Biochemical Pharmacology, Vol. 28, pp. 3221–3225

0006-2952/79/1101-3221 $02.00/0

CYTOTOXICITIES OF THE L AND D ISOMERS OF PHENYLALANINE MUSTARD IN L1210 CELLS

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(*Received* 29 *January* 1979; *accepted* 9 *April* 1979)

Abstract—The cytotoxicity of D-phenylalanine mustard (medphalan) to murine L1210 leukemia cells in culture was reduced by both the D and L isomers of leucine. L-Leucine only partially protected L1210 cells from medphalan cytotoxicity at drug concentrations above 10 μM, suggesting that medphalan uptake occured via both an amino acid carrier and an as yet undetermined agency, possibly passive diffusion. At equitoxic concentrations of L-phenylalanine mustard (melphalan) and medphalan, L-leucine reduced medphalan cytotoxicity by only one-sixth that obtained with melphalan. Analysis of melphalan and medphalan inhibition of the initial rate of L-leucine transport indicated a melphalan K_i of 0.085 mM, a value one-seventh that of medphalan (K_i, 0.635 mM).

The cytotoxicity of melphalan, L-phenylalanine mustard, to murine L1210 leukemia cells in culture is reduced in growth medium containing amino acids [1]. Investigation of the effect of single amino acids on melphalan cytotoxicity indicated that leucine was the most effective naturally occuring amino acid in reducing drug toxicity [1] and that this was accompanied by a reduction in drug uptake [2,3]. Kinetic analysis indicated that melphalan uptake is mediated by an amino acid transport system of the leucine (L) type and suggested that the protection afforded L1210 cells from melphalan cytotoxicity by amino acids is related to their affinities for transport as compared to melphalan [3]. Further studies indicated that melphalan is transported by two separate high-affinity leucine carriers [4]. Medphalan, D-phenylalanine mustard, was reported to have a therapeutic index similar to that of melphalan and to be effective against a wide variety of experimental neoplasms [5]. In order to gain insight into the role played by carrier transport in phenylalanine mustard cytotoxicity, the effect of leucine on the two isomers was compared.

MATERIALS AND METHODS

Bovine serum albumin was obtained as serum fraction V from Miles Laboratories, Inc. (Elkhart, IN). Fetal calf serum was purchased from Flow Laboratories (Rockville, MD) and RPMI-1630 medium and Dulbecco's phosphate-buffered saline were supplied by the NIH Media Unit. Gentamicin (50 mg/ml) and Fungizone (250 μg/ml) were purchased from Microbiological Associates (Bethesda, MD) and the Grand Island Biological Co. (Grand Island, NY), respectively. Unlabeled melphalan was obtained from the Burroughs Wellcome Co. (Research Triangle Park, NC). Medphalan was obtained from Dr. Harry Wood of the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. L-Leucine was purchased from CalBiochem (San Diego, CA) and D-leucine was obtained from the J. P. Greenstein Collection (Bethesda, MD). The silicone oil, Versilube F-50 (specific gravity 1.045 at 25°; viscosity 70 centistokes at 25°) was obtained from the Harwick Chemical Corp. (Cambridge, MA).

L-[4,5-^{3}H]Leucine (57.4 Ci/mmole) and inulin-[carboxyl-^{14}C] (1.55 mCi/g) were purchased from the New England Nuclear Corp. (Boston, MA).

Melphalan (11 m Ci/mmole), labeled in the chloroethyl groups with ^{14}C, was synthesized by Mr. Morris Leaffer under contract with the Stanford Research Institute (Menlo Park, CA). Radiochemical purity was 97 per cent as determined by thin-layer chromatography on silica gel in *n*-butanol–acetic acid-water (7:2:1). 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. Unlabeled and labeled melphalan exhibited similar transport characteristics as determined by isotope dilution [3] and both had similar cytotoxic potency to L1210 cells [3].

Cell growth and cytotoxicity assays. The conditions used for maintenance of murine L1210 leukemia cells have been described elsewhere [1,3]. Briefly, cells were grown in RPMI-1630 medium supplemented with 20% heat-inactivated fetal calf serum. They were harvested at the logarithmic phase of growth, washed three times in PBS* containing 0.1 mM BSA and 0.25% glucose and suspended in the same medium at 1.0×10^5 to 1.2×10^5 cells/ml. The cytotoxicity of melphalan or medphalan was assessed after a 35 min exposure to the drug, washing and clonal growth of surviving cells in soft nutrient-agar according to the procedure of Chu and Fischer [6] with minor modifications [1,3]. Colonies were scored after 14 days at 37°. All data are corrected for the cloning efficiency (90–100 per cent) of L1210 cells washed and incubated in the appropriate medium.

*Transport of melphalan and amino acids by L*1210

* Abbreviations: PBS, phosphate-buffered saline (pH 7.4); BSA, bovine serum albumin; and PAG, phosphate-buffered saline containing 0.1 mM BSA and 0.25% glucose (pH 7.4).

cells. Logarithmic phase L1210 cells (5×10^5–10×10^5 cells/ml) were harvested by centrifugation at 300 *g* for 5 min and washed three times by suspension and centrifugation in transport medium, composed of Dulbecco's PBS containing 0.1 mM BSA and 0.1% glucose. They were suspended in the appropriate volume of medium and the uptake of melphalan or leucine was initiated by the addition of labeled material, as indicated in the individual experiments. 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 *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 per cent. Tips containing the cell pellet were cut off and the pellets were solubilized in 0.2 N NaOH and counted on a Beckman liquid scintillation counter. Data are corrected for trapped extracellular label using inulin as a marker. Inhibition constants were obtained from Hunter–Downs plots [7,8] in order to reduce the contribution of passive diffusion common to analysis of inverted plots [9].

Cells were maintained at 37° during all phases of the transport study and experiments were completed within 1 hr of their removal from growth medium. Control populations were found to be 90–100 per cent viable as determined by clonal growth after the transport study.

RESULTS

Reduction of melphalan and medphalan cytotoxicities by leucine. The LD_{90} concentration for medphalan, 14 μM, was 9-fold higher than that for melphalan, 1.6 μM (Fig. 1). Medphalan cytotoxicity to L1210 cells was reduced by both the L and the D isomers of leucine, but this reduction is substantially less than the reduction in melphalan cytotoxicity provided by L-leucine. L-Leucine decreased the cytotoxic potency of melphalan by a factor of 20 while reducing medphalan cytotoxicity by a factor of only 3, resulting in a convergence of the dose–response curves. Thus, leucine essentially eliminated the marked difference in the cytotoxic potencies of melphalan and medphalan.

The L and D isomers of leucine differentially reduced medphalan cytotoxicity, the former being considerably more effective than the latter (Fig. 2). The protection curves for both isomers of leucine were similar, with each isomer exhibiting a region in which increases in its concentration were no longer accompanied by decreased cytotoxicity to medphalan.

Inhibition of L-leucine transport by melphalan and medphalan. The greater reduction in medphalan cytotoxicity by the L rather than the D isomer of leucine (Fig. 2) suggested that medphalan might enter cells via the leucine-preferring amino acid transport system also responsible for melphalan transport. However, in order to demonstrate a reduction in transport of L-leucine by medphalan, it was necessary to use substrate concentrations in the nanomolar range, as no inhibition could be demonstrated with higher concentrations. The K_i for medphalan inhibition of L-leucine transport (0.635 mM) was 7-fold higher than that for melphalan (Fig. 3), a ratio closely approximating that for the inhibition of L-leucine transport by D-leucine (Fig. 4),

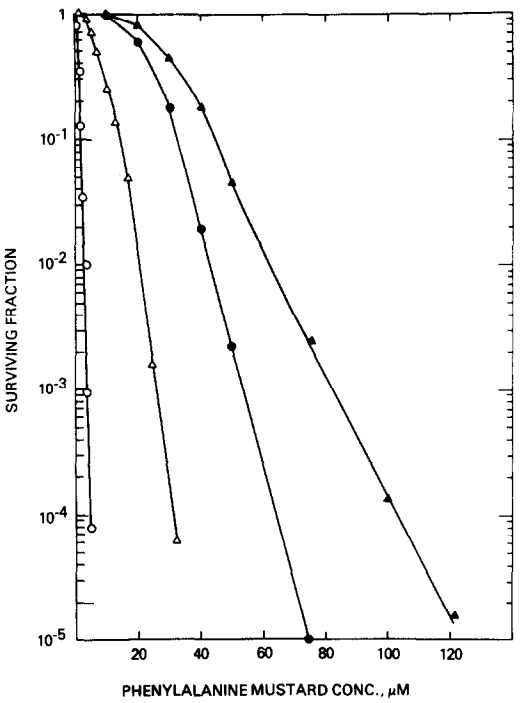

Fig. 1. Comparison of melphalan and medphalan cytotoxicities to murine L1210 leukemia cells; effect of L-leucine. Cells (1.0×10^5 to 1.2×10^5 cells/ml), prepared as described in Materials and Methods, were exposed for 35 min at 37° in PAG medium to the indicated concentration of either melphalan (○) or medphalan (△) alone or to melphalan (●) or medphalan (▲) and 1 mM L-leucine. Cells were harvested and prepared for clonal growth in soft nutrient-agar according to the method of Chu and Fischer [6] with minor modifications [1,3]. Experimental points represent the mean of three separate determinations.

and is in agreement with the increased K_m values of the D isomers of amino acids reported for the transport carrier in the Ehrich ascites tumor cell [10] and in segments of rat small intestine [11].

These results indicate that medphalan is reactive with the leucine preferring transport system of L1210 cells and suggest that medphalan uptake occurs, in part, via the leucine carrier. The failure of leucine to completely block medphalan cytotoxicity (Fig. 2) at an amino acid concentration (1 mM) which would presumably saturate the leucine carrier (5–25 times the D-leucine K_i; 30–150 times the K_m for L-leucine) also suggests that medphalan is partially transported via an agency not reactive with the L or D isomers of leucine.

Reduction in melphalan uptake by medphalan. Examination of the effect of medphalan on the uptake of the D_{37} concentration of melphalan (1.1 μM), the concentration which reduces the surviving fraction to 37 per cent of control, indicated that melphalan uptake is reduced by medphalan (Fig. 5). This reduction in melphalan transport by medphalan approached a limiting value of 50 per cent and contrasted with the complete reduction in melphalan uptake by leucine. The failure of medphalan to reduce melphalan uptake by more than 50 per cent may be related to the specificities of the two leucine-preferring transport systems existing in this cell type [4].

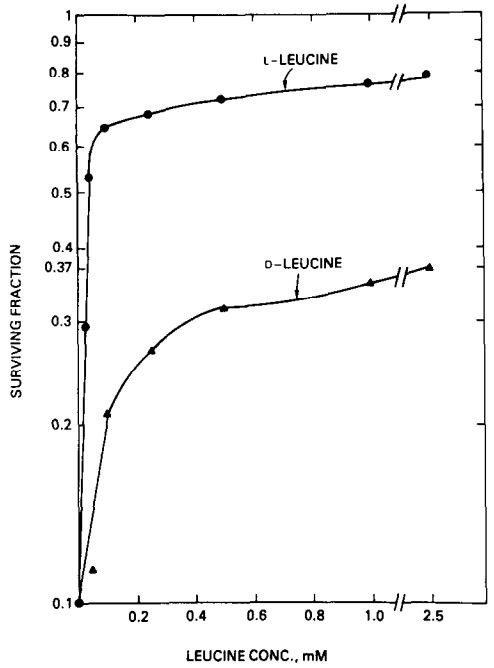

Fig. 2. Interference with medphalan cytotoxicity by the L and D isomers of leucine. Cells (1.0×10^5–1.2×10^5 cells/ml), prepared as described in Materials and Methods, were exposed to 14 μM medphalan, the LD_{90} dose, for 35 min at 37° in PAG medium containing the indicated concentration of either L-leucine (●) or D-leucine (▲). Cells were harvested and the experiment was continued as described in the legend to Fig. 1. Experimental points represent the mean of three separate determinations.

DISCUSSION

Significance of transport mechanism to host toxicity and therapy. It is apparent from the results described here that a portion of medphalan transport into L1210 cells is mediated by the leucine-preferring amino acid transport system. However, they do not permit specific assignment of medphalan transport to one or both of the leucine carriers responsible for melphalan transport [4]. These results also suggest a relationship between such carrier-mediated transport and cytotoxicity since the 10-fold difference in the cytotoxic potencies of melphalan and medphalan is nearly eliminated by leucine. The greater cytotoxic potency of melphalan to L1210 cells may be related to the ability of this cell type to concentrate melphalan 10-fold against a concentration gradient [12], and suggests that medphalan is not concentrated to the same extent as melphalan. These results, as well as those described previously [12], also suggest that it is carrier-mediated uptake which is responsible for such accumulation since concentrative uptake of melphalan is abolished by leucine.

These observations raise the possibility that medphalan may be used as an alternative to melphalan in instances where the concentration of competitive amino acids such as leucine is high. In addition, the recent report [13] that tumors exhibit preferential accumulation of D amino acids suggests the possibility that medphalan may exhibit more tumor specificity than melphalan.

Recent work in this laboratory has confirmed the reduced host toxicity of medphalan [5] and, in addition, has demonstrated that it does not possess the precipitous dose–response toxicity curve of melphalan. The observations that melphalan accumulates in the intes-

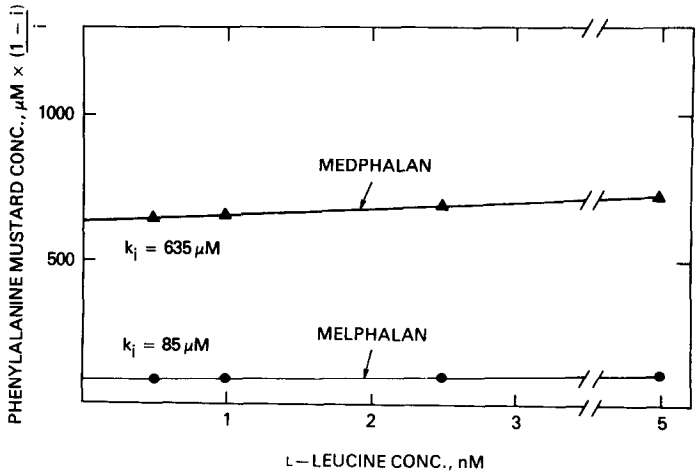

Fig. 3. Inhibition of L-leucine transport by melphalan and medphalan. Five hundred μl of cells (5.0×10^6 cells/ml) in transport medium was incubated with 500 μl of transport medium containing 0.5, 1, 3.5 or 5 nM L-leucine either with or without 100 μM melphalan (●) or 100 μM medphalan (▲). L-Leucine transport was terminated at 24 sec by centrifugation of cells through silicone oil at 12,000 *g*. The data are plotted according to the method of Hunter and Downs [7,8] where *i* is the fractional inhibition of L-leucine transport occurring in the presence of melphalan or medphalan. Assays were performed in triplicate.

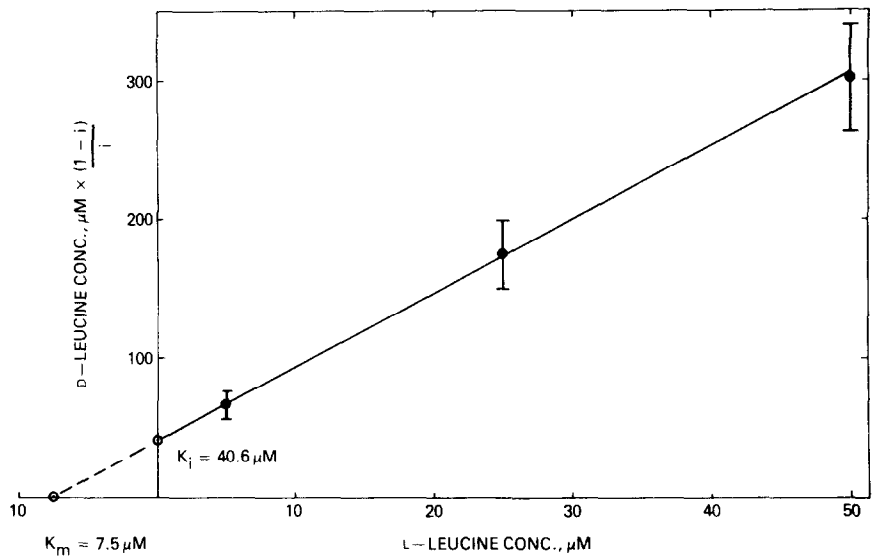

Fig. 4. Inhibition of L-leucine transport by D-leucine. Five hundred μl of cells (2.0×10^6 cells/ml) in transport medium was incubated with 500 μl of transport medium containing 5, 25 or 50 μM L-leucine either with or without 10, 25, 50 or 100 μM D-leucine. L-Leucine transport was terminated at 24 sec by centrifugation of cells through silicone oil at 12,000 g. The data are plotted according to the method of Hunter and Downs [7,8] where i is the fractional inhibition of L-leucine transport occurring in the presence of D-leucine. The line of best fit for D-leucine inhibition of L-leucine transport was derived from the linear regression equation $y = 5.45x + 40.61$. The D-leucine K_i is 40.6 μM and the L-leucine K_m is 7.5 μM. Points on the graph represent the means ± S.D.; N = 3.

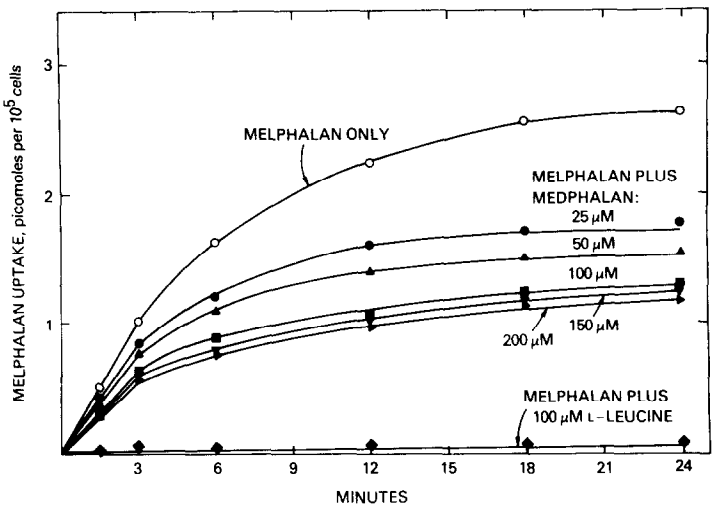

Fig. 5. Inhibition of melphalan uptake by medphalan. Five hundred μl of cells (2.0×10^6 cells/ml) in transport medium was added to 500 μl of medium containing either 1.1 μM [^{14}C]melphalan alone (○), [^{14}C]melphalan and 25 μM (●), 50 μM (▲), 100 μM (■), 150 μM (▼) or 200 μM (►) medphalan, or [^{14}C]melphalan and 100 μM L-leucine (♦). Melphalan uptake was terminated at the indicated time by centrifugation of the cells through silicone oil at 12,000 g. Assays were performed in triplicate.

tine of the dog [14] and that its toxicity in mice is most likely due to such accumulation [15] indicate the importance of studies on the transport characteristics and cytotoxicity of medphalan in sensitive tissues of the tumour bearing host.

REFERENCES

1. D. T. Vistica, J. N. Toal and M. Rabinovitz, *Cancer Treat. Rep.* **60**, 1363 (1976).
2. D. T. Vistica, J. N. Toal and M. Rabinovitz, *Proc. Am. Assn. Cancer Res.* **18**, 26 (1977).
3. D. T. Vistica, J. N. Toal and M. Rabinovitz, *Biochem. Pharmac.* **27**, 2865 (1978).
4. D. T. Vistica, *Biochim. biophys. Acta* **550**, 309 (1979).
5. L. H. Schmidt, R. Fradkin, R. Sullivan and A. Flowers, *Cancer Chemother. Rep.* Suppl. 2, 1 (1965).
6. M. Chu and G. A. Fischer, *Biochem. Pharmac.* **17**, 753 (1968).
7. J. L. Webb, *Enzyme and Metabolic Inhibitors*, Vol. 1, pp. 149–91. Academic Press, New York (1963).

8. A. Hunter and G. E. Downs, *J. biol. Chem.* **157**, 427 (1945).
9. K. D. Neame and T. G. Richards, *Elementary Kinetics of Membrane Carrier Transport*, pp. 16–40. Blackwell Scientific Publications, London (1972).
10. D. L. Oxender and H. N. Christensen, *J. biol. Chem.* **238**, 3686 (1963).
11. L. R. Finch and F. J. R. Hird, *Biochim. biophys. Acta* **43**, 278 (1960).
12. D. T. Vistica and M. Rabinovitz, *Biochem. biophys. Res. Commun.* **86**, 929 (1979).
13. O. Tamemasa, R. Goto and T. Suzuki, *Gann* **69**, 517 (1978).
14. R. L. Furner, L. B. Mellett, R. K. Brown and G. Duncan, *Drug Metab. Dispos.* **4**, 577 (1976).
15. J. L. Millar, B. N. Hudspith, T. J. McElwain and T. A. Phelps, *Br. J. Cancer* **38**, 137 (1978).