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Characterization of the Multiple Transport Routes for Methotrexate in L1210 Cells Using Phthalate as a Model Anion Substrate

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Summary. *o*-Phthalate is actively transported into L1210 cells and the primary route for cell entry is the same transport system which mediates the influx of methotrexate and other folate compounds. The identity of the influx route has been established by the following observations: (A) Phthalate influx is competitively inhibited by methotrexate and the inhibition constant (K_i) is comparable to the K_t for half-maximal influx of methotrexate; (B) Various anions inhibit the influx of phthalate and methotrexate with comparable K_i values; (C) The influx of phthalate and methotrexate both fluctuate in parallel with changes in the anionic composition of the external medium; and (D) A specific covalent inhibitor of the methotrexate transport system (NHS-methotrexate) also blocks the transport of phthalate. In contrast, the efflux of phthalate does not occur via the methotrexate influx carrier, but rather by two separate processes which can be distinguished by their sensitivities to bromosulfophthalein. Efflux via the bromosulfophthalein-sensitive route constitutes 75% of total efflux and is enhanced by glucose and inhibited by oligomycin. The inability of phthalate to exit via the methotrexate influx carrier is due to competing intracellular anions which prevent phthalate from interacting with the methotrexate binding site at the inner membrane surface.

Key Words methotrexate · phthalate transport · methotrexate transport · transport mechanisms · L1210 cells

Introduction

L1210 mouse leukemia cells contain a single, high affinity transport system for the accumulation of methotrexate and other folate compounds [4, 5, 16]. A characteristic feature of methotrexate influx via this system is that it is inhibited competitively by a broad spectrum of anions [4–6, 8–12]. Conversely, extracellular anions are required for efflux via this system, and, moreover, various structurally unrelated anions as well as folate compounds can fulfill this anion requirement [9, 12]. These observations led to the conclusion that the methotrexate influx carrier mediates transport via an anion exchange mechanism and that gradients of exchange anions act as the driving force for methotrexate uptake [4, 9–14]. An exchange mechanism is also supported by the observation that anion gradients promote the accumulation of methotrexate in plasma membrane vesicles derived from L1210 cells [18].

Efflux of methotrexate in L1210 cells proceeds via the methotrexate influx carrier and also via two additional routes which can be distinguished by their sensitivities to bromosulfophthalein [14]. The proportion of efflux that proceeds via these routes is highly dependent upon the energetic state of the cell. The methotrexate influx carrier is the predominant route in energy-depleted cells, while the carrier-system which can be inhibited by bromosulfophthalein is the most active in energized cells. Steady-state levels of methotrexate are thus determined by the combined ability of the influx carrier to utilize anion gradients for the concentrative uptake of methotrexate and opposing energy-dependent processes which facilitate the return of substrate to the cell exterior. In the present study, *o*-phthalate (1,2-dicarboxybenzene) has been evaluated as a model substrate for divalent anion transport systems of L1210 cells. The results show that the influx of [^{14}C]phthalate occurs primarily via the same system which facilitates the uptake of methotrexate, whereas this system does not contribute to phthalate efflux. Efflux, instead, proceeds primarily via a second transport process which has several properties in common with the bromosulfophthalein-sensitive component of methotrexate efflux.

Abbreviations

MES, 2-(N-morpholino)ethanesulfonate; HEPES, 4-2(2-hydroxyethyl)-1-piperazine-ethanesulfonate; HBS, HEPES-buffered saline; MHS, Mg-HEPES-sucrose buffer; NHS-methotrexate, N-hydroxysuccinimide ester of methotrexate.

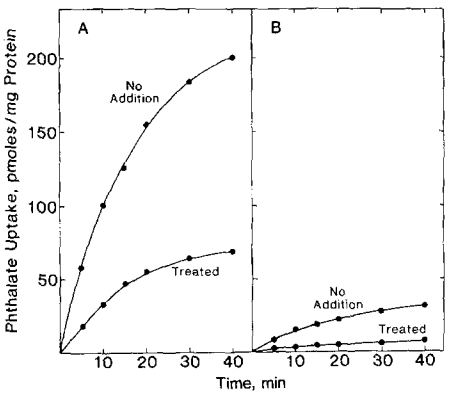

Fig. 1. Phthalate uptake by untreated cells and by cells pretreated with NHS-methotrexate in two different buffer systems. L1210 cells were pretreated with NHS-methotrexate and transport was measured as described in Materials and Methods. (*A*) MHS, pH 7.4; (*B*) HBS, pH 7.4

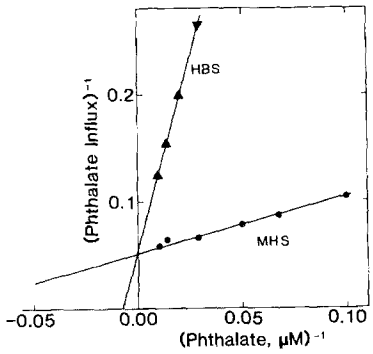

Fig. 2. Double-reciprocal plot of phthalate influx versus phthalate concentration in MHS and HBS. Influx units, pmol/min/mg protein

Materials and Methods

Chemicals

o-[7-^{14}C]Phthalic acid (5 mCi/mmol) was obtained from ICN. Radiochemical purity was 99% by thin-layer chromatography on cellulose sheets using 20 mM MES-200 mM NaCl, pH 6.0, as the solvent. [3',5',7-^{3}H]Methotrexate (300 mCi/mmol) was obtained from Amersham and purified by thin-layer chromatography on cellulose sheets with 20 mM Na-HEPES-200 mM NaCl, pH 7, as the solvent. NHS-methotrexate was prepared as described previously [7, 12] by dissolving methotrexate (2.2 mg), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (7.8 mg), and N-hydroxysuccinimide (5.8 mg) in 2.0 ml anhydrous dimethylsulfoxide and allowing the mixture to stand for 60 min at 23°C. The concentration of NHS-methotrexate was determined from the extinction coefficient for methotrexate of 18.9 at 302 nm and pH 7. All other chemicals were of the highest purity available commercially and were used without further purification.

Cells

L1210 mouse leukemia cells were grown as described previously [7], washed with the desired buffer, and resuspended to a density of 3×10^7 cells/ml. Buffers employed as suspending media for cells were as follows: HBS, HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4 with NaOH); and MHS, Mg-HEPES-sucrose (20 mM HEPES, 225 mM sucrose, pH 7.4 with MgO).

Treatment with NHS-Methotrexate

Treatment with NHS-methotrexate was accomplished by exposing freshly harvested cells (in either HBS or MHS, pH 7.4) to 10 μM NHS-methotrexate for 5 min at 23°C. The cells were then centrifuged, washed with 25 ml of the desired buffer, and resuspended to 3×10^7/ml in the same buffer.

Influx Measurements

Phthalate influx was determined in assay mixtures containing untreated or NHS-methotrexate-treated cells (3×10^7), the desired additions, and 20 nmol of [^{14}C]phthalate in 1.0 ml of buffer. After incubation at 37°C for 5 min, the cells were chilled to 4°C, collected by centrifugation at $1000 \times g$ (5 min, 4°C), washed with 4 ml of ice-cold 0.15 M NaCl, resuspended in 0.5 ml water, and analyzed for radioactivity. Uptake at 4°C served as the control. Methotrexate influx was determined similarly, except that the assay mixtures contained 2.0 nmol of [^{3}H]methotrexate. Intracellular concentrations of [^{14}C]phthalate were calculated on the basis of a cell volume of 5×10^{-10} ml [8].

Efflux Measurements

Substrate loading was achieved by combining cells (6×10^8) and [^{14}C]phthalate (400 nmol) in 20 ml of the desired buffer and incubating the mixture for 15 min at 37°C. The cells were recovered by centrifugation (at 4°C), and either washed and resuspended directly in the buffer (to 3×10^7/ml) or treated with NHS-methotrexate by suspending in buffer containing 10 μM NHS-methotrexate and incubating for 5 min at 23°C. After centrifugation, the latter cells were resuspended in buffer as above. The desired compounds were then added, and the cells were incubated for various times at 37°C, diluted with 7 ml of ice-cold saline, collected by centrifugation, and analyzed for associated radioactivity.

Results

General Properties of Phthalate Uptake by L1210 Cells

L1210 cells have the capacity to transport the divalent anion, phthalate. Uptake with time at 20 μM [^{14}C]phthalate is shown in Fig. 1 for cells suspended in either of two buffer systems. In an anion-deficient buffer (MHS) (Fig. 1*A*), phthalate transport proceeded at an initial rate which was linear for 5

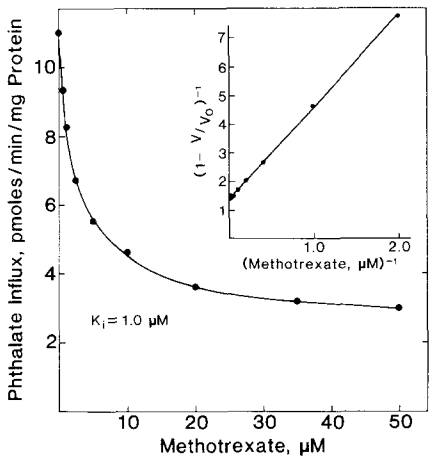

Fig. 3. Concentration dependence for the inhibition of phthalate influx by methotrexate. Inset, double-reciprocal plot of 1 minus the fraction of influx remaining as a function of inhibitor concentration. Buffer, MHS

Table 1. Inhibition of phthalate and methotrexate influx by various anions[a]

Anion	K_i for influx	
	[^{14}C]Phthalate (μM)	[^{3}H]Methotrexate (μM)
Methotrexate	1.0	0.7
Phthalate	22	20
Thiamine pyrophosphate	6.5	5.5
Bromosulfophthalein	2.8	2.4
Phosphate	700	500

[a] K_i values were derived from Dixon plots of the inverse of the transport rate versus inhibitor concentration (*see* Fig. 3). A K_i of 15 μM for [^{14}C]phthalate and 0.7 μM for [^{3}H]methotrexate and substrate concentrations of 20 and 2.0 μM, respectively, were employed in the calculations. Buffer, MHS.

min at 37°C and then diminished thereafter but did not reach a plateau value after 40 min. At the latter time, intracellular levels of phthalate (200 pmol/mg protein) corresponded to a concentration gradient of approximately threefold. When these same cells were pretreated with NHS-methotrexate, an irreversible inhibitor of the methotrexate influx carrier of L1210 cells [7], influx and steady-state levels of phthalate were reduced by 70% (Fig. 1*A*). Phthalate influx and the extent of uptake was sixfold lower (Fig. 1*B*) when transport was measured in a saline buffer (HBS), although the majority of uptake (80%) remained sensitive to NHS-methotrexate. A double-reciprocal plot of influx as a function of phthalate concentration (Fig. 2) showed that the K_t for half-maximal influx was the component of the transport process which was affected by buffer composition. The K_t for phthalate in MHS was 15 μM, compared with 140 μM in HBS. V_{max} for phthalate in both buffers was 19 pmol/min/mg protein.

The influx of phthalate is also inhibited by methotrexate (Fig. 3). Inhibition was observed at methotrexate concentrations in the micromolar range, although a complete loss of transport activity was not achieved at high levels of the inhibitor. Maximum inhibition was 73% (Y-intercept = 1.37) as determined from a replot of the data (inset, Fig. 3). A K_i for methotrexate of 1.0 μM was determined for the inhibitor-sensitive portion of influx using the Dixon equation and a K_t for phthalate of 15 μM. Other anions which inhibit methotrexate influx in L1210 cells also inhibit the influx of phthalate. These anions include phthalate, thiamine pyrophosphate, bromosulfophthalein, and phosphate and their K_i values for both transport processes are listed in Table 1. Parallel inhibition of phthalate and methotrexate transport was observed.

Characteristics of the Methotrexate-Insensitive Component of Phthalate Influx

Transport measurements in cells pretreated with NHS-methotrexate (Fig. 1) or in the presence of methotrexate (Fig. 3) showed that in MHS buffer a portion of phthalate influx (27%) proceeded via a second methotrexate-insensitive route. In contrast, bromosulfophthalein inhibited a much greater portion (95%) of total phthalate influx. Inhibition of the methotrexate-insensitive portion of phthalate influx by bromosulfophthalein was confirmed by uptake measurements performed with cells pretreated with NHS-methotrexate (filled circles, Fig. 4). Initial influx under these conditions was reduced by 80% over a range of bromosulfophthalein concentrations. The K_i for bromosulfophthalein was 7.0 μM. When cells treated with NHS-methotrexate were transferred to HBS (filled triangles, Fig. 4), phthalate influx was much lower and little or no inhibition by bromosulfophthalein was observed. Probenecid (inset, Fig. 4) also inhibited the same methotrexate-insensitive influx route for phthalate. The K_i for probenecid was 200 μM and maximum inhibition of total influx was 80%.

Characteristics of Phthalate Efflux

The efflux of phthalate from cells suspended in MHS is shown in Fig. 5. Efflux proceeded with a $t_{1/2}$

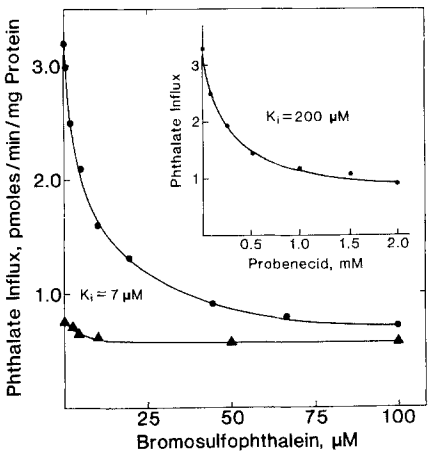

Fig. 4. Concentration dependence for the inhibition of phthalate influx by bromosulfophthalein in cells pretreated with NHS-methotrexate. Buffers: filled circles, MHS; filled triangles, HBS. Inset, corresponding plot of the inhibition of phthalate influx by increasing amounts of probenecid

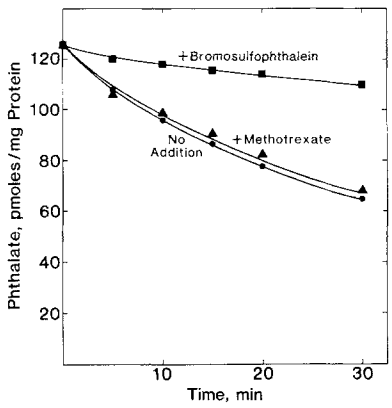

Fig. 5. Effect of methotrexate and bromosulfophthalein on phthalate efflux. Buffer, MHS

of 35 min in buffer alone or upon addition of methotrexate, while a decrease to one-fourth of the control level occurred in the presence of bromosulfophthalein. A plot of initial efflux versus bromosulfophthalein concentration (Fig. 6) showed that bromosulfophthalein inhibited efflux with a K_i of 5.0 μM and that maximum inhibition was 75%. Probenecid (inset, Fig. 6) inhibited efflux to a similar maximum as bromosulfophthalein but with a higher K_i of 130 μM. In separate measurements (*data not shown*), phthalate efflux was found to be unaffected by treatment with NHS-methotrexate, while a slight increase (1.5-fold) was observed upon transfer of the cells to HBS. When the metabolic state of the cell was analyzed for an effect on phthalate efflux, glucose was found to stimulate efflux by 1.6-fold, while an inhibition of 50% was observed with oligomycin (Table 2). Efflux in the presence of bromosulfophthalein was not affected by glucose or oligomycin (Table 2), indicating that changes in the energetic state of the cell affect phthalate efflux only via the bromosulfophthalein-sensitive route.

Discussion

Transport of methotrexate across the plasma membrane of L1210 cells is a complex process. Influx and efflux of methotrexate proceed via different transport routes [14] which show different sensitivities to various anions, drugs, and metabolic perturbing agents [1–3, 12, 14, 17]. The single system which mediates the cell entry of methotrexate is inhibited competitively by a broad spectrum of anions but it is relatively insensitive to short-term alterations in the metabolic state of the cell, while efflux, which proceeds via at least three routes, has a more diverse response to various anions and modulators of energy metabolism. The combined effect of various additions to the cells has been to differentially inhibit or stimulate influx or efflux and produce alterations in the steady-state levels of methotrexate. Conditions giving rise to increased uptake of methotrexate have been of interest since this potentially enhances the effectiveness of this drug as a cancer chemotherapeutic drug. An important observation in resolving the complexity of the methotrexate transport process has been that L1210 cells exhibit a high structural stability to various ionic environments. This stability has allowed transport measurements to be performed in certain nonphysiological buffers [8, 9, 12–14]. Cells in these latter buffers become depleted of energy reserves, conditions which preferentially inhibit the alternative efflux routes for methotrexate [1, 14]. The result has been that influx, efflux and steady-state levels of methotrexate are determined almost entirely by the properties of the high-affinity methotrexate carrier system. Studies on the mechanism of transport via this system were then possible and the results provided substantial evidence for an electroneutral and obligatory anion exchange process [9–14]. Exchange anions thus contribute in two ways to the concentrative uptake of methotrexate: (A) An electroneutral exchange allows the methotrexate dianion to enter the cells against the membrane potential; and (B) An obligatory exchange provides a means for coupling methotrexate uptake to anion gradients. Studies on the alternative efflux routes for methotrexate have also been possible since the contribution to efflux by the high-affinity transporter can be blocked completely and irreversibly by an active ester of methotrexate [7, 12, 14]. The

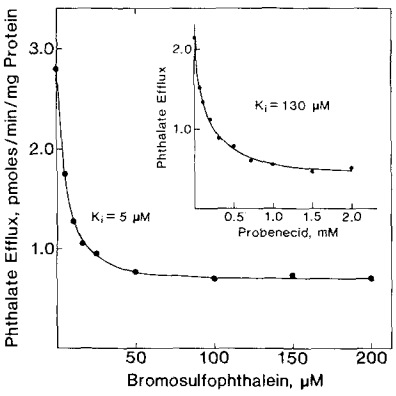

Fig. 6. Concentration dependence for the inhibition of phthalate influx by bromosulfophthalein. Efflux was calculated from a plot of phthalate released from cells versus time (*see* Fig. 5). Inset, corresponding plot of the inhibition of phthalate influx by increasing amounts of probenecid. Buffer, MHS

remaining efflux is mediated by at least two additional components, one of which is sensitive to inhibition by low levels of bromosulfophthalein [14].

The susceptibility of the methotrexate influx carrier to inhibition by anions was one of the initial indications that transport via this system proceeds via an anion exchange mechanism [4]. The basis for this broad inhibition was suggested subsequently to result from the ability of this transport system to mediate an exchange of extracellular methotrexate with intracellular anions of diverse structure [12]. A broad anion-binding capacity is proposed to have developed to accommodate the structural diversity of these exchange anions, although specificity has been maintained by retaining a much higher affinity for folate compounds. Anion substrates for the methotrexate exchange carrier were identified by their ability to promote methotrexate efflux [12]. Anions having this property include folate compounds, various small organic or inorganic monovalent or divalent anions, a few nucleotides, thiamine pyrophosphate and phthalate. The extent of enhancement did not vary significantly between many of the anionic substrates, indicating that the transport of methotrexate and other anion substrates proceeds at a generally similar rate. Calculations of availability within the cell and affinity for the carrier protein indicate that phosphate, AMP, chloride, bicarbonate and lactate are the most likely anions to participate in this exchange process *in vivo* [9, 11, 12]. Direct uptake measurements have confirmed that phosphate [11] and sulfate [10] are anion substrates for the methotrexate carrier, and that these anions are transported via this system at about the same rate as methotrexate.

Efflux analysis had shown that phthalate is a substrate for the methotrexate carrier system [12]

Table 2. Effect of glucose and oligomycin on phthalate efflux[a]

Addition	Concentration (μM)	Initial flux	
		−BSP[b]	+BSP
		(pmol/min/mg protein)	
None	—	0.87	0.27
Glucose	5000	1.43	0.26
Oligomycin	2	0.43	0.23

[a] Initial efflux was determined from a plot of the release of [^{14}C]phthalate versus time at 37°C (*see* Fig. 5). Cell load, 38 pmol/mg protein. Loading and efflux buffer, HBS. Bromosulfophthalein concentration, 50 μM.
[b] BSP, bromosulfophthalein.

and this was established further in the present study by direct measurements using [^{14}C]phthalate. Evidence that phthalate is transported by the methotrexate influx carrier was derived from the following observations: (A) Phthalate influx is inhibited by methotrexate with a K_i (1.0 μM) which is comparable to that for the K_t for influx of methotrexate (*cf.* Fig. 3 and Table 1); (B) Influx of both phthalate (Figs. 1 and 2) and methotrexate [8, 13] show a comparable sensitivity to the ionic composition of the medium; (C) An active ester of methotrexate (NHS-methotrexate) irreversibly inhibits both phthalate (Fig. 1) and methotrexate [7] influx; (D) Various anions which inhibit the influx of methotrexate inhibit phthalate influx to a similar extent (Table 1); and (E) Methotrexate [14] and phthalate (Fig. 2) influx both proceed with the same V_{max}. The amount of phthalate influx which occurred via the methotrexate carrier was approximately 75% in either of two buffer systems. An additional influx route for phthalate, which was sensitive to inhibition by both bromosulfophthalein (K_i = 7 μM) and probenecid (K_i = 130 μM) (Fig. 4), was also identified in this study, although it was expressed only in cells suspended in an anion-deficient buffer (MHS). In HEPES-buffered saline (HBS), this bromosulfophthalein-sensitive route was inoperative (Fig. 4).

Efflux of phthalate does not proceed via the methotrexate influx carrier but instead via two distinct routes which can be separated by their sensitivities to bromosulfophthalein. An involvement in phthalate efflux by the methotrexate influx carrier was excluded by the observations that efflux is not inhibited by NHS-methotrexate, nor is it enhanced by methotrexate in an anion-deficient buffer (Fig. 5). The inability of phthalate to utilize the methotrexate carrier system for efflux can be explained by calculations of its probable binding affinity at the inner membrane surface. The cell contains a broad

spectrum of anions which compete for the methotrexate binding site when the latter is directed to the cell interior and as a consequence anions are transported out of the cell at a much higher apparent K_t than for influx. For methotrexate, the K_t for efflux is 70 μM [14], compared with an influx K_t of 1 to 5 μM depending on the buffer employed [8]. The predicted K_t for phthalate efflux via the methotrexate influx carrier would thus be in the range of 1 mM, a value too high to allow significant movement of phthalate (intracellular concentration, *ca.* 40 μM) via this system. Phthalate efflux proceeds primarily via a component which is inhibited by bromosulfophthalein (Figs. 5 and 6) and by oligomycin (Table 2) and is stimulated by glucose (Table 2). Since half-maximal inhibition of efflux by bromosulfophthalein occurs at a relatively low concentration of 5 μM (Fig. 6), mediation of phthalate efflux by a carrier protein which binds bromosulfophthalein with a high affinity is indicated. Moreover, the close correlation between inhibition of the minor bromosulfophthalein-sensitive component of phthalate influx and the principal efflux carrier by bromosulfophthalein and probenecid (*cf.* Figs. 4 and 6) indicates that the same transport system mediates both of these processes. The bromosulfophthalein-sensitive efflux route for phthalate also has properties in common with one of the major routes for methotrexate efflux [14]. Common features between these processes include half-maximal inhibition at similar concentrations of bromosulfophthalein [12] and probenecid [15], stimulation by glucose [14], and inhibition by oligomycin [15]. In saline buffers, the bromosulfophthalein-sensitive transport route is unable to mediate an appreciable influx of either phthalate (Fig. 4) or methotrexate [14], yet it readily facilitates their efflux, indicating that the primary function of this system is to act as an anion efflux porter. A broad specificity is also indicated since phthalate and methotrexate differ substantially in molecular size and structure.

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