Transport of Melphalan by Sensitive and Resistant L1210 Cells

William R. Redwood and Michael Colvin
Pharmacology Laboratory, The Johns Hopkins Oncology Center, Baltimore, Maryland 21205

ABSTRACT

The in vitro transport of L-\(\text{p-\{di-2-chloroethylamino\}}\)\(^{[\text{14C}]\text{phenylalanine}}\) by i.p. grown mouse L1210 leukemias has been studied in melphalan-sensitive and melphalan-resistant cell populations. As has been reported previously, uptake of the drug is a temperature-sensitive, partly sodium-dependent, active process with cell:medium accumulation in excess of 5:1 at 37°C and a process which is competitively inhibited by L-phenylalanine. Efflux studies demonstrated that at least 75% of the intracellular radioactivity was free to leave the cell.

At low concentrations of melphalan (3 to 10 \(\mu\)M), the sensitive cells were characterized by an apparent \(K_m\) for transport of 11.1 \(\pm\) 2.1 (S.E.) \(\mu\)M and an apparent \(V_{max}\) of 25.6 \(\pm\) 9.9 pmol/10\(^6\) cells/min while at high concentrations of melphalan (14 to 80 \(\mu\)M), the corresponding values were 90 \(\pm\) 16 \(\mu\)M and 158 \(\pm\) 15 pmol/10\(^6\) cells/min. These data are consistent with previous reports that there are at least two distinct carrier systems which transport melphalan.

Transport studies on melphalan-resistant L1210 cells revealed both a lower velocity of drug uptake and a lower intracellular accumulation of the drug at equilibrium than in sensitive L1210 cells. At high concentrations of melphalan (14 to 80 \(\mu\)M), a kinetic analysis indicated that in the resistant cells there was a 3-fold increase in the apparent \(K_m\) for transport with no appreciable change in the \(V_{max}\). At low concentrations of melphalan (3 to 10 \(\mu\)M), there was a similar decrease in the transport of melphalan by the resistant cells as compared to sensitive cells. However, in the presence of 1 mM \(\text{dl-\beta-2-aminobicyclo[2,2,1]heptane-2-carboxylic acid, a specific inhibitor of the lower-affinity L system, there was no significant difference between melphalan transport in resistant and sensitive cells at the low concentration. These studies suggest that the nature of the alteration in the melphalan transport in the resistant L1210 cell may be a specific mutation in the lower-affinity L system rendering melphalan transport less efficient.

INTRODUCTION

Melphalan (L-phenylalanine mustard) is an antitumor-alkylating agent which is in extensive clinical use (2, 5, 13). In 1977, Goldenberg et al. (7) reported that the transport of melphalan into L5178Y lymphoblasts in vitro was a carrier-mediated process, and Vistica et al. (16–18) have recently found a similar carrier system in L1210 cells. Melphalan is active against the murine L1210 leukemia in vivo, and a melphalan-resistant variant has been isolated by Schabel et al. (12). The purpose of this study was to examine melphalan transport into L1210 ascites cells and to determine if the melphalan-resistant subpopulation had altered transport characteristics since mutation of a specific carrier protein is a known mechanism of drug resistance (8).

MATERIALS AND METHODS

\(^{[\text{14C}]\text{Melphalan}}\) (specific activity, 8.0 mCi/mmol) was obtained from the Drug Development Branch of the National Cancer Institute. Radiochemical purity was found to be 97% by thin-layer chromatography on silica gel using n-butyl alcohol:acetic acid:water (4:1:1). L-Leucine, L-glutamine, p-hydroxymercuribenzoate, and gramicidin D were obtained from the Sigma Chemical Co., St. Louis, Mo. BCH was obtained from New England Nuclear, Boston, Mass.

L1210 cells were grown i.p. in C57BL x DBA/2 F1 (BD2F1) mice and were harvested on the fifth day following inoculation of 10\(^6\) cells. Melphalan-sensitive L1210 cells were originally obtained from Dr. Richard Adamson of the National Cancer Institute and have been maintained in passage at Johns Hopkins for 5 years. Melphalan-resistant L1210 cells were provided by Dr. Frank Schabel of the Southern Research Institute, Birmingham, Ala. and have been maintained in mouse passage with melphalan treatment during each passage for approximately 1 year. That these cells are highly resistant to melphalan is indicated in the data shown in Table 1. Harvested cells were washed 3 times in cold PBS (Grand Island Biological Co., Grand Island, N. Y.). The same medium adjusted to pH 7.4 at 37°C and containing 10 mM glucose was used for the transport studies. Trypan blue exclusion was used to monitor cell viability, and only cell suspensions containing greater than 95% viable cells were used for the drug uptake measurements. Cell size was determined with a Coulter electronic particle analyzer equipped with a channel analyzer (Models ZB and C1000; Coulter Electronics, Hialeah, Fla.).

Transport studies were carried out by incubating L1210 cells at 0.5 to 2.5 \(\times\) 10\(^7\) cells/ml with varying concentrations of \(^{[\text{14C}]\text{melphalan}}\) (3 to 80 \(\mu\)M) in a total volume of 0.75 ml. In order to minimize hydrolysis, it was necessary to prepare the melphalan solutions immediately prior to use. The radiolabeled drug supplied as a powder was dissolved in ethanol:1 M HCl [5:1 (v/v)] and was diluted to the desired concentration in PBS immediately prior to use. At zero times, the radiolabeled drug was rapidly injected into the cell suspensions, and the incubation mixtures were layered over 0.5-mI solutions of silicon oil and light mineral oil [84:16 (v/v)] in 1.5-mi microcentrifuge tubes maintained at 37°C. The drug uptake was terminated by centrifugation of the cells through the oil layer (19) at 12,000 \(\times\) g for 2 min in a Beckman Microfuge B (Beckman Instruments, Inc., Palo Alto, Calif.). In all experiments, the first 4 samples were taken at 25- to 30-second intervals, and the initial uptake velocity was calculated from the linear portion of the curve.

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2 Recipient of a Special Postdoctoral Fellowship from the American Cancer Society. To whom requests for reprints should be addressed.
3 The abbreviations used are: \(^{[\text{14C}]\text{melphalan}}, \text{L-p-\{di-2-chloroethylamino\}-}\)\(^{[\text{14C}]\text{phenylalanine}}, \text{BCH, DL-\beta-2-aminobicyclo[2,2,1]heptane-2-carboxylic acid; PBS, Dulbecco’s phosphate-buffered saline.}
through these initial points and the origin. Binding of [14C]-melphalan to the L1210 cell surface was measured by incubating the cells at 0° with varying concentrations of the drug. Metabolic inhibitors of drug transport were preincubated with the L1210 cells for 15 min at 37° prior to addition of the [14C]melphalan. After decantation of the oil, the cell pellet was dissolved in 0.25 M NaOH, and the radioactivity was measured by liquid scintillation counting. An estimation of the quantity of aqueous solution that was being transported through the oil layer by the centrifugation of the L1210 cells was made using a solution of methoxy[3H]inulin (731 μCi/mg; New England Nuclear, Boston, Mass.) as a marker for extracellular fluid.

Efflux studies were conducted by preincubation of L1210 cells with [14C]melphalan for 20 min at 37° followed by separation of the cells by centrifugation through silicon oil and light mineral oil and resuspension of the cells in fresh PBS at 37°. Serial samples were collected at 2-min intervals, cells were separated by centrifugation, and radioactivity was measured in both the supernatant and cell fractions. Thin-layer chromatography studies of the radiolabeled compound contained in the cell effluent were carried out to characterize the radioactivity.

The drug uptake data were processed by linear regression analysis, and the reciprocal initial velocities were plotted against reciprocal melphalan concentration to determine the apparent \( K_m, V_{max}, \) and \( K \) (in the presence of leucine) for the transport process. The Lineweaver-Burk plots

\[
\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{S} + \frac{1}{V_{max}}
\]

were analyzed by linear regression, and the S.E. of the slope and intercept on the y axis were obtained. \( K \) was determined from the expression \([1 + (1/K)K_m/V_{max}]\) for the slopes of the Lineweaver-Burk plots in the presence of L-leucine. Comparison of the statistical significance of the difference of means was by 2-tailed t test.

RESULTS

**Time Course of [14C]Melphalan Uptake.** The uptake of [14C]melphalan by L1210 cells was linear with time for the first 3 to 4 readings within 1 to 2 min of mixing, but nonlinearity occurred rapidly and was more marked at high drug concentrations. Representative uptake data for short incubations are shown in Chart 1. No significantly greater uptake of [14C]melphalan was observed after 10 min, indicating a rapid saturation of the intracellular compartment. The cell:medium ratio of [14C]melphalan, corrected for the surface binding of [14C]melphalan, is plotted in Chart 2 as a function of the concentration of free drug. L1210 cell volume was measured as 3.73 ± 1.79 (S.D.) \( \times 10^{-13} \) L/cell for melphalan-sensitive cells and 3.86 ± 1.65 \( \times 10^{-13} \) L/cell for melphalan-resistant cells. Uptake of [14C]melphalan was strongly dependent upon temperature. At 37°, cell:medium ratios in excess of 5:1 were routinely observed after 10-min incubation with [14C]melphalan, but at 0°, no uptake could be demonstrated. However, at 0°, it was possible to demonstrate the rapid surface binding of [14C]melphalan to L1210 cells. A zero-degree control for such binding was run with each experiment, and this value was subtracted from each time recording for [14C]melphalan uptake.

Comparison of radioactivity attributable to [3H]inulin in both the cell pellet and supernatant indicated that the amount of extracellular fluid associated with the cell pellet was 7.1 ± 0.2 \( \times 10^{-14} \) L/cell. In a typical drug uptake experiment, 10% of

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**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dose</th>
<th>% of ILS*</th>
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</thead>
<tbody>
<tr>
<td>Sensitive L1210</td>
<td>10 mg/kg</td>
<td>136 (4/5 &gt; 60 days)</td>
</tr>
<tr>
<td></td>
<td>20 mg/kg</td>
<td>226 (4/5 &gt; 60 days)</td>
</tr>
<tr>
<td>Resistant L1210</td>
<td>10 mg/kg</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>20 mg/kg</td>
<td>3</td>
</tr>
</tbody>
</table>

* ILS, increase in life span.
the $[^{14}C]$melphalan bound to the cell at 0° was attributable to the extracellular fluid. This contamination contributed only 2% to the $[^{14}C]$melphalan uptake observed after 5-min incubation at 37°.

**Influence of Inhibitors of $[^{14}C]$Melphalan Uptake.** The addition of cold melphalan caused a significant reduction in the transport of the radiolabeled drug. In the presence of a fixed ratio of cold melphalan to radiolabeled drug of 7.5:1, the measured uptake of $[^{14}C]$melphalan was $25.6 \pm 2.9\%$ of the control values in the absence of cold drug over the concentration range of 14 to 40 $\mu M$ melphalan.

Table 2 lists the effects of various metabolic inhibitors on $[^{14}C]$melphalan transport. Of the metabolic inhibitors, $p$-hydroxymercuribenzoate, 2,4-dinitrophenol, NaCN, and gramicidin D.

Table 2 also lists the values found for $[^{14}C]$melphalan transport into L1210 cells in the presence of L-leucine, L-glutamine, and BCH, a specific inhibitor of the L-amino acid (leucine-preferring) system in Erhlich ascites cells (4). Lineweaver-Burk plots of the transport of $[^{14}C]$melphalan in the presence of L-leucine are presented in Chart 3 together with the control plot obtained in the absence of the inhibitor. The coefficients and S.E. of the coefficients of these lines are given in the chart legend. The correlation coefficients of the lines approach unity, and there is no statistically significant difference in the values for the intercepts on the y axis. It would therefore appear that $[^{14}C]$melphalan transport into L1210 ascites cells obeys simple Michaelis-Menten kinetics over the range of 14 to 80 $\mu M$ and that L-leucine acts as a competitive inhibitor. The mean value for the inhibitory coefficient $K_i$ for L-leucine is 73.9 $\pm 8.9\%$. 

**Kinetics of $[^{14}C]$Melphalan Transport at High Concentrations.** A comparison of the uptake of $[^{14}C]$melphalan by melphalan-resistant L1210 cells with melphalan-sensitive cells is shown in Chart 4. The cell:medium ratio of $[^{14}C]$melphalan in the resistant cells was typically about 70% of that of sensitive cells after 10-min incubation with the drug. The initial velocity of $[^{14}C]$melphalan uptake was significantly lower in the melphalan-resistant than in the control cells as illustrated by the corresponding Lineweaver-Burk plots in Chart 5. The coefficients and S.E. of the coefficients of the 2 linear regression lines of Chart 5 are provided in the chart legend. From these data, it can be seen that there is no significant difference between the intercepts on the y axis for the 2 populations of L1210 cells. The apparent kinetic parameters for $[^{14}C]$melphalan transport in the concentration range of 14 to 80 $\mu M$ are as follows: sensitive, $K_m = 90 \pm 16\%$ and $V_{max} = 158 \pm 45$ pmol/10⁶ cells/min; resistant, $K_m = 287 \pm 83\%$ and $V_{max} = 214 \pm 97$ pmol/10⁶ cells/min.

**Influence of Inhibitors of $[^{14}C]$Melphalan Transport.** Begleiter et al. (1) have recently reported that there are 2 distinct carrier systems mediating the uptake of $[^{14}C]$melphalan into L5178Y lymphoblasts in vitro. These are the Na⁺-independent L system (leucine preferring) which operates op-
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Sodium Dependence of Melphalan Uptake. Goldenberg et al. (1, 7) have reported that melphalan transport into L5178Y lymphoblasts in vitro is sodium dependent. Uptake studies of \[^{14}C\]melphalan by L1210 cells were carried out in the balanced salt medium described by Martin (9) in which Na\(^+\) was replaced by choline, and pH 7.4 was maintained with 20 mM Tris buffer. The cellular radioactivity measured after 10 min incubation was found to be lower in the Na\(^+\)-free medium compared to the control Na\(^+\)-containing medium at both high (20 \(\mu\)M) and low (4 \(\mu\)M) concentrations of melphalan. This Na\(^+\) dependence of melphalan uptake was also seen in the melphalan-resistant cells. At 20 \(\mu\)M melphalan in Na\(^+\)-free medium, the sensitive cells contained 55.4 \pm 4.4% of the melphalan in sensitive cells in control medium while the melphalan-resistant cells contained 50.2 \pm 1.0% of the melphalan in the resistant cells in control medium. These values were not significantly different. At 4 \(\mu\)M melphalan in Na\(^+\)-free medium, the corresponding values for sensitive and resistant cells are 48.6 \pm 0.2% and 42.0 \pm 0.6%, respectively. There is a statistically significant difference (\(p < 0.02\)) between the effect of Na\(^+\) depletion upon sensitive and resistant cells at the low concentrations of melphalan.

Efflux of \[^{14}C\]Melphalan from L1210 Cells. The time course for efflux of \[^{14}C\]melphalan from melphalan-resistant and control L1210 cells is illustrated in Chart 7. The 2 populations of cells were preincubated at 37\(^\circ\)C for 20 min with 57 \(\mu\)M \[^{14}C\]melphalan, and the drug uptake was measured and corrected for surface binding. Melphalan-resistant cells contained 101 pmol/10\(^6\) cells. Melphalan-sensitive L1210 cells

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The transport of melphalan into cultured Li210 cells is mediated by 2 distinct carrier systems.

**DISCUSSION**

While this work was in progress, Vistica et al. (16–18) have reported that the transport of melphalan into cultured Li210 cells is carrier mediated and have studied amino acid-conferred protection against melphalan in cultured Li210 cells and in Li210 cells in vivo. The present investigation confirms that in i.p. grown Li210 cell populations, melphalan transport is carrier mediated. Transport of the drug into these Li210 cells is an uphill process with intracellular accumulations at least 5-fold greater than in the external medium (Chart 2) and is markedly temperature dependent with no accumulation of the drug observed at 0°C. Efflux studies have demonstrated that after 20-min incubation, at least 75% of the intracellular radioactivity was free to leave the cell. Marked inhibition of [14C]melphalan uptake was observed in Li210 cells treated with the sulfhydryl-binding agent p-hydroxymercuribenzoate. Significant inhibition of drug transport also occurred in the presence of 2,4-dinitrophenol or cyanide, inhibitors of cellular ATP production.

The small but significant inhibition of [14C]melphalan transport observed with gramicidin D may be due to a decrease in intracellular ATP caused by interference with the alkali-metal ion gradients. Christensen et al. (4) have shown that there is an inhibition of the uphill amino acid transport by the Na+-independent L system in Ehrlich cells after treatment with gramicidin D in Na+-containing media. An alternative explanation is that there is a contribution to [14C]melphalan uptake from a Na+-dependent carrier, and this cotransport is indirectly inhibited by the reduction in Na+ ion gradient caused by the addition of gramicidin D. The recent reports by Begleiter et al. (1) and Vistica (16) that there are 2 different carrier systems operating to transport [14C]melphalan into Li5178Y cells have also been considered. The present observation that there is a statistically significant difference in the Lineweaver-Burk equations for the high- and low-concentration ranges suggests that the transport of [14C]melphalan into Li210 ascites cells is mediated by 2 distinct carrier systems similar to the transport systems in Li5178Y lymphoblasts (1). The low-affinity system is analogous to the Na+-independent, leucine-prefering system of the Ehrlich cell (3). Application of the Neal Analysis (10) for 2 independent saturable transport systems to the data of Charts 5 and 6 for sensitive and resistant cells is shown in Table 3.

The transport studies of melphalan into melphalan-resistant Li210 cells have demonstrated both a lower velocity of melphalan uptake and a lower intracellular accumulation of the drug at equilibrium than in sensitive Li210 cells. Kinetic analysis at concentrations of 14 to 80 μM melphalan indicates that there is no difference between the apparent Vmax of the melphalan carrier in the resistant and control Li210 cells but that there is a 3-fold increase in the apparent Km for melphalan transport in the melphalan-resistant cells (Chart 5). This finding...
is evidence for a decrease in the affinity of the carrier for melphalan in the melphalan-resistant cells. Efflux studies of [14C]melphalan at high concentration (57 μM) have revealed no difference in the efflux of the drug from melphalan-resistant compared with control L1210 cells. This finding rules out the possibility that the drug resistance could be due to an enhanced efflux from the cell.

At low concentrations of melphalan (3 to 10 μM), the initial velocity of drug uptake is also lower in the melphalan-resistant cells compared to the control cells, but this difference is eliminated when both cell populations are exposed to BCH (Chart 6). BCH is known to be a specific inhibitor of the leucine-prefering carrier of Ehrlich cells (4, 6) and other cells (11). Measurement of the Na⁺ dependence of melphalan transport in sensitive and resistant cells revealed that there was a greater proportional inhibition of [14C]melphalan uptake by the resistant cells at low melphalan concentration (4 μM) in Na⁺-free medium. This suggests that in the resistant cells, there is a proportionally larger contribution to melphalan transport from a Na⁺-dependent carrier at low melphalan concentration. The elimination of the transport differences between sensitive and resistant cells at low concentrations of melphalan in the presence of BCH and the data on Na⁺ effect at low melphalan concentrations suggest that the transport mutations may be limited to the Na⁺-independent, low-affinity, high-velocity L system. Against this interpretation is the fact that the Km's of the sensitive and resistant cells at low (3 to 10 μM) concentrations of melphalan were different even after application of the Neal correction. The use of the Neal correction should remove the contributions of the L system to the kinetics of uptake at low melphalan concentrations.

The demonstration that the melphalan-resistant L1210 cells transport the drug less efficiently does not necessarily mean that this alteration represents the mechanism of the resistance. However, this alteration is certainly a potentially important factor in the mechanism of resistance, and the degree of transport alteration is similar to that found in other drug-resistant lines (8, 14).

One might expect that the high degree of resistance indicated in Table 1 would require a more impaired transport than has been found if this is the major mechanism of resistance. In this regard, Skipper et al. (15) have demonstrated that a mixture of drug-resistant and drug-sensitive cells of up to 90% sensitive cells showed significant resistance in vivo to the agents studied, and complete resistance occurred with a mixture of 50% resistant and 50% sensitive cells. Thus, it seems possible that our resistant cell population is actually a mixture of sensitive and resistant cells and that the resistant cells have a more complete impairment of drug uptake than the average we have measured. Thus, the presence of a small percentage of such highly resistant cells would result in the high degree of resistance which these cells show in vivo. To completely characterize transport and other potential mechanisms of resistance, it may be necessary to study cloned populations of tumor cells.

REFERENCES

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