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Identification of Efflux Systems for Large Anions and Anionic Conjugates as the Mediators of Methotrexate Efflux in L1210 Cells

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ABSTRACT. Two ATP-dependent efflux systems for methotrexate have been identified in inside-out vesicles from an L1210 mouse cell variant with a defective influx carrier for methotrexate. Transport at 40 μM [^{3}H]methotrexate was separated by inhibitors into two components comprising 62 and 38% of total transport activity. The predominant route was inhibited by low concentrations of indoprofen (K_i = 2.5 μM), 4-biphenylacetic acid (K_i = 5.3 μM), and flurbiprofen (K_i = 5.2 μM), whereas the second component showed a high sensitivity to the glutathione conjugates of bromosulfophthalein (K_i = 0.08 μM), ethacrynic acid (K_i = 0.52 μM), and 1-chloro-2,4-dinitrobenzene (K_i = 0.77 μM). Bilirubin ditaurate was a potent inhibitor of both transport components (K_i = 1.5 and 0.17 μM, respectively). Separation of transport activities without interference from the other route was achieved by adding an excess (100 μM) of either the glutathione conjugate of ethacrynic acid or biphenylacetic acid. Double-reciprocal plots of transport at various substrate concentrations gave K_m values of 170 and 250 μM for methotrexate transport via the anion-sensitive and conjugate-sensitive routes, respectively. A comparison of inhibitor specificities indicated that the anion-sensitive transport activity in vesicles represents efflux system II for methotrexate in intact cells and is the same system identified previously in vesicles as an anion/anion conjugate pump. The conjugate-sensitive activity corresponds to efflux system I for methotrexate in intact cells and is the same system identified in vesicles as the high-affinity glutathione conjugate pump. BIOCHEM PHARMACOL 51;7:975–982, 1996.

KEY WORDS. methotrexate efflux; glutathione conjugate efflux; anion efflux; methotrexate; dinitrophenyl *S*-glutathione; ethacrynic acid; bilirubin ditaurate; biphenylacetic acid; inside-out vesicles.

ATP-dependent extrusion systems have been identified in mammalian cells which can accommodate compounds with similarities in general features such as size, charge, hydrophobicity, or conjugation [1–4]. A broad substrate specificity for efflux pumps contrasts with most import processes, which often have a high selectivity for one or a few related substrates. Efflux proteins often belong to the ABC superfamily of ATP-binding transport proteins [5], which are characterized by two homologous domains composed of six transmembrane sequences and an ATP binding site [4, 5]. Substrates for export proteins include large hydrophobic compounds [1, 4], glutathione conjugates [2, 3, 6–11], anions [12–15], and anionic conjugates [16–18]. Resistance of tumor cells to various drugs can result from the up-regulation of efflux proteins such as P-glycoprotein, the MDR1 gene product [1, 4], and MRP, a multidrug resistance-associated protein [19–22].

The divalent anion, methotrexate, had been shown in early studies to enter and exit L1210 mouse cells primarily via different routes as determined by responses to metabolic inhibitors [23] and probenecid [24, 25]. The reduced-folate carrier was found to mediate nearly all influx of methotrexate, whereas efflux could be separated into a small contribution by the influx carrier and two unidirectional components [14, 26–28]. The major unidirectional route (system I) accounted for 70% of total unidirectional efflux, exhibited a high sensitivity to indomethacin, prostaglandin A_1, reserpine, ethacrynic acid, and BSP†, and also had the ability to efflux cholate [14]. The second route (system II) comprised 30% of the total and could be distinguished by its high sensitivity to BPAA, indoprofen, and flurbiprofen [28]. A variant L1210/C7 cell line has been isolated which lacks system I, and expresses elevated activity for system II [27]. Potent inhibition of efflux system I by CDNB, ethacrynic acid, and other electrophiles with the potential for intracellular reaction with glutathione suggested that a

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† *Abbreviations:* BSP, bromosulfophthalein; CDNB, 1-chloro-2,4-dinitrobenzene; GS-conjugate, glutathione *S*-conjugate; DNP-SG, 2,4-dinitrophenyl S-glutathione; BPAA, 4-biphenylacetic acid; DTE, dithioerythritol; BSP-SG, GS-conjugate of BSP; and EA-SG, GS-conjugate of ethacrynic acid.

primary physiological function of efflux system I may be to expel GS-conjugates [28].

L1210 cells contain at least two ATP-dependent efflux systems for DNP-SG which have been identified as high- and low-affinity uptake activities in inside-out vesicles [11]. The high-affinity system (K_m = 0.63 μM) was correlated with efflux pumps for DNP-SG in other cells by its broad sensitivity to leukotriene C_4 and other GS-conjugates. DNP-SG and leukotriene C_4 are also transported by MRP [20, 22], which suggests that MRP and the high-affinity system may represent the same transport activity. The low-affinity system for DNP-SG in L1210 cells (K_m = 450 μM) responds poorly to various GS-conjugates, but has a high sensitivity to anions and anion conjugates such as bilirubin ditaurate, BPAA, and indoprofen.

The present study was initiated to investigate methods for detecting efflux routes for methotrexate in inside-out vesicles from L1210 cells and to determine whether relationships exist between the efflux systems for methotrexate and DNP-SG. The results demonstrated that substantial ATP-dependent uptake of methotrexate occurs at neutral pH in inside-out vesicles from a variant L1210/R81 cell line with minimal influx activity for methotrexate [29, 30]. Vesicles from L1210/R81 cells contain two ATP-dependent transport systems which can be correlated by their inhibitor specificities to efflux systems for methotrexate in intact cells and to transport systems for DNP-SG in inside-out vesicles.

MATERIALS AND METHODS

Chemicals

[3′,5′,9-^{3}H]Methotrexate (20 Ci/mmol), (Moravek Biochemicals) was purified and stored as described previously [14]. [^{3}H]DNP-SG was synthesized enzymatically [11] in a reaction mixture containing CDNB (2 μmol), glutathione (0.25 μmol), 250 μCi [glycine-2-^{3}H]glutathione (New England Nuclear), and 2 U of glutathione S-transferase. Synthesis, purification, and quantitation of DNP-SG, EA-SG, and BSP-SG were performed as described previously [11]. Glutathione S-transferase, glutathione, ATP, creatine phosphate, creatine kinase, methotrexate, BSP (sulfobromophthalein), CDNB, ethacrynic acid, flurbiprofen, indoprofen, BPAA, and DTE were obtained from the Sigma Chemical Co. Bilirubin ditaurate was purchased from United States Biochemicals. Immobilized wheat germ agglutinin was prepared [31] by coupling wheat germ agglutinin (Boehringer-Mannheim) with CNBr-activated Sepharose 4B (Sigma).

Cells

Parental L1210 mouse leukemia cells and the L1210/R81 subline (a gift from Dr. James H. Freisheim) were grown in RPMI 1640 medium containing 3% fetal bovine serum (Gibco) and 100 U/mL of penicillin and 100 μg/mL of streptomycin. Culture flasks (2 L), which contained medium (1 L) and an inoculum of 10^8 cells, were capped and incubated (with gentle shaking) for 48 hr at 37°. Cells were harvested by centrifugation at 4° (500 *g*), and washed with PBS (5 mM sodium-phosphate and 150 mM NaCl, pH 7.4) prior to storage at −80°.

Preparation of Inside-Out Vesicles

Plasma membrane vesicles were prepared as described previously [11] by the method of Schaub *et al.* [32]. Briefly, frozen cells were thawed, diluted with ice-cold hypotonic buffer, and stirred gently for 16 hr at 4°. Crude plasma membranes were recovered from the cell lysate, suspended in hypotonic buffer, homogenized with 20 strokes in a Potter-Elvehjem homogenizer, layered onto 38% sucrose, and centrifuged at 100,000 *g* for 30 min at 4°. The turbid layer at the interface was collected, and vesicles were formed by five passages through a 27-gauge needle. The sample was then enriched for inside-out vesicles by a slow passage through a column of wheat germ agglutinin linked to CnBr-activated Sepharose 4B. The unabsorbed fraction was recovered by centrifugation at 100,000 *g*, suspended to 4 mg protein/mL in storage buffer (10 mM Tris-HCl, 250 mM sucrose, and 1 mM DTE, pH 7.4), aliquoted into 1.0-mL portions, and stored at −80°. Protein was determined by the method of Bradford [33] using bovine serum albumin as the standard.

Transport Determinations in Inside-Out Vesicles

Assay mixtures were prepared at 4° (in 12 × 75 mm siliconized glass tubes) and consisted of inside-out vesicles (150–200 μg), 1.0 mM ATP, 10 mM $MgCl_2$, ATP-regenerating system (10 mM creatine phosphate and 12 U of creatine kinase), [^{3}H]methotrexate or [^{3}H]DNP-SG, the desired inhibitors, and assay buffer (10 mM Tris-250 mM sucrose, pH 7.4) in a final volume of 150 μL. After incubation for 10 min at 37° (unless otherwise indicated), uptake was stopped by placing on ice and diluting with 2.5 mL of ice-cold assay buffer. The vesicles were collected by rapid filtration onto Millipore HAWP 0.45 μM filters, washed with four 2.5-mL portions of ice-cold assay buffer, placed in scintillation vials containing 8 mL of scintillation fluid (Scintisafe, Fisher), allowed to stand for 16 hr at 23°, and analyzed for radioactivity. Samples incubated at 37° without ATP and containing 1 mM vanadate served as the control. Inhibitor studies were performed at eight to twelve inhibitor concentrations, and IC_{50} values for half-maximal transport inhibition were calculated from a Dixon plot of the data. Inhibition constants (K_i values) were calculated using the Dixon equation: −x intercept (IC_{50}) = K_i [1 + s/K_m], where s equals substrate concentration. K_m and V_{max} values were determined from a Lineweaver–Burk plot of the data. Experimental points were performed in duplicate, and kinetic values were derived from two or more separate determinations. Measurements were repeated until standard deviations were less than 30%.

Analysis of Vesicle-Associated Radioactivity

The radioactivity accumulated by quadruplicate standard assay samples that had been incubated for 10 min at 37° with 40 μM [^{3}H]methotrexate and 1.0 mM ATP (where indicated) were collected onto four separate filters, washed according to the standard procedure, submerged together in 1.0 mL of water, and placed at −20°. After 16 hr, the thawed samples were mixed vigorously and centrifuged (5 min at 24,000 *g*), and the supernatants were removed for lyophilization. Dried samples were dissolved in 200 μL of water, applied to a Rainin MicroSorb-MV C18 column, and separated as described previously for DNP-SG [11] using a linear gradient of 100% A (aqueous 0.1% trifluoroacetic acid) to 100% B (0.1% trifluoroacetic acid in 90% acetonitrile). [^{3}H]Methotrexate eluted at the 18th mL during the 30-mL separation. Chromatography fractions (1.0 mL) were collected in scintillation vials, dried (72 hr at 37°), and analyzed for radioactivity in 8 mL of ScintiSafe. Recovery of total radioactivity ranged from 80 to 100%.

RESULTS

General Characteristics of Methotrexate Transport into Inside-Out Vesicles

Inside-out vesicles were prepared as described previously [11] and purified from right-side-out vesicles by passage through a column of wheat germ agglutinin linked to CNBr-activated Sepharose 4B [34]. Vesicle preparations were 80 ± 5% inside-out, as determined by an acetylcholinesterase accessibility assay [35]. Purified vesicles from L1210 cells exhibited relatively low ATP-dependent uptake of [^{3}H]methotrexate (Fig. 1). Linear uptake was observed for only a short time interval (2 min) at 37°, and a maximum was reached after 10 min.

Transport activity improved substantially when uptake was measured with inside-out vesicles from an L1210/R81 cell line with a defective influx carrier for methotrexate [29, 30]. ATP-dependent uptake in this case remained approximately linear for 10 min (Fig. 1), and reached a plateau between 20 and 30 min (not shown). In various preparations, ATP-dependent transport at 40 μM methotrexate proceeded at a rate of 3.7 ± 0.8 pmol/min/mg protein (N = 12). In the absence of ATP, samples incubated from 0 to 10 min at 37° showed no time-dependent uptake of methotrexate, but the level of trapped and/or non-specifically bound substrate in samples lacking ATP represented a moderate 30–40% of total ATP-dependent uptake after 10 min at 37°. An HPLC analysis of the radioactivity accumulated after 10 min at 37° in either the absence or presence of 1.0 mM ATP revealed that authentic methotrexate represented greater than 90% of the total radioactivity. When the [^{3}H]methotrexate concentration was increased by 5-fold to 200 μM, ATP-dependent uptake remained approximately linear for 10 min at 37°. ATP-dependent uptake after 25 min at 37° decreased in a linear fashion with increasing medium osmolarity (from 250 to 1000 mM sucrose), indicating that uptake represented the accumulation of free intravesicular methotrexate and not methotrexate bound to the membrane or to residual trapped dihydrofolate reductase.

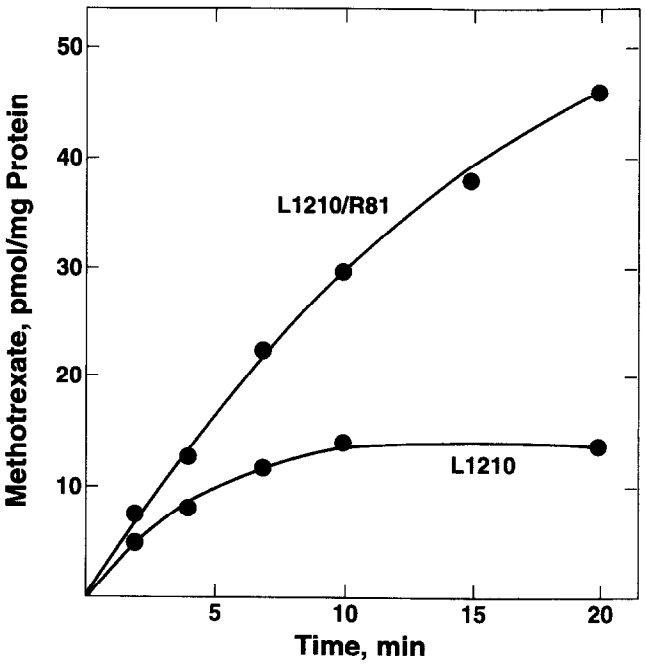

FIG. 1. Time dependence at 37° for ATP-dependent uptake of [^{3}H]methotrexate by inside-out vesicles from L1210 and L1210/R81 cells. [^{3}H]Methotrexate, 40 μM; ATP, 1.0 mM; each experiment performed twice.

Inhibitor Response of the ATP-Dependent Transport of Methotrexate Into Vesicles from L1210/R81 *Cells*

Since prior studies had indicated that methotrexate efflux may proceed via the same two routes utilized by L1210 cells for the efflux of GS-conjugates [11], specific inhibitors of the high- and low-affinity efflux systems for DNP-SG were assessed for the ability to inhibit the ATP-dependent transport of methotrexate. GS-conjugates were employed as representative inhibitors of the high-affinity system, whereas BPAA, indoprofen, and flurbiprofen were selected as specific inhibitors of the low-affinity system. Bilirubin ditaurate was employed as a potential inhibitor of both transport systems.

BSP-SG and EA-SG produced a biphasic inhibition of the ATP-dependent transport of [^{3}H]methotrexate (Fig. 2A). Transport was reduced at low concentrations of BSP-SG and EA-SG, but inhibition reached a maximum between 35 and 42%. An average maximum inhibition of 38% was obtained in various experiments with BSP-SG at concentrations up to 5 μM and with EA-SG at concentrations up to 100 μM. Half-maximal inhibition (IC_{50}) occurred at concentrations of BSP-SG and EA-SG of 0.09 and 0.64 μM, respectively, and combinations of BSP-SG and EA-SG did not produce a further reduction in methotrexate transport. A maximal inhibition of 38% was also observed with DNP-SG (IC_{50} = 0.90 μM).

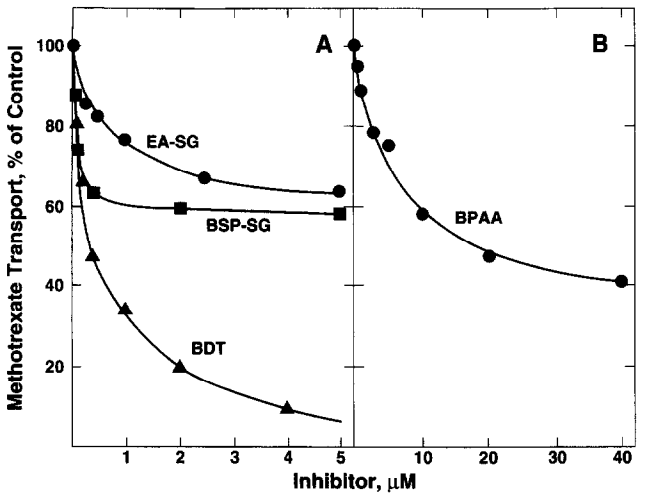

FIG. 2. Inhibition of ATP-dependent transport of [^{3}H]methotrexate by inside-out vesicles from L1210/R81 cells at various concentrations of inhibitors. (A) Effects of BSP-SG, EA-SG, and bilirubin ditaurate (BDT). (B) Effect of BPAA. [^{3}H]Methotrexate, 40 μM; ATP, 1.0 mM; control transport rate in (A), 3.9 pmol/min/mg protein (three experiments); control transport rate in (B), 3.0 pmol/min/mg protein (two experiments); transport interval, 10 min, 37°.

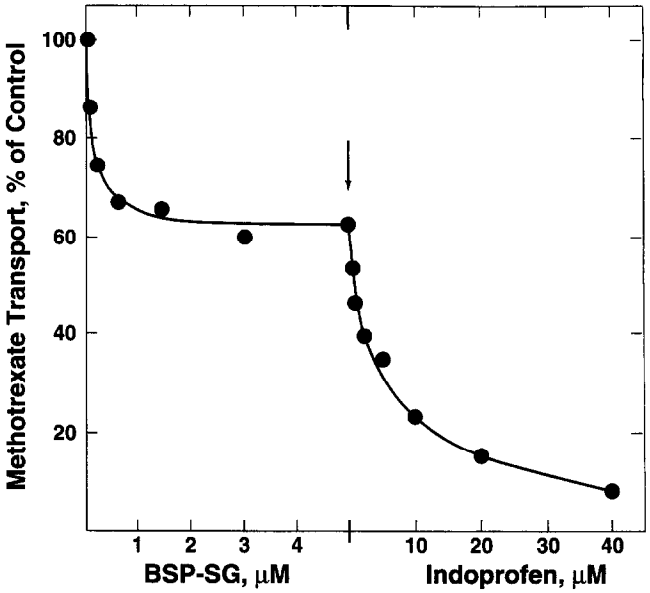

FIG. 3. Inhibition of methotrexate transport by combinations of BSP-SG and indoprofen. Transport at 40 μM [^{3}H]methotrexate was measured at increasing concentrations of BSP-SG up to 5.0 μM, and then with BSP-SG held constant at 5.0 μM, at increasing concentrations of indoprofen up to 40 μM. The arrow signifies the change from BSP-SG alone to BSP-SG plus indoprofen. ATP, 1.0 mM; control transport rate, 4.5 pmol/min/mg protein (two experiments); transport interval, 10 min, 37°.

BPAA also produced a partial inhibition of [^{3}H]methotrexate transport (Fig. 2B), but the inhibition maximum was higher than had been observed with GS-conjugates (Fig. 2A). An inhibition maximum of 60–65% was reached at concentrations of BPAA between 40 μM (Fig. 2B) and 100 μM (not shown). The IC_{50} for half-maximal inhibition by BPAA was 6.5 μM. A similar maximal inhibition of 60–65% (mean, 62%) was obtained with indoprofen (IC_{50} = 3.0 μM) and flurbiprofen (IC_{50} = 6.4 μM), and combinations of BPAA, indoprofen, and flurbiprofen did not produce a further decline in methotrexate transport. Conversely, combinations of BPAA or indoprofen with BSP-SG or EA-AG reduced methotrexate transport by greater than 90%. This is illustrated in Fig. 3 for vesicles that were exposed sequentially to increasing concentrations of BSP-SG (up to 5.0 μM) and then to indoprofen (up to 40 μM). BSP-SG alone reduced transport to a maximum of about 40%, whereas the further addition of indoprofen to samples already containing 5.0 μM BSP-SG reduced transport by greater than 90%. The IC_{50} for indoprofen in this combination experiment (4.2 μM) was similar to the IC_{50} value obtained in the absence of a GS-conjugate.

Bilirubin ditaurate produced a nearly complete inhibition of the ATP-dependent transport of methotrexate (Fig. 2A), indicating that this inhibitor blocks both the conjugate- and anion-sensitive components. Two transport routes with differing sensitivities to bilirubin ditaurate were separated subsequently using inhibitor combinations (Fig. 4). Inhibition by bilirubin ditaurate was measured either with BPAA added in excess (100 μM) to block the major anion-sensitive component (Fig. 4A), or with EA-SG added in excess (100 μM) to block the component sensitive to GS-conjugates (Fig. 4B). The transport component remaining in the presence of 100 μM BPAA (Fig. 4A) was more sensitive to bilirubin ditaurate (IC_{50} = 0.20 μM), whereas the component remaining in the presence of excess EA-SG (Fig. 4B) was less sensitive to bilirubin ditaurate (IC_{50} = 1.8 μM). The lines drawn in panels A and B of Fig. 4 were derived from the Dixon equation for a single transport component with the indicated IC_{50} values. The close fit of the data points to these lines indicated that the assay conditions had isolated each of the transport routes.

Kinetics of the ATP-Dependent Transport Systems for Methotrexate

The two transport activities for methotrexate were separated by the addition of EA-SG or BPAA and then measured at various substrate concentrations. In each case, linear double-reciprocal plots were obtained (Fig. 5). The anion-sensitive activity (measured in the presence of EA-SG) exhibited a K_m of 170 μM and a V_{max} of 27 pmol/min/mg protein, whereas the conjugate-sensitive component (measured in the presence of BPAA) had a K_m of 250 μM and a V_{max} of 22 pmol/min/mg protein.

Inhibition constants (K_i) for inhibitors of each transport system were calculated from IC_{50} values for half-maximal inhibition and K_m values for methotrexate transport. K_i values for inhibitors of the conjugate-sensitive system are listed in Table 1, and are compared with K_i values for these same compounds as inhibitors of the high-affinity transport system for GS-conjugates [11]. Comparable inhibition was

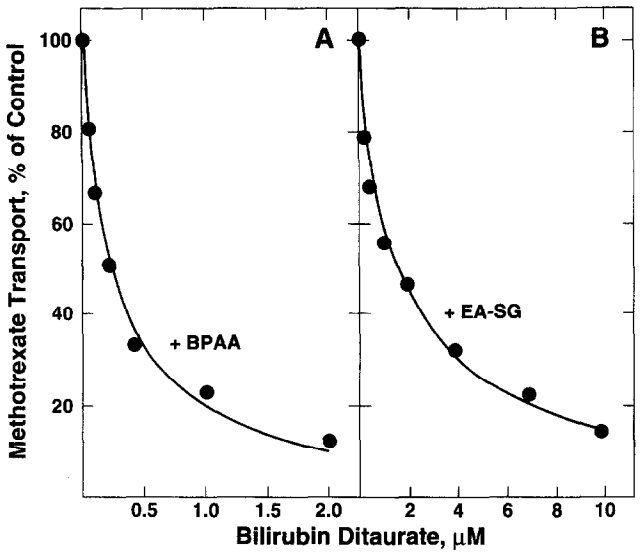

FIG. 4. Inhibition by bilirubin ditaurate of the conjugate-sensitive and anion-sensitive portions of ATP-dependent transport of [^{3}H]methotrexate. (A) Inhibition by bilirubin ditaurate of the conjugate-sensitive system (anion-sensitive system blocked with 100 μM BPAA). Curve represents the response calculated from a Dixon plot for a single transport component with an IC_{50} for bilirubin ditaurate of 0.20 μM. (B) Inhibition by bilirubin ditaurate of the anion-sensitive system (conjugate-sensitive system blocked with 100 μM EA-SG). Curve represents the response calculated from a Dixon plot for a single transport component with an IC_{50} for bilirubin ditaurate of 1.8 μM. [^{3}H]Methotrexate, 40 μM; ATP, 1.0 mM; control transport rate in (A), 1.6 pmol/min/mg protein (two experiments); control transport rate in (B), 2.5 pmol/min/mg protein (two experiments); assay interval, 10 min, 37°.

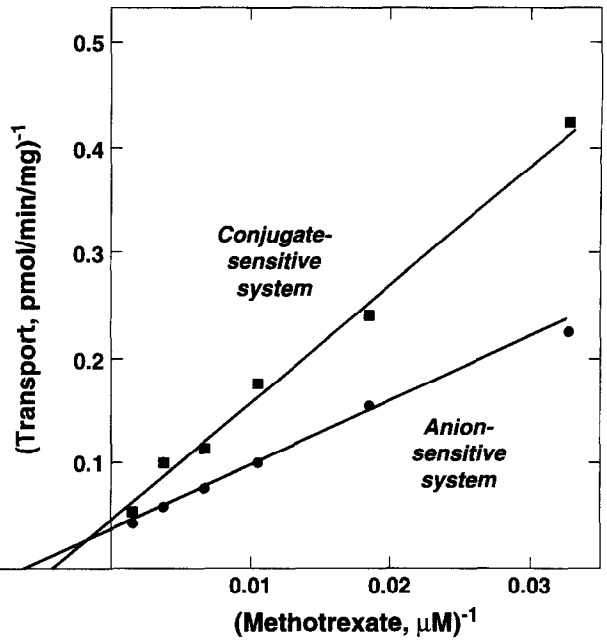

FIG. 5. Double-reciprocal plots of transport versus [^{3}H]methotrexate concentration by the conjugate-sensitive and anion-sensitive transport systems. The conjugate-sensitive system was measured in the presence of 100 μM BPAA, whereas the anion-sensitive system was measured in the presence of 100 μM EA-SG. ATP, 1.0 mM; transport interval, 10 min, 37°; each experiment performed twice.

observed with BSP-SG, EA-SG, DNP-SG, and bilirubin ditaurate. A similar comparison of K_i values for inhibitors of the anion-sensitive transport system for methotrexate and the low-affinity transport system for GS-conjugates is shown in Table 2. An essentially identical response by these latter two systems was obtained with bilirubin ditaurate, indoprofen, BPAA, and flurbiprofen.

ATP-Dependent Transport of DNP-SG by Inside-Out Vesicles from L1210/R81 *Cells*

Inside-out vesicles from L1210/R81 cells (with a defective influx carrier for methotrexate) were employed to characterize the ATP-dependent transport systems for methotrexate since measurements of transport kinetics in parental vesicles were hindered by a rapid saturation of uptake after a relatively short time interval (Fig. 1). However, it was possible that changes in methotrexate efflux had arisen during the selection of the L1210/R81 variant. To determine whether the efflux routes in L1210/R81 vesicles were comparable to those in parental cells, the ATP-dependent transport of an alternative substrate (DNP-SG) was compared in vesicles from L1210 and L1210/R81 cells. Transport at 20 μM [^{3}H]DNP-SG by L1210/R81 vesicles (44 ± 6 pmol/min/mg protein) (N = 4) was somewhat higher than in vesicles from L1210 cells (31 ± 3 pmol/min/mg protein) (N = 4), but the relative contribution to total DNP-SG transport by the high- and low-affinity systems did not vary with vesicle source. At 20 μM [^{3}H]DNP-SG, the high-affinity system (inhibitable by 100 μM EA-SG) accounted for 55 and 54% of transport in vesicles from parental and L1210/R81 cells, respectively, whereas corresponding contributions by the low-affinity system (inhibitable by 100 μM BPAA) were 38 and 42%. Hence, no significant dif-

TABLE 1. Comparison of the inhibitor sensitivity of the conjugate-sensitive transport system for methotrexate and the high-affinity transport system for DNP-SG in inside-out vesicles

Inhibitor	K_i (μM)	
	Conjugate-sensitive methotrexate transport	High-affinity DNP-SG transport
Bilirubin ditaurate	0.17	0.10
BSP-SG	0.08	0.09
EA-SG	0.52	0.44
DNP-SG	0.77	0.60

Inhibition of the ATP-dependent conjugate-sensitive transport system for methotrexate by BSP-SG, EA-SG, and DNP-SG was measured twice in assay mixtures containing 40 μM [^{3}H]methotrexate and various concentrations of inhibitor (see Fig. 2A). Inhibition constants (K_i) were calculated using the Dixon equation from IC_{50} values based on a maximum inhibition of 38% and a K_m for methotrexate transport of 250 μM (see Fig. 5). Inhibition by bilirubin ditaurate was measured similarly, except that 100 μM BPAA was added to block the anion-sensitive system. K_i values for the high-affinity transport system for DNP-SG are from Ref. 11.

TABLE 2. Comparison of the inhibitor sensitivity of the anion-sensitive transport system for methotrexate and the low-affinity transport system for DNP-SG in inside-out vesicles

Inhibitor	K_i (μM)	
	Anion-sensitive methotrexate transport	Low-affinity DNP-SG transport
Bilirubin ditaurate	1.5	1.5
Indoprofen	2.5	3.0
BPAA	5.3	5.5
Flurbiprofen	5.2	6.0

Inhibition of the ATP-dependent anion-sensitive transport system for methotrexate by indoprofen, BPAA, and flurbiprofen was measured twice in assay mixtures containing 40 μM [^{3}H]methotrexate and various concentrations of inhibitor (see Fig. 2B). Inhibition constants (K_i) were calculated using the Dixon equation from IC_{50} values based on a maximum inhibition of 62% and a K_m for methotrexate transport of 170 μM (see Fig. 5). Inhibition by bilirubin ditaurate was measured similarly, except that 100 μM EA-SG was added to block the conjugate-sensitive system. K_i values for the low-affinity transport system for DNP-SG are from Ref. 11.

ferences could be detected between L1210 and L1210/R81 cells when DNP-SG was employed as a substrate for the two anion efflux systems.

DISCUSSION

L1210 mouse cells contain two ATP-dependent transport systems for the unidirectional efflux of methotrexate. These two systems were detected and characterized using purified inside-out vesicles from L1210/R81 cells. Transport activity was also observed in vesicles from parental cells (Fig. 1), but the presence of a normal reduced-folate influx carrier for methotrexate apparently interfered with the ATP-dependent accumulation of methotrexate. The influx carrier functions bidirectionally [36], and hence could have facilitated the exit of methotrexate from inside-out vesicles of parental but not variant cells. In addition, phosphate compounds (ATP and creatine phosphate) present in the assay medium may have further enhanced the exit of methotrexate since the influx carrier can utilize anion gradients to facilitate methotrexate transport [37]. L1210/R81 cells are a reasonable substitute for parental cells in the analysis of anion efflux systems since vesicles from both cells contain comparable levels of high- and low-affinity transport systems for DNP-SG. In prior studies, a different influx-deficient L1210/R24 cell line was shown to have an ATP-dependent transport activity for methotrexate which was sensitive to BSP and had a pH optimum of about pH 5.8 [38].

Inhibitor responses were employed to separate the two ATP- dependent transport systems for methotrexate in L1210/R81 vesicles. An anion-sensitive component accounting for 62% of vesicular transport was detected by its sensitivity to certain monovalent anions, whereas a component sensitive to GS-conjugates accounted for the remaining 38% of total methotrexate transport (Figs. 2 and 3). A clear separation and a kinetic analysis of these two transport systems was possible since inhibitors of the transport systems for anions (indoprofen and BPAA) and for conjugates (BSP-SG and EA-SG) were highly system specific (Figs. 2–4). The anion-sensitive system exhibited a K_m of 170 μM, whereas the conjugate-sensitive system had a K_m of 250 μM (Fig. 5). The high K_m values for methotrexate relative to K_i values for various inhibitors (see Tables 1 and 2) indicate that methotrexate is bound by these systems with a relatively low affinity, but the combination of two transport routes with moderate V_{max} values can account for the significant capacity of intact L1210 cells to mediate the efflux of methotrexate. Prior studies had also reported a relatively high K_m for methotrexate efflux. In inside-out vesicles from L1210/R24 cells, a single K_m of 46 μM was reported for the ATP-dependent uptake of methotrexate at pH 6.1 [38], whereas a K_m of 470 μM was reported for the ATP-dependent uptake of methotrexate at neutral pH in vesicles from human erythrocytes [39].

The ATP-dependent transport systems for methotrexate in vesicles can be assigned to unidirectional efflux systems for methotrexate in intact cells by common responses to inhibitors. Efflux system II in intact cells [26–28] was correlated with the anion-sensitive transport system in vesicles since both activities respond comparably to the same low levels of indoprofen, BPAA, and flurbiprofen (Table 2). Similarly, apparent sensitivity to GS-conjugates is a common feature of efflux system I in intact cells [28] and the conjugate-sensitive system for methotrexate in vesicles. Inhibition of the vesicle transport system by GS-conjugates was demonstrated directly (see Figs. 2 and 3), whereas sensitivity to GS-conjugates by efflux system I in cells had been implied by the response of this system to low concentrations of electrophiles which are converted to GS-conjugates [28].

The conjugate-sensitive transport of methotrexate and the high-affinity transport of DNP-SG are mediated by the same transport system on the basis of common binding features. Comparisons of inhibitor sensitivities showed that these two ATP-dependent transport activities respond almost identically to low concentrations of BSP-SG, EA-SG, DNP-SG, and bilirubin ditaurate (Table 1). Comparable values were also observed for the K_i of methotrexate (300 μM) for inhibition of high-affinity DNP-SG transport [11] and the K_m for methotrexate transport (250 μM) via the conjugate-sensitive system (Fig. 5). A common efflux system would also explain prior findings that an L1210/C7 efflux variant with a defect in methotrexate efflux system I in intact cells [27] also exhibits a defect in high-affinity GS-conjugate transport in isolated vesicles [11]. A shared efflux system for methotrexate and GS-conjugates had also been suggested from transport measurements in inside-out vesicles from human erythrocytes [39]. Methotrexate transport in this system was inhibited by DNP-SG and other compounds that also inhibit GS-conjugate transport.

Inhibitor responses indicate that the anion-sensitive ATP-dependent transport of methotrexate is mediated by the same system identified previously in vesicles as an export pump for anions and anion conjugates [11]. These two

transport activities show substantially identical responses to inhibition by low concentrations of bilirubin ditaurate, indoprofen, BPAA, and flurbiprofen (Table 2). Moreover, the affinity of the anion/anion-conjugate system for methotrexate (K_i = 150 μM) [11] is comparable to the K_m for methotrexate transport via the anion-sensitive system (170 μM) (Fig. 5). Methotrexate also fits the general binding specificity of the anion/anion-conjugate exporter for either large monovalent or divalent anions [11].

Efflux systems for methotrexate are possible targets for improving the efficacy of methotrexate in killing tumor cells since a block in efflux could improve toxicity by increasing intracellular concentrations of methotrexate. However, in L1210 cells the presence of two unidirectional efflux systems with different inhibitor sensitivities, and an additional small contribution to efflux from the influx carrier [25, 40], complicates the use of efflux inhibitors as a means to enhance methotrexate toxicity. CCRF-CEM human lymphoblastic cells contain only efflux system II for methotrexate [41] and, hence, could represent a more suitable target for efflux inhibitors, but the influx carrier of CCRF-CEM cells contributes to total efflux to a greater extent than in L1210 cells.

Overproduction of an efflux pump for methotrexate could result in methotrexate resistance, but this mode of resistance has not been generally observed [42]. It is possible that enhanced efflux has been underestimated as a resistance mechanism due to difficulties in detection. Decreased influx and overproduction of dihydrofolate reductase are common mechanisms of resistance to methotrexate, but in cells containing either of these latter changes, efflux measurements become difficult to perform. Diminished influx hinders the ability to load cells for efflux measurements, and enhanced binding to dihydrofolate reductase decreases the level of free methotrexate available to efflux pumps. An alternative approach would be to develop substrates for efflux pumps which have routes of uptake different from methotrexate and do not bind to dihydrofolate reductase. One such compound is cholate, which enters L1210 cells slowly by diffusion and is a shared substrate with methotrexate for efflux system I [14]. Alternatively, enhanced efflux may be an uncommon resistance mechanism since the over-production of an efflux system for methotrexate could cause the depletion of cellular anions such as nucleotides. A case for nucleotide efflux is supported by the identification of an efflux pump for cyclic AMP, which appears to be a GS-conjugate pump by its sensitivity to prostaglandin A_1 [12, 15]. This same transport system also appears to mediate the efflux of cholate [15].

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