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Temperature dependence and effect of membrane lipid alteration on melphalan transport in L1210 murine leukemia cells

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Melphalan (L-phenylalanine mustard) is an aromatic alkylating agent derived from phenylalanine. It is an important drug for the treatment of human malignancies. There has been renewed basic interest in the drug because of evidence that its transport into tumor cells is mediated by two different carrier-mediated systems [1–3]. The major objective of our study was to examine the response of melphalan transport to a change in the ordered-fluid transition state of the membrane. This was done by measuring the initial rate of transport as a function of temperature. In addition, we have utilized a method previously reported by us for altering the type of lipids in the membranes of L1210 leukemia cells, using experimental diets fed to the host animal [4]. This alteration in membrane lipids results in a change in fluidity as measured by electron spin resonance; therefore, changes in transport rate could be related to this phase transition. We have reported previously that the kinetic parameters of transport of the anti-leukemic drug, methotrexate, are affected by these lipid alterations [4]. Since melphalan transport, like that of methotrexate, is carrier-mediated, we anticipated there would be a similar effect of membrane lipid alteration on melphalan transport. Moreover, this approach offered an opportunity to study the effect of lipid modification on the two separate carrier components of the melphalan transport system.

Materials and methods

Male DBA/2 mice (Jackson Laboratories, Bar Harbor, ME) were fed either a predominantly saturated-fat diet (basal fat-deficient mixture supplemented with 16% coconut oil), a diet rich in polyunsaturates (basal fat-deficient mixture plus 16% sunflower oil) or a rodent chow (Ralston Purina Co., St. Louis, MO). The fat-deficient base was purchased from Teklad Test Diets, Madison, WI. The coconut oil was purchased from the Ruger Chemical Company, Inc., Hillside, NJ, and the sunflower oil from Cargill Industries, Minneapolis, MN. The exact fatty acid composition of these diets has been reported [5]; briefly, the sunflower oil contains 88% unsaturates, (mostly linoleic acid), whereas the coconut oil contains 90% saturated fatty

acids. After 4 weeks of feeding, 1×10^5 L1210 cells were injected intraperitoneally into the mice, and the diets were continued during the 7-day tumor growth period. Cells were harvested, washed, and counted as described previously [6]. Fatty acid composition was determined as previously reported [4].

[^{14}C]Melphalan, *p*-(di-2-chloroethyl)amino-L-[ring-U-^{14}C]phenylalanine, synthesized by the Stanford Research Institute, Menlo Park, CA, was supplied by Dr. Robert Engle, National Cancer Institute. Unlabeled melphalan was purchased from the Burroughs Wellcome Co., Greenville, NC. For transport studies, $10–15 \times 10^6$ cells were incubated with the desired concentration of melphalan in 1 ml buffered salt solution consisting of 132 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 16 mM Na_2HPO_4 and 5.6 mM glucose, pH 7.2. Time course determinations were made at 0.5, 1, 2, 5, 10, 20, 30 and 60 min. Uptake was terminated by rapid addition of 8 ml of ice-cold buffered salt solution followed by immediate centrifugation at 2000 *g* for 2 min at 0°. The resulting cell pellets were washed twice and dissolved in Soluene-350 (Packard Instrument Co., Downers Grove, IL). [^{3}H]Inulin (Amersham Corp., Arlington Heights, IL) was used to determine that more than 95% of the intracellular [^{14}C]melphalan was retained during the washing procedure. The solubilized pellets were counted in Dimilume-30 (Packard Instrument Co., Downers Grove, IL) on a Beckman LS-3133T liquid scintillation spectrometer. In each experiment, the amount of melphalan taken up at 0° was determined, and this value was subtracted from the 37° uptake values to correct for rapid nonspecific adsorption to the cell surface [1, 7]. For kinetic experiments, an incubation time of 2 min was used, with substrate concentrations from 2 to 100 μM.

Temperature dependence of transport was studied using 100 μM melphalan. Points were obtained at 2° intervals from 10 to 40°. Incubations were for 2 min following a 5-min equilibrium period for the cells and medium at the respective temperature. The lines and break points were assigned by the method of least-squares and represented the best correlations obtained by fitting the data from both directions around the break points. The analysis of Neal [8] was used to calculate the parameters of the individual components of transport with biphasic kinetics. Since the exact contribution of diffusion is unknown, the values obtained are apparent constants for the carrier-mediated processes.

Results

Fatty acid alteration and kinetics of melphalan uptake. The proportions of fatty acids and classes of fatty acids contained in L1210 cell phospholipids are shown in Table 1. There was a considerably higher proportion of polyunsaturates including linoleic acid (18:2) and arachidonic acid (20:4) in the phospholipids of the L1210 cells from the animals fed the sunflower oil diet. Conversely, the cells from animals fed the coconut oil diet contained higher percentages of monounsaturates, especially oleic acid (18:1).

The time courses of uptake by L1210 cells of 10 μM melphalan at 37° were similar for the L1210 cells from the two diet groups, being linear for 2 min during which time the uptake increased to 4.75 nmoles/10^8 cells and then approached an equilibrium at 6.8 nmoles/10^8 cells. All later experiments including kinetic measurements and temperature-dependent studies were carried out for 2 min. There was an initial rapid binding phase that was evident when the initial linear portions of the curve were extrapolated back to 0. This rapid binding has been noted for uptake of melphalan [1] and methotrexate [4, 7]. Uptake at 0° remained linear and increased as a function of melphalan concentration (2–100 μM) but it never exceeded 1.5 nmoles/10^8 cells. The relationship of concentration of melphalan to uptake over the concentration range 2–100 μM demonstrated biphasic Michaelis–Menten kinetics as previously reported for L1210 leukemia [3, 9] and other cells [10]. The apparent values for the L1210 cells from animals fed sunflower oil rich diets were $K'_m = 1.2 \pm 0.5$ μM, $V'_{max} = 0.9 \pm 0.2$ nmole per 10^8 cells per min (high-affinity component) and $K'_m = 128.7 \pm 50.5$ μM, $V'_{max} = 25.2 \pm 7.9$ nmoles per 10^8 cells per min (low-affinity component). There was no significant difference in the K'_m or V'_{max} for melphalan uptake between the cells from the two diet groups.

Table 1. Fatty acid composition of L1210 phospholipids*

Fatty acid	Carbon atoms:double bonds†	Percentage composition	
		Sunflower diet‡	Coconut diet
Individual acids			
Palmitic	16:0	15.8 ± 0.5	15.1 ± 0.3
Palmitoleic	16:1	1.2 ± 0.3	5.1 ± 0.5
Stearic	18:0	23.7 ± 1.2	17.8 ± 0.3
Oleic	18:1	13.8 ± 0.6	40.0 ± 0.7
Linoleic	18:2	23.2 ± 0.9	8.4 ± 0.1
Eicosadienoic	20:2	3.7 ± 0.1	3.1 ± 0.1
Arachidonic	20:4	12.8 ± 0.1	6.1 ± 0.1
Docosatetraenoic	22:4	3.8 ± <0.1	0.4 ± <0.1
Other§		0.5 ± 0.1	0.8 ± 0.2
Classes			
Polyenoics		45.2 ± 1.2	19.4 ± 0.1
Monoenoics		15.6 ± 0.6	45.9 ± 0.4
Saturates		40.2 ± 0.7	34.7 ± 0.5

* L1210 leukemia cells from animals fed the 16% sunflower or 16% coconut rich diets were obtained and the lipids were extracted with $CHCl_3$:CH_3OH. The saponifiable lipids were obtained by alkaline hydrolysis and the fatty acids were methylated and separated by GLC. Shown are the mean ± S.E. of the percentage of total fatty acids from four determinations.

† Number of carbon atoms:number of double bonds.

‡ Differences in the two diet groups were significant at the $P < 0.001$ level in all cases except stearic ($P < 0.005$), eicosadienoic ($P < 0.01$), palmitic, and other (not significant).

§ Includes myristic (14:0), eicosaenoic (20:1), eicosatrienoic (20:3), docosanoic (22:0), docosapentaenoic (22:5), and docosahexaenoic (22:6).

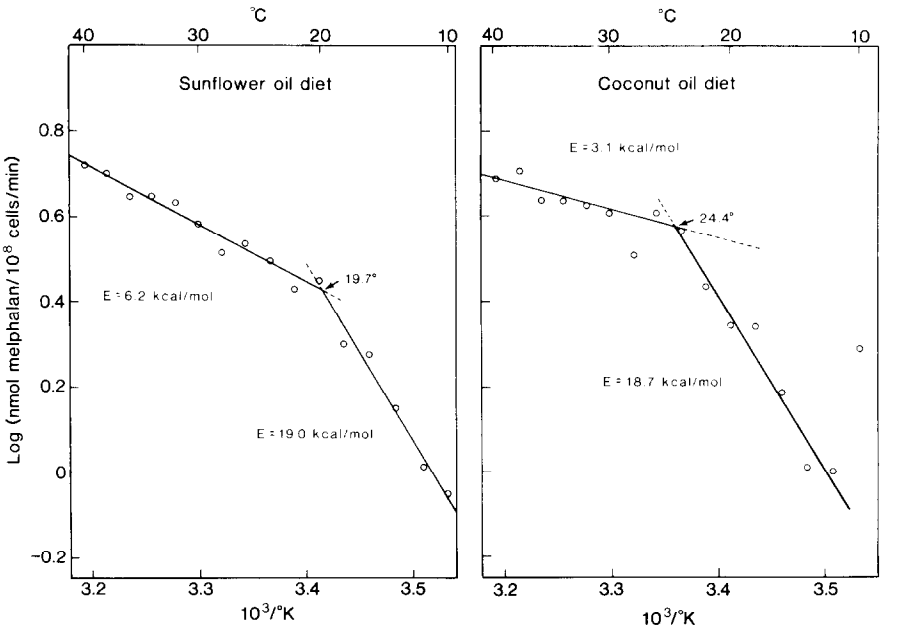

Fig. 1. Representative Arrhenius plots of temperature dependence of melphalan transport by L1210 cells from animals fed sunflower or coconut oil. Cells were incubated for 2 min in buffered salt solution containing 100 μM melphalan, at 2° intervals from 10–40°. Data were corrected for 0° uptake. E, activation energy.

Temperature dependence. The uptake of melphalan at various temperatures by L1210 cells from animals fed sunflower oil rich diets is shown in Fig. 1. The data in the form of an Arrhenius plot from a representative experiment demonstrate that initial transport was greater at higher temperatures. A discontinuity in the line on the Arrhenius plot delineates a single transition temperature. A representative experiment using L1210 cells from animals fed the coconut oil rich diet is shown in Fig. 1. Like the curves for transport of melphalan by cells from mice fed sunflower oil, it was biphasic with a downward discontinuity. However, the transition temperature was higher. The results of determinations on cells from three separate groups of animals are shown in Table 2. The mean transition temperature for the leukemic cells from animals fed the sunflower oil diets was almost 4° lower than that for the cells from the coconut fed animals ($P < 0.05$). The energies of activation were not affected by the lipid alteration.

Table 2. Effect of membrane lipid alteration of L1210 leukemia cells on transition temperatures and activation energies for melphalan uptake*

	Transition temperature (°)	E_a†	E_b
Sunflower oil	18.7 ± 0.5	7.6 ± 1.4	20.9 ± 1.1
Coconut oil	22.5 ± 1.2	5.7 ± 1.6	20.4 ± 2.5
P value	< 0.05	NS	NS

* L1210 cells were incubated for 2 min in buffered salt solution containing 100 μM melphalan at 2° intervals from 10 to 40°. Data were corrected at 0° uptake. The transition temperatures and activation energies were obtained from Arrhenius plots. Shown are the means ± S.E. of determinations on cells from three separate groups of animals fed each diet and studied at different times.

† Activation energies above (E_a) or below (E_b) the transition temperatures are given in kcal/mole. NS = not significant.

Unaltered L1210 cells from animals fed standard chow had a transition temperature of 22.7° and activation energies of 5.4 and 22.0 kcal/mole.

Discussion

The temperature dependence of melphalan uptake has not heretofore been studied extensively. In one study, uptake of the drug by L5178Y cells was markedly temperature-dependent; the cell/medium ratio at 4° never exceeded 0.2, compared to almost 2 at 37° [10]. Furthermore, an Arrhenius plot was reported to be linear between 0° and 37° (energy of activation 11.6 kcal/mole) [10]. We carried out an extensive study of the temperature dependence of melphalan transport using the L1210 murine leukemia cell as a model. The initial transport of melphalan by intact L1210 cells was markedly dependent upon temperature, with a greater rate of transport at higher temperatures. The Arrhenius plot for the transport process was biphasic. This two-phase curve could have been due to a lipid phase transition from ordered to fluid state. Alternatively, it could have been due to the interaction of the two separate transport systems. If the breakpoint had been due to a change in lipid phase, then an alteration of the lipid composition of the plasma membranes might have resulted in a shift of the transition temperature. We have, therefore, utilized diets rich in polyunsaturated or saturated fat fed to the host animal to bring about lipid changes in the L1210 cell. We have shown previously that a diet rich in 16% sunflower seed oil results in an increase in polyunsaturated fats in the L1210 plasma membrane as compared to membranes of animals fed coconut oil which were enriched in saturated and monounsaturated fatty acids [4]. Furthermore, this alteration in lipid composition led to an increase in fluidity and a shift in the lower transition temperatures from 22.0° to 18.5° when the 12-nitroxide stearic acid probe was used [4]. Therefore, in the present study the temperature dependence of melphalan transport by L1210 cells from animals fed either the sunflower or coconut oil diets was studied. The transition temperature for transport by the cells from mice fed the sunflower seed oil diet, which was rich in polyunsaturates, was 18.7 ± 0.5°. In con-

trast, the transition temperature was 22.5 ± 1.2° for cells from animals fed the coconut oil diet. This 3.8° difference, which was associated with a lipid alteration, suggests that the transition temperature was due to a lipid phase change. The values of the transition temperature for melphalan transport of cells from the sunflower fed animals (18.7°) were similar to the lower transition temperature of the electron spin resonance studies (19.5° for 5-nitroxide stearic acid and 18.5° for 12-nitroxide stearic acid) [4]. Likewise, the cells from animals fed the coconut oil diets had transition temperatures for melphalan transport (22.5°) which were similar to the transition temperatures of the electron spin resonance study (22.0° for both probes). This is further evidence that the break in the Arrhenius plot for melphalan transport by the L1210 cell probably was due to a lipid phase change. It has been reported that fatty acid alteration of Ehrlich cells resulted in changes in transition temperatures for phenylalanine transport without affecting the kinetic parameters [11]. This amino acid is transported by system L which is the low-affinity system responsible for transport of a majority of melphalan at higher concentrations.

We have shown previously that alteration of the fatty acid composition and fluidity of plasma membranes has a marked effect on the transport of methotrexate by intact L1210 cells [4]. The K'_m for transport of methotrexate by L1210 cells from animals fed a polyunsaturated rich diet was 2.9 ± 0.4 μM compared to 4.1 ± 0.1 μM for cells from animals fed a more saturated-fat rich diet ($P < 0.02$). Since the transport of melphalan, like that of methotrexate, is an active carrier-mediated process, we examined the effect of fatty acid composition on the kinetic parameters of melphalan transport. There were no differences in K'_m or V'_{max} of either component of melphalan transport. This lack of effect of membrane lipid composition and fluidity on melphalan transport as compared to methotrexate transport cannot be explained by data currently available.

In summary, the transport of melphalan by L1210 lymphoblastic leukemia cells was markedly temperature dependent, and the Arrhenius plot demonstrated a biphasic pattern. Modification of the lipid composition of the cell phospholipids had a significant effect on the transition temperature for melphalan transport even though the K'_m and V'_{max} were the same in both types of cells. This shift in transition temperature demonstrates that the discontinuity in the Arrhenius plot was the result of a lipid phase transition and not of an interaction of the two carrier-mediated transport processes. These data indicating differential membrane lipid effects on the transport mechanism provide further evidence of a relationship between lipids and membrane drug transport.

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Effects of clofibrate and ethanol on the pathways of initial fatty acid oxidation

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Clofibrate administration to rats increases peroxisomal oxidation many-fold and perhaps mitochondrial oxidation [1–4]. Clofibrate also stimulates a variety of hepatic microsomal enzymatic activities [5].

A microsomal fraction of liver catalyzes the hydroxylation of the terminal carbon of fatty acids. The resulting omega-hydroxylated fatty acids are then oxidized to their corresponding dicarboxylic acids [6–8]. We have developed a method for estimating the contribution of omega-oxidation relative to beta-oxidation in the initial oxidation of fatty acids [9]. The changes produced by clofibrate on fatty acid oxidation by the different hepatic subcellular fractions prompted us to examine whether or not clofibrate changes the proportion of fatty acids undergoing omega-oxidation in the rat. Ethanol has profound effects upon lipid metabolism including the inhibition of fatty acid oxidation [10]. We therefore elected also to assess the effect of ethanol administration on the relative contribution of omega-oxidation to fatty acid oxidation.

Our estimations depend upon the fate of the omega-carbon of a fatty acid [9, 11]. Thus, if [16-^{14}C]palmitic acid undergoes beta-oxidation, its terminal two carbons will yield [2-^{14}C]acetyl CoA. The hexadecanedioic acid formed from it by omega-oxidation will have one of its carboxyls