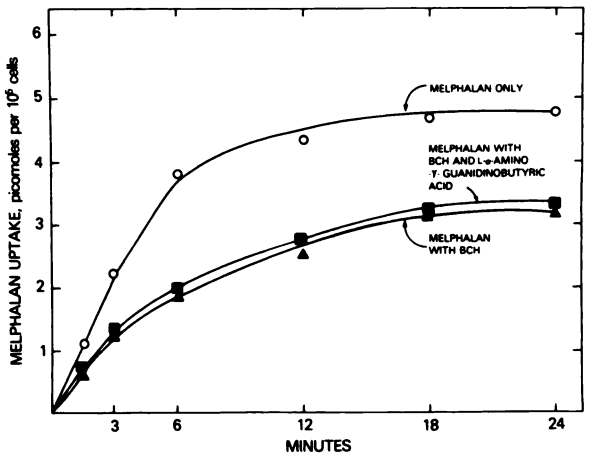

FIG. 3. *Failure of α-amino-γ-guanidinobutyric acid to increase intracellular melphalan via System L*

Murine L1210 leukemia cells, prepared in sodium ion-free transport medium as described in legend to Fig. 1, were exposed to 6.5 μM [^{14}C]-melphalan alone (○), to [^{14}C]-melphalan and 100 μM BCH (▲), or to [C^{14}]-melphalan and 100 μM each of BCH and α-amino-γ-guanidinobutyric acid (■). The experiment was continued as described in legend to Fig. 1.

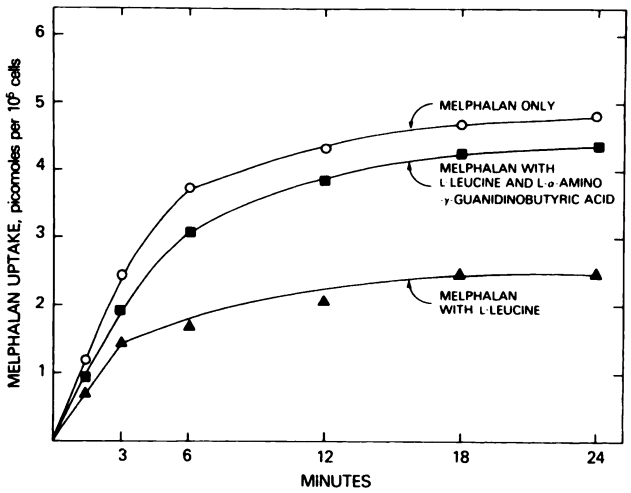

FIG. 4. *α-Amino-γ-guanidinobutyric acid increases intracellular melphalan via the monovalent cation-dependent leucine transport system*

Murine L1210 leukemia cells, prepared in transport medium containing sodium ions as described in legend to Fig. 1, were exposed to 6.5 μM [^{14}C]-melphalan, 2 mM BCH, and 50 mM AIB (○); to [^{14}C]-melphalan, BCH, AIB, and 100 μM L-leucine (▲); or to [^{14}C]melphalan, BCH, AIB, and 100 μM each of L-leucine and α-amino-γ-guanidinobutyric acid (■). The experiment was continued as described in legend to Fig. 1. BCH was included in the final incubation medium in order to block that part of melphalan uptake (50%) which occurs via System L.

To obtain further evidence that the increase in intracellular melphalan provided by the arginine homologue occurs via the monovalent cation-dependent, leucine-preferring transport system, the effect of leucine on the transport of the homologue was examined. Leucine inhibited the transport of L-α-amino-γ-guanidinobutyric acid with an apparent K_i of 20 μM (Fig. 5), a value which closely approximates the affinity of this transport system for leucine (K_m = 10 μM) (9). These results suggest that the arginine homologue interacts directly with the transport system and indicates that the increase in intracellular melphalan provided by the homologue can be attributed to its interaction with the monovalent cation-dependent, high-affinity, leucine-preferring transport system (9) and not to its interaction with System L.

Acceleration of melphalan exodus by leucine and its antagonism by L-α-amino-γ-guanidinobutyric acid. Melphalan exodus from murine L1210 leukemia cells into transport medium devoid of amino acids occurs rapidly, and only 50% of the drug is retained by the cells after 18 min (Fig. 6). Melphalan exodus is accelerated by leucine (Fig. 6), an effect which is antagonized by the arginine homologue.

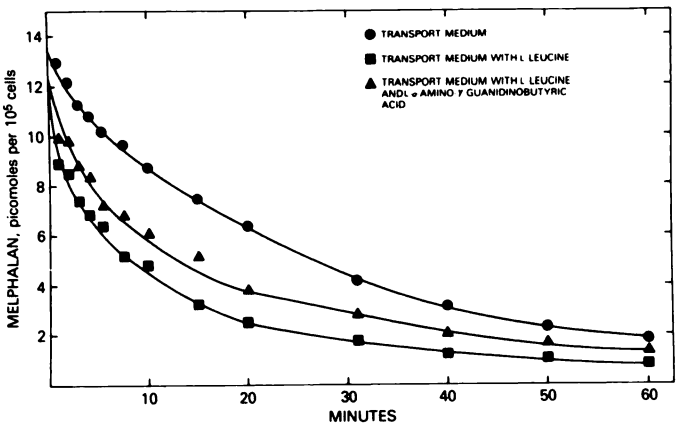

FIG. 6. *Acceleration of melphalan exodus by leucine and its antagonism by α-amino-γ-guanidinobutyric acid*

Murine L1210 leukemia cells, in transport medium containing sodium, were exposed to 6.5 μM [^{14}C]-melphalan for 15 min at a cell density of 1.0 × 10^6 cells/ml. The cell suspension was centrifuged at 300 × *g* for 5 min and the pellets were then resuspended in fresh transport medium only (○), in transport medium containing 10 mM L-leucine (■), or in transport medium containing 10 mM each of L-leucine and L-α-amino-γ-guanidinobutyric acid (▲). The experiment was continued as described in legend to Fig. 1.

DISCUSSION

Previous studies indicated that α-amino-γ-guanidinobutyric acid, the lower homologue of arginine, increased the cytotoxicity of melphalan only in the presence of leucine by increasing the intracellular concentration of the drug (11). The small increases in intracellular melphalan provided by the arginine homologue resulted in large increases in cytotoxicity, an effect which is due to the sharp dose-response curve of the drug (1). Thus, small increases in intracellular melphalan, especially within a critical region corresponding to uptake of 2–5 pmoles of melphalan per 10^5 cells, is accompanied by large increases in cytotoxicity (2). This correlation of intracellular melphalan levels with the cytotoxicity of the drug provided insight into why α-amino-γ-guanidinobutyric acid was so effective in promoting melphalan cytotoxicity in the presence of leucine and was the basis for studies *in vivo* which demonstrated that the arginine homologue reduced the interference of melphalan therapy by leucine (13).

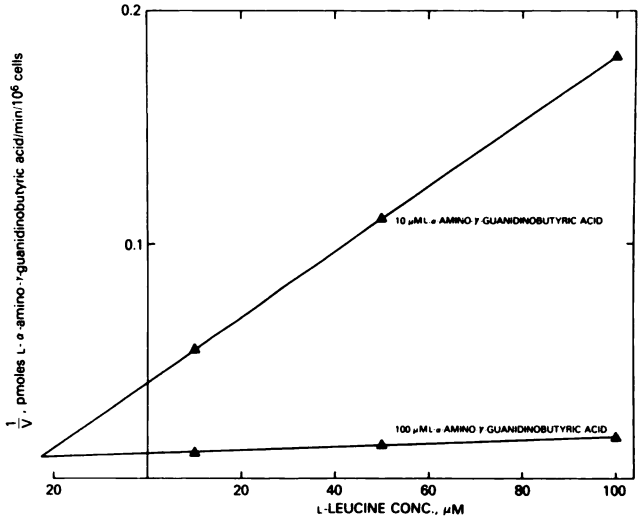

FIG. 5. *Dixon plot for competitive inhibition of L-α-amino-γ-guanidinobutyric acid transport by L-leucine*

Murine L1210 leukemia cells, prepared in transport medium containing sodium ions as described in legend to Fig. 1, were exposed to either 10 μM or 100 μM L-α-amino-γ-guanidinobutyric acid with or without 10 μM, 50 μM, or 100 μM L-leucine. The final incubation medium contained 2 mM BCH and 50 mM AIB. L-α-Amino-γ-guanidinobutyric acid uptake was terminated at 2 min by centrifugation of 1.0 × 10^6 cells through silicone oil at 12,000 × *g* for 1 min. Tips containing the cell pellets were removed and processed as described under Materials and Methods.

The results described in the present study indicate that the observed increase in intracellular melphalan provided by α-amino-γ-guanidinobutyric acid can be attributed to the interaction of the homologue with the monovalent cation dependent, high-affinity, leucine-preferring amino acid transport system (9). This conclusion is supported by the following observations: (a) the homologue is inactive in transport medium with BCH under conditions in which both leucine transport systems are operative or in which only the presumed System L is operative, and (b) the observed increase in intracellular drug found (2 pmoles/10^5 cells) under conditions in which both leucine transport systems are operative is identical with that found when melphalan uptake by System L is blocked by the model substrate BCH, forcing drug uptake to occur via the monovalent cation-dependent leucine transport system. These studies also provide insight into the mechanism of melphalan exodus. It is apparent that leucine, the most effective competitor for both amino acid transport systems involved in melphalan uptake (3–5), is ineffective in reducing melphalan exodus under conditions described in the present study. In fact, melphalan exodus into medium containing leucine is accelerated as compared with melphalan exodus into an amino acid-free environment. It would appear from these results

that a major portion of drug exodus may occur via a pathway not responsible for melphalan and leucine influx. However, the ability of α-amino-γ-guanidinobutyric acid to antagonize the increase in melphalan exodus provided by leucine suggests the potential involvement of an amino acid carrier in the exodus of the drug.

REFERENCES

1. Vistica, D. T., J. N. Toal, and M. Rabinovitz. Amino acid-conferred resistance to melphalan (NSC-8806). I. Structure-activity relationship in cultured L1210 murine leukemia cells. *Cancer Treat. Rep.* **60**:1363–1367 (1976).
2. Vistica, D. T., J. N. Toal, and M. Rabinovitz. Amino acid-conferred protection against melphalan: characterization of melphalan transport and correlation of uptake with cytotoxicity in cultured L1210 murine leukemia cells. *Biochem. Pharmacol.* **27**:2865–2870 (1978).
3. Vistica, D. T. Cytotoxicity as an indicator for transport mechanism: evidence that melphalan is transported by two leucine-preferring carrier systems in the L1210 murine leukemia cell. *Biochim. Biophys. Acta* **550**:309–317 (1979).
4. Goldenberg, G. J., H. Y. P. Lam, and A. Begleiter. Active carrier-mediated transport of melphalan by two separate amino acid transport systems in LPC-1 plasmacytoma cells *in vitro*. *J. Biol. Chem.* **254**:1057–1064 (1979).
5. Begleiter, A., H. Y. P. Lam, J. Grover, E. Froese, and G. J. Goldenberg. Evidence for active transport of melphalan by two amino acid carriers in L5178Y lymphoblasts *in vitro*. *Cancer Res.* **39**:353–359 (1979).
6. Christensen, H. N. *Biological Transport,* Ed 2. W. A. Benjamin, Inc., Menlo Park, Calif., 174–211 (1975).
7. Tager, H. S., and H. N. Christensen. 2-Aminonorbornane-2-carboxylic acid: preparation, properties, and identification of the four isomers. *J. Am. Chem. Soc.* **94**:968–972 (1972).
8. Christensen, H. N., M. E. Handlogten, I. Lam, S. Tager, and R. Zand. A bicyclic amino acid to improve discriminations among transport systems. *J. Biol. Chem.* **244**:1510–1520 (1969).
9. Vistica, D. T., and B. Schuette. Substrate specificity of a high-affinity, monovalent cation-dependent amino acid carrier. *Biochem. Biophys. Res. Commun.* **90**:247–252 (1979).
10. Thomas, E. L., and H. N. Christensen. Nature of the cosubstrate action of Na^+ and neutral amino acids in a transport system. *J. Biol. Chem.* **246**:1682–1688 (1971).
11. Vistica, D. T., J. N. Toal, and M. Rabinovitz. Amino acid-conferred protection against melphalan: interference with leucine protection of melphalan cytotoxicity by the basic amino acids in cultured murine L1210 leukemia cells. *Mol. Pharmacol.* **14**:1136–1142 (1978).
12. Oxender, D. L., and H. N. Christensen. Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cell. *J. Biol. Chem.* **238**:3686–3699 (1963).
13. Vistica, D. T., A. Rabon, and M. Rabinovitz. Effect of L-α-amino-γ-guanidinobutyric acid on melphalan therapy of the L1210 murine leukemia. *Cancer Lett.* **6**:345–350 (1979).

Send reprint requests to: Dr. David T. Vistica, Laboratory of Medicinal Chemistry and Biology, National Cancer Institute, Building 37, Room 6B-09, Bethesda, Md. 20205.