Vol. 86, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1979 Pages 929-932

CONCENTRATIVE UPTAKE OF MELPHALAN, A CANCER CHEMOTHERAPEUTIC AGENT WHICH IS TRANSPORTED BY THE LEUCINE-PREFERRING CARRIER SYSTEM

David T. Vistica and Marco Rabinovitz

Laboratory of Medicinal Chemistry and Biology,
National Cancer Institute
Bethesda, Maryland 20014

Received January 4, 1979

SUMMARY

The transport of melphalan, L-phenylalanine mustard, proceeded uphill against a concentration gradient and resulted in a distribution ratio of approximately 10. Concentrative uptake was temperature sensitive and was inhibited by the metabolic inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and L-leucine, a natural substrate of the transport carriers. These results indicate that melphalan transport is an energy requiring process and that naturally occurring competitive substrates such as leucine markedly reduce concentrative uptake of the drug.

INTRODUCTION

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 occurring 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 transport is partially dependent upon sodium and is mediated by two separate high-affinity leucine carriers (4). In order to gain insight into the relationship between drug uptake and cytotoxicity, a study was undertaken to determine whether melphalan transport occurs via facilitated diffusion or whether the uptake of the drug occurs via an active, energy requiring process.

0006-291X/79/030929-04$01.00/0

Copyright © 1979 by Academic Press, Inc.
All rights of reproduction in any form reserved.

MATERIALS AND METHODS

Materials. 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. L-leucine and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were purchased from Calbiochem (San Diego, California). Thiourea was obtained from the Sigma Chemical Company (St. Louis, Missouri). 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, Massachusetts). Inulin-[carboxyl-^{14}C] (1.55 mCi/gram), thiourea-[^{14}C] (55.5 mCi/mmole) and tritiated water (25 mCi/gram) were purchased from New England Nuclear Corp. (Boston, Massachusetts). Labelled thiourea was diluted with unlabelled material and was used at a final concentration of 100 µM.

Melphalan (11 mCi/mmole), labelled 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). Labelled 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. Intracellular radioactivity was identified as unhydrolyzed melphalan by thin layer chromatography on silica gel 60 in n-butanol:acetic acid:water (4:1:1, v/v) as described previously (3).

Methods.

Cell Growth and Transport Studies.

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 3 times in transport medium composed of Dulbecco's phosphate buffered saline containing 0.1 mM bovine serum albumin either with or without 0.1% glucose (pH 7.4) and suspended in the same medium at 2.0 x 10^6 cells/ml. The uptake of labelled melphalan, thiourea or tritiated water was initiated by addition of labelled 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 x g for 1 minute 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 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 with the use of inulin as a marker.

Cells were maintained at 37° during all phases of the transport study and experiments were completed within 1 hour of their removal from growth medium. Control populations were found to be 90-100% viable as determined by clonal growth (1,3) after the transport study.

Estimation of Intracellular Water Space and Calculation of Distribution Ratios.

Intracellular water was estimated by the method of Wohlhueter et al. (5) with the use of tritiated water. The intracellular water space for L1210 cells was 0.95 µl/10^6 cells. Thiourea was used as an index of facilitated or passive diffusion as distribution ratios for it averaged 1.4, indicating that it is not concentrated to any significant extent. Distribution ratios for melphalan were normalized to that obtained with thiourea.

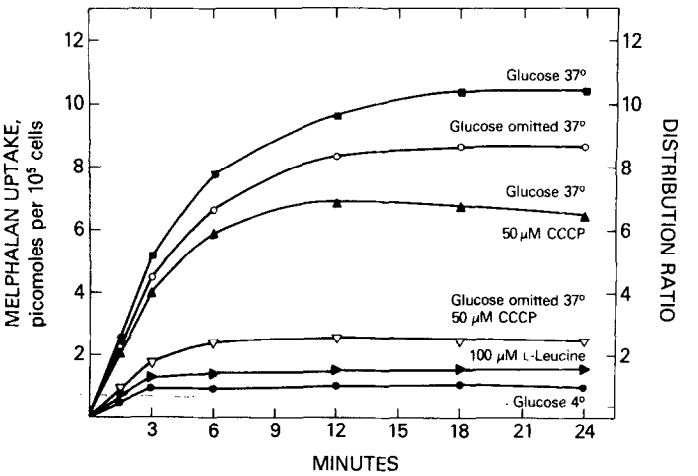

Figure 1. Concentrative Uptake of Melphalan and its Sensitivity to Temperature, CCCP and Leucine. L1210 cells (1.3 x 10^6 cells/ml) were incubated in transport medium under the conditions illustrated in the figure. Incubations with CCCP were carried out for 30 minutes prior to addition of drug. [^{14}C]-Melphalan or [^{14}C]-melphalan and L-leucine were then added to yield a drug concentration of 6.5 μM, a minimal 100% cytotoxic concentration (1), and a cell suspension of 1.0 x 10^6 cells/ml. Melphalan uptake was terminated by centrifugation of 200 μl aliquots of the cell suspension through silicone oil at the indicated time points. Cell pellets were prepared for liquid scintillation counting as described in Methods.

RESULTS AND DISCUSSION

Melphalan uptake proceeded uphill against a concentration gradient and achieved a distribution ratio of approximately 10 (Figure 1). Concentrative uptake is abolished by incubation of cells with drug at 4°C and by leucine, the native substrate of the leucine carriers. In the absence of glucose, CCCP, an inhibitor of oxidative phosphorylation, reduces concentrative uptake indicating that melphalan transport is an active, energy requiring process. The failure of CCCP to abolish concentrative uptake of melphalan in the presence of glucose suggests that both glycolysis and oxidative processes may participate in the generation of cellular energy utilized for drug uptake.

The observation that the drug is concentrated by tumor cells for which it has chemotherapeutic potency *in vivo* (6) and that this is mediated by natural carrier substrates (2-4), has distinct therapeutic consequences. A reduction

in the concentrative uptake of melphalan may contribute to the development of resistant cell populations due to exposure of cells to sub-optimal levels of drug.

REFERENCES

1. Vistica, D.T., Toal, J.N. and Rabinovitz, M. (1976) Cancer Treat. Repts. 60, 1363-1367.
2. Vistica, D.T., Toal, J.N. and Rabinovitz, M. (1977) Proc. Amer. Assoc. Cancer Res. 18, 26.
3. Vistica, D.T., Toal, J.N. and Rabinovitz, M. Biochem. Pharmacol., In Press.
4. Vistica, D.T., Biochim. et Biophys. Acta, In Press.
5. Wohlheuter, R.M., Marz, R., Graff, J.C. and Plagemann, P.G.W. (1976) J. Cell. Physiol. 89, 605-612.
6. Schmidt, L.H., Fradkin, R., Sullivan, R. and Flowers, A. (1965) Cancer Chemotherapy Repts., Supplement 2, 1.