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Efficient Utilization of the Reduced Folate Carrier in CCRF-CEM Human Leukemic Lymphoblasts by the Potent Antifolate N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523) and Its B-Ring Analogues

*Joel E. Wright, Chitra M. Vaidya, Ying-Nan Chen and Andre Rosowsky**

DANA-FARBER CANCER INSTITUTE AND DEPARTMENT OF BIOLOGICAL CHEMISTRY AND MOLECULAR PHARMACOLOGY, HARVARD MEDICAL SCHOOL, BOSTON, MA 02115, U.S.A.

ABSTRACT. The potent nonpolyglutamatable dihydrofolate reductase inhibitor N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523) and six of its B-ring (5-deaza, 8-deaza, and 5,8-dideaza) analogues were compared in terms of their ability to: (a) inhibit the growth of CCRF-CEM human leukemic lymphoblasts, and (b) utilize the reduced folate carrier (RFC) in these cells as measured in a competition assay of [^{3}H]methotrexate ([^{3}H]MTX) influx. The IC_{50} values of the hemiphthaloylornithine derivatives against CCRF-CEM cells after 72 hr of drug exposure varied from 0.64 to 1.3 nM as compared with 14 nM for MTX and 4.4 nM for aminopterin (AMT). The K_i values of these compounds in the [^{3}H]MTX influx assay were in the 0.3 to 0.7 μM range as compared with a K_i of 5.4 μM for AMT and a K_t of 7.1 μM for MTX. As a group, the affinities of these compounds for the RFC were approximately 10-fold greater than those of their respective glutamate analogues. These results indicate that, in addition to their previously reported tight binding to dihydrofolate reductase, a property contributing to the high potency of PT523 and its B-ring analogs as inhibitors of tumor cell growth is their strong affinity for the RFC. BIOCHEM PHARMACOL **60**;1:41–46, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523) analogues; reduced folate carrier; dihydrofolate reductase

Analogues of the potent anticancer drug AMT† (**1**, Fig. 1) in which the glutamate side chain is replaced by N^{δ}-hemiphthaloylornithine are of interest because this substitution results in enhanced potency against a variety of tumor cells in culture [1–7]. In contrast to classical antifolates with a glutamate side chain, the antifolate effect of these compounds is not dependent on metabolic activation by FPGS. For this reason, they may be useful in the treatment of certain AMT and MTX resistant tumors with *less* FPGS activity than is present in sensitive progenitor cells of dose-limiting host tissues such as the marrow [8] or gut epithelium [9].

In an earlier study, the ability of the hemiphthaloylornithine analogues to inhibit the growth of a human tumor cell line (SCC25) derived from a previously untreated patient with head-and-neck squamous cell carcinoma were correlated with their affinity for human DHFR [7]. Another prior investigation showed that the lead compound in the series, N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523, **2**, Fig. 1), was a strong competitive inhibitor of [^{3}H]MTX unidirectional influx [6]. Similar results were obtained when PT523 was tested as a competitive inhibitor of the influx of [^{14}C](6*R*)-5,10-dideaza-5,6,7,8-tetrahydrofolic acid ([^{14}C]DDATHF) into CCRF-CEM cells [4]. Both MTX [10, 11] and DDATHF [12] are known to be substrates for the RFC transport system in these cells. DDATHF may also utilize membrane folate-binding protein (mFBP) receptors for uptake in specially adapted cell lines that either naturally express high levels of mFBP, such as MA104 monkey kidney epithelial cells [12], or are adapted for growth in medium containing physiologic (i.e. nanomolar) folic acid concentration, as exemplified by the murine leukemia line L1210-FBP [12]. Owing to amino substitution at the 4-position, neither MTX nor PT523 is an efficient substrate for this alternative mode of cellular entry. Because of the novel mode of action of PT523 as a water-soluble nonpolyglutamatable DHFR inhibitor, and because the drug has been found in several animal models

* Corresponding author: Andre Rosowsky, Ph.D., Department of Adult Oncology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115. Tel. (617) 632-3117; FAX (617) 632-2410; E-mail: andre_rosowsky@dfci.harvard.edu

† *Abbreviations:* AMT, aminopterin; FPGS, folylpolyglutamate synthetase; DHFR, dihydrofolate reductase; RFC, reduced folate carrier; MTX, methotrexate; LSC, liquid scintillation counting; and HBSS, HEPES balanced salt solution.

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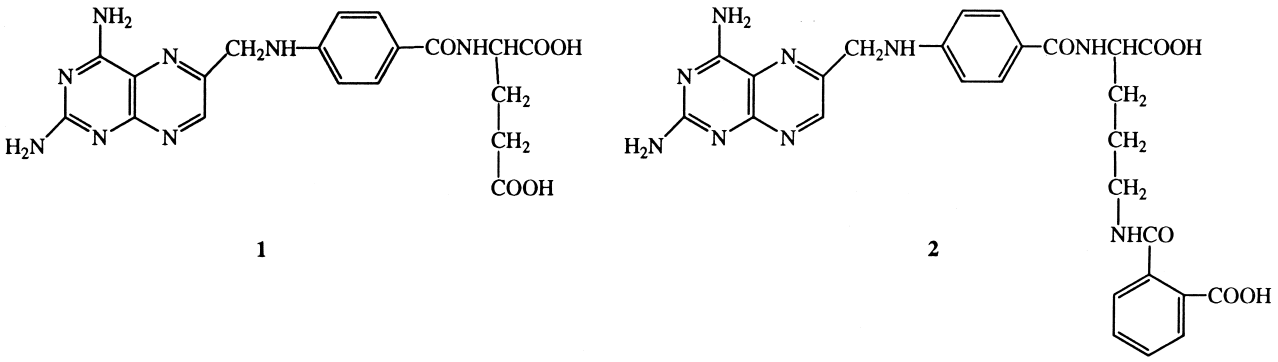
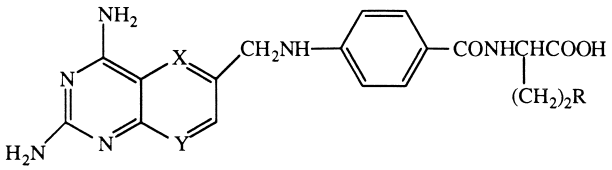


FIG. 1. Chemical structures of aminopterin (AMT, 1) and N^{α}-(4-amino-4-deoxy)-N^{δ}-hemiphthaloyl-L-ornithine (PT523, 2).

to give tumor growth delays somewhat greater than those produced by MTX at higher doses [13], PT523 was selected recently by the National Cancer Institute for accelerated preclinical development.

In the present work we have continued our examination of the relationships between the cytostatic activity of non-polyglutamatable antifolates and their affinity for membrane RFC. To that end, we have determined the K_i values of a group of B-ring analogues of PT523 (structures **3–8**, Fig. 2) as inhibitors of RFC-mediated [^{3}H]MTX influx into CCRF-CEM cells, and have assessed the activity of these compounds as inhibitors of the growth of CCRF-CEM cells in culture. As will be discussed below, all the B-ring analogues of PT523 were similar to the parent compound as RFC ligands and, in two examples where side-by-side comparisons could be made (structures **9** and **11**, Fig. 2), were approximately 10-fold better than the corresponding B-ring analogues with glutamate side chains.



	X	Y	R
(3)	CH	N	$CH_2NHCOC_6H_4$(ortho-COOH)
(4)	CMe	N	"
(5)	N	CH	"
(6)	CH	CH	"
(7)	CMe	CH	"
(8)	CCl	CH	"
(9)	CH	N	COOH
(10)	CMe	N	"
(11)	N	CH	"
(12)	CH	CH	"
(13)	CMe	CH	"
(14)	CCl	CH	"

FIG. 2. Chemical structures of B-ring analogues of PT523 and AMT.

MATERIALS AND METHODS

Drugs and Radiochemicals

PT523 (**2**) and its B-ring analogues (**3–8**) were synthesized at the Dana-Farber Cancer Institute as described [1, 2, 7]. 5-Deazaaminopterin (**9**) [14] and 8-deazaaminopterin (**11**) [15] were provided by Dr. Edward Taylor (Princeton University) and Dr. Raymond Blakley (St. Jude Children's Research Hospital). [3′,5′,7-^{3}H]MTX ([^{3}H]MTX, specific activity 15 Ci/mmol) was purchased from Moravek Biochemicals, and was determined to be >99% radiochemically pure by HPLC. Tritium analysis by LSC was carried out in a Redi-Safe mixture (Fisher) using a Beckman model LS1801 instrument.

Cytotoxicity Assays

CCRF-CEM human leukemic lymphoblasts [16] (American Type Culture Collection) were maintained in suspension culture in 175 cm^2 vented flasks at 37° in a 5% CO_2 humidified atmosphere in RPMI 1640 medium (Fisher) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Sigma). For cytotoxicity assays, cells at an initial density of 5.5×10^4/mL were incubated in 24-well microtiter plates with 0.1 to 1000 nM drugs (half-log increments) or in the absence of drug (controls) for 72 hr. A 0.5-mL aliquot from each well was suspended in Isoton II, and cell numbers were determined electronically with a Coulter model Z_{BI} counter gated for 10- to 30-μm diameters. The counts showed close correspondence with viable cell numbers obtained with a hemocytometer after staining with trypan blue. Background values were obtained by counting cells that had been treated for 72 hr with 1 μM PT523, a concentration *ca.* 10^3 times greater than the IC_{50}. Ten replicate validation trials showed that these cells were completely permeable to trypan blue and that >98% were smaller than 10 μm. The survival fraction (SF) was calculated from the formula SF = (treated cell number − background)/(control cell number − background). A semilog graph of drug concentrations versus SF values was plotted with the aid of a program using

Microsoft Excel, and the IC_{50} was determined by interpolation. Each IC_{50} determination was repeated three times on different days.

Transport Assays

For determination of the K_t for MTX influx into CCRF-CEM cells, 20.0 μL of a 66.7 μM [^{3}H]MTX stock solution (specific activity 15.0 Ci/mmol) and 10.1 to 62.4 μL of nonradioactive 1.00 mM MTX were added to appropriate amounts of HBSS buffer (20 mM HEPES, 107 mM NaCl, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2 \cdot 2H_2O$, 1.0 mM $MgCl_2$, and 7.0 mM D-glucose, adjusted to pH 7.4 with NaOH) to give a final volume of 3.2 mL. The initial [^{3}H]MTX concentrations were thus 3.33, 4.00, 5.00, 6.67, 10.0, and 20.0 μM. For the determination of the IC_{50} of the B-ring analogues of PT523 as inhibitors of MTX influx, the assay solutions contained 5.0 μL of [^{3}H]MTX stock solution (see above), 7.6 μL of nonradioactive 1.00 μM MTX, and 3.33 to 80.0 μL of 100 μM inhibitor in 1.0 mL (final volume) of HBSS. For the determination of the K_i for competitive inhibition of MTX influx via the RFC, the assay mixture consisted of 20.0 μL of [^{3}H]MTX stock solution (see above), 12–63 μL of nonradioactive 1.00 mM MTX, and 12.8 μL of 200 μM inhibitor in 3.2 mL (final volume) of HBSS. The initial inhibitor concentration was 0.800 μM, and those of [^{3}H]MTX were the same as were used to obtain the K_t.

For the determination of K_t, IC_{50}, and K_i values, log phase cultures of 3×10^8 cells (≥98% trypan blue excluding) were washed with 10 mL of HBSS at 37°, and centrifuged at 800 *g* for 5 min. The 200- to 300-μL cell pellet was resuspended with 450–550 μL of HBSS, and 100-μL aliquots were distributed into six 15-mL conical tubes. The tubes were kept at 37° for 5–10 min to allow the cells to become conditioned to the uptake buffer. The assay solutions containing [^{3}H]MTX alone or [^{3}H]MTX plus inhibitor (see above) were prewarmed to 37°, and 900-μL aliquots were mixed with the cells at 1-min intervals. For K_t and K_i determinations, the final [^{3}H]MTX concentrations were thus 3.0, 3.6, 4.5, 6.0, 9.0, and 18 μM. For IC_{50} determinations, the corresponding inhibitor concentrations were 0, 0.3, 0.6, 1.8, 3.6, and 7.2 μM. The K_t for [^{3}H]MTX influx was obtained from a Lineweaver–Burk plot of concentration-dependent rates in the absence of inhibitor. The K_i was calculated for each compound from the relationship $K_i = (K_t)(I)/(K_{app} - K_t)$ for competitive inhibition, where K_{app} is the inverse of the x-axis intercept of the corresponding Lineweaver–Burk plot for data obtained in the presence of a fixed concentration (I) of inhibitor as described in the literature [17].

A 10-μL aliquot of each mixture was withdrawn and counted by LSC in order to allow the final [^{3}H]MTX concentration to be expressed in pmol · count^{-1} · min. Exactly 1 min after addition of the assay solution, active transport was quenched by adding 10 mL of ice-cold PBS solution. The cells were washed twice with PBS as described above, and the final pellet was rediluted with HBSS to *ca.* 550 μL. A 10-μL aliquot was used for cell density determination, and another 500 μL was analyzed for radioactivity by LSC. The 1-min method was validated by comparing the results with those obtained for full [^{3}H]MTX uptake progress curves with and without PT523 inhibition (nine time points: 1, 2, 3, 5, 10, 15, 20, 30, and 40 min; four concentrations: 2, 4, 6, and 8 μM; three replicates). Uptake was linear over the 4 min and thus was assumed to represent the unidirectional phase. All determinations were corrected for nonspecific binding measured at 0–4° (<4% at 8 μM after 40 min; <1.3% at 18 μM after 1 min). The two methods gave statistically similar results by Student's *t*-test at $P = 0.002$.

RESULTS AND DISCUSSION

The K_i values of PT523 (**2**) and its B-ring analogues (**3–8**) as inhibitors of [^{3}H]MTX influx via the RFC system are given in Table 1, along with the K_t for [^{3}H]MTX influx in the absence of inhibitor. The K_t/K_i ratio, an indicator of binding affinity relative to MTX, and the IC_{50} values for CCRF-CEM cell growth inhibition during 72 hr of continuous drug exposure are also presented. In addition, the K_i values we recently reported for these compounds as inhibitors of human DHFR [7, 19] are shown for the sake of a more complete structure–activity correlation. All the hemiphthaloylornithine derivatives displayed a high affinity for the RFC of CCRF-CEM cells as judged from their K_i values, which were in the 0.3 to 0.7 μM range versus a K_t of 7.1 μM for MTX. Thus, the K_t/K_i ratio varied from 10:1 in the case of PT523 (**2**) to 23:1 in the case of 5,8-dideazaPT523 (**6**), which is likewise one of the analogues most tightly bound to DHFR. Lineweaver–Burk plots (not shown) indicated that all the compounds behaved as competitive inhibitors, suggesting that they bind to the same site on the RFC protein as MTX and the reduced folates. The K_i values for PT523 and MTX obtained in this study were similar to those reported earlier for CEM-7A cells, whose RFC protein is kinetically similar to that of CCRF-CEM cells but is more highly expressed on the cell surface [6].

We have not investigated whether alternative entry routes such as the recently identified low pH transporter in murine [20, 21] and human [22] cells play a significant role for our compounds. However, we do not consider it likely that this transporter is important under the conditions of our experiments, which were carried out at pH 7.4.

Although the K_i values of all the B-ring analogues of PT523 to the RFC protein were quite similar, it should be noted that differences may exist in the ease with which the RFC actually translocates the various B-ring analogues across the cell membrane, even though the free-energy differences for binding to the *trans* epitope of the carrier are relatively small.

Because the K_i values for B-ring analogues **4–7** as inhibitors of MTX influx were lower than the K_i of PT523,

TABLE 1. RFC-mediated influx, DHFR inhibition, and CEM cell growth inhibition by PT523 (2) and its B-ring analogs (3–8)

Compound	[^{3}H]MTX influx* IC_{50} (μM)	[^{3}H]MTX influx* K_i (μM)	[^{3}H]MTX influx* K_t/K_i	DHFR inhibition K_i† (pM)	Cell growth‡ IC_{50} (nM, 72 hr)
PT523 (**2**)	1.42	0.71 ± 0.12	10	0.35 ± 0.13	1.3 ± 0.20 (12)
5-DeazaPT523 (**3**)	1.25	0.59 ± 0.18	12	0.41 ± 0.11	0.93 ± 0.14 (16)
5-Me-5-deazaPT523 (**4**)	0.93	0.43 ± 0.15	17	0.40 ± 0.10	0.67 ± 0.03 (22)
8-DeazaPT523 (**5**)	1.02	0.40 ± 0.08	18	0.19 ± 0.006	0.94 ± 0.04 (16)
5,8-DideazaPT523 (**6**)	0.66	0.31 ± 0.05	23	0.09 ± 0.03	0.64 ± 0.04 (23)
5-Me-5,8-dideazaPT523 (**7**)	1.00	0.34 ± 0.06	21	0.10 ± 0.008	0.82 ± 0.09 (18)
5-Cl-5,8-dideazaPT523 (**8**)	0.85	0.48 ± 0.11	15	0.11 ± 0.05	0.67 ± 0.12 (22)
MTX		7.1 ± 0.72 (K_t)	1.0	5.2 ± 0.45	14 ± 2.6 (1.0)
AMT	11.0	5.4 ± 0.09§	1.3	3.1 ± 0.73	4.4 ± 0.10 (7.9)‖
5-DeazaAMT (**9**)	6.19	3.1 ± 0.13	2.3	ND	16 ± 3.0 (0.9)
8-DeazaAMT (**11**)	8.72	3.96 ± 0.49§	2.2	ND	5.0 ± 0.95 (3.0)‖

*K_t in the ratio K_t/K_t was obtained from the x-axis intercept in a Lineweaver–Burk plot for [^{3}H]MTX influx in the absence of inhibitor. The term K_i was calculated for each compound from the relationship $K_i = (K_t)(I)/(K_{app} - K_t)$, where K_{app} is the inverse of the x-axis intercept for the double-reciprocal plot for [^{3}H]MTX influx in the presence of a fixed concentration (I) of the competitive inhibitor [17]. K_i values listed are means ± SD of at least 3 experiments on different days.

†Data are taken from Ref. 7 (K_i for PT523 and MTX also reported in Ref. 19).

‡The IC_{50} values are means ± SD of at least 3 experiments on different days. Numbers in parentheses are normalized potencies relative to MTX (1.0).

§The K_t values for influx of [^{3}H]AMT and **11** into CCRF-CEM cells in serum-free RPMI 1640 containing 20 mM $NaHCO_3$, 2.0 mM L-glutamine, and 25 mM HEPES, pH 7.4, are reported to be 5.1 ± 1.4 and 3.4 ± 1.2 μM [18].

‖The IC_{50} for growth inhibition of CCRF-CEM cells after 48 hr of continuous treatment in RPMI 1640 containing 10% fetal bovine serum and 2 mM L-glutamine are reported to be 5.2 ± 1.2 and 3.5 ± 0.5 nM [18].

it was of interest to compare the K_i values of all our compounds with those reported in the literature for the corresponding glutamate analogues **9–14** (Fig. 2). The K_i values of 5-deazaAMT (**9**) and 5-methyl-5-deazaAMT (**10**) as inhibitors of [^{3}H]MTX influx into L1210 cells are reported to be almost the same as the K_i of AMT itself, 1.2 μM [23], whereas in another paper from the same group the K_i values of 5,8-dideazaAMT (**12**), 5-methyl-5,8-dideazaAMT (**13**), and 5-chloro-5,8-dideazaAMT (**14**) are reported to be 5.7, 4.8, and 2.2 μM [24]. The K_t for MTX influx into the murine cells was reported in the latter study [24] to be 3.5 μM. Although the K_i of 8-deazaAMT (**11**) has been reported only for CCRF-CEM cells rather than for L1210 cells [18], the K_i values of the other B-ring analogues of AMT all can be seen to lie in the 1–6 μM range. When K_t/K_i is calculated from these data, the ratio does not exceed 3.2:1.

To address the possibility that the apparent difference in K_i and K_t/K_i for the glutamate and hemiphthaloylornithine analogues was due to the fact that different cells were used, we also compared the K_i values of AMT, **9**, and **11** in CCRF-CEM cells. As shown in Table 1, the K_i values of these glutamyl derivatives were higher than those of the corresponding hemiphthaloylornithine analogues even when the comparison was made in the same cell line. From these results it seems clear that the compounds with a hemiphthaloylornithine side chain collectively have an affinity for the RFC that is several times higher than that of the classical glutamate analogues.

Qualitative similarities appear to exist among the hemiphthaloylornithine and glutamate analogues as substrates for the RFC. For example, **5** was a better substrate than PT523, for the RFC of CCRF-CEM cells, just as **11** has been reported [18], and is now confirmed, to be a better substrate than AMT for influx into these cells. Similarly, the K_i values of **3** and **4** are close to the K_i of PT523, just as those of **9** and **10** are reported to be essentially identical to the K_i of AMT [23]. In our hands, **5** was a slightly better inhibitor of [^{3}H]MTX influx into CCRF-CEM cells than PT523. Others have reported that the K_m of **11** is lower than that of AMT [18], but the difference was not statistically significant. Our K_i value (95% confidence interval = 3.4 to 4.5 μM) for **11** indicates that its utilization of the RFC is indeed slightly more efficient than that of AMT (95% confidence interval = 5.3 to 5.5 μM). However, the K_i values for the hemiphthaloylornithine analogues do not exactly parallel those of the corresponding glutamate series. For example, the reported K_i values of the 5,8-dideaza analogues of AMT are as much as 6-fold higher in L1210 cells than the K_i of AMT [24], whereas we find much less difference between the corresponding hemiphthaloylornithine derivatives in CCRF-CEM cells. Since we have not investigated the influx of PT523 analogues into L1210 cells, we can only speculate as to whether these incongruities reflect structural differences between the human and murine RFC protein, differences in the spatial orientation of hemiphthaloylornithine versus glutamate analogues when they bind to the human protein, or a combination of both.

Because PT523 and its B-ring analogs cannot be polyglutamated, their activity must depend upon cellular penetration to levels sufficient to suppress reduction of dihydrofolate to tetrahydrofolate. Although steady-state levels of the drugs could not be determined because of the lack of availability of the analogs in tritiated form, the 10-fold decrease in the influx K_i that we observed for the hemiph-

thaloylornithine derivatives relative to MTX would be likely to produce higher free intracellular drug levels relative to MTX at concentrations below the influx K_t for MTX.

We have reported previously that PT523 and its B-ring analogs are very potent inhibitors of DHFR, with K_i values in the 0.1 to 0.4 pM range [7, 19]. In terms of structure–activity correlation, it is of interest to note that 5,8-dideazaPT523 (**6**) and 5-methyl-5,8-dideazaPT523 (**7**), which have the highest DHFR affinity of all the compounds in this group, with K_i values of 0.09 and 0.1 pM, respectively, also have a very strong affinity for the RFC, suggesting that the two proteins may be similar in terms of their interaction with 4-amino antifolates. We also reported previously that PT523 and its B-ring analogs are potent inhibitors of the growth of a human squamous cell carcinoma line (SCC25) in culture, with IC_{50} values ranging from 0.33 to 1.8 nM for a 72-hr exposure [7]. The range of IC_{50} values (0.67 to 1.3 nM) shown by these compounds against CEM cells is in excellent agreement with the results we obtained against SCC25 cells.

The present studies suggest that, in addition to their previously observed high affinity for DHFR, efficient cellular uptake via the RFC probably contributes to the unusually high potency of PT523 and its B-ring analogs as inhibitors of the growth of human tumor cells. Influx experiments using other ring and side-chain analogues of PT523 are currently in progress in our laboratory in the hope that elucidation of the structure–activity relationships among this more diverse series of structures will further broaden our understanding of the interaction of antifolates with the RFC. Because of the important role of efflux in the net cellular accumulation of antifolates, which appears to occur via more than one pathway [25], it would also be of interest to investigate whether the same structure–activity relationships exist for the efflux of these compounds as for their influx. Although they were outside the scope of the work described here, such studies could be done, for example, with inside-out vesicles of CCRF-CEM cells as others have done to study MTX efflux from L1210 cells [26] and P-glycoprotein-mediated drug efflux from CCRF-CEM cells [27].

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