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STRUCTURAL PREFERENCES AMONG FOLATE COMPOUNDS AND THEIR ANALOGUES FOR ATPase-MEDIATED EFFLUX BY INSIDE-OUT PLASMA MEMBRANE VESICLES DERIVED FROM L1210 CELLS

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Abstract—Our prior studies with inside-out plasma membrane vesicles from L1210 cells (Schlemmer SR and Sirotnak FM, *J Biol Chem* **267**: 14746–14752, 1992) identified an outwardly directed, translocating ATPase as mediating the majority of methotrexate (MTX) efflux in these cells. In the current studies, we examined by competitive inhibition with [^{3}H]MTX as permeant some of the structural features that determine preferences among folate compounds and their analogues as permeants for this ATPase. The results show that folate compounds are preferred over simple quinazolines (5,8-dideaza-pteridines), and IL5-CH_3-folateH_4, and probably other 5-substituted folates are preferred over folic acid. In the latter regard, the observed equivalence in preference to IL5-CH_3-folateH_4 of the 4-oxa-pyridopyrimidine, lometrexol (DDATHF), probably relates to its close similarity to folateH_4. The results also suggest that the 4-position in the case of folate analogues, but not in the case of the quinazoline analogues, is an important determinant with 4-amino preferred over 4-oxa. They also suggest that the N10 position on the bridge region in both series of compounds, and probably for the pyridopyrimidine lometrexol, is not an important determinant. In contrast to results seen with the simple quinazolines, the 2-CH_3 desamino quinazoline ZEN D1694, modified as well by a 2-benzyl to thienyl replacement on the side chain, was highly preferred. The same relative differences seen among some of these analogues as inhibitors of [^{3}H]MTX efflux in inside-out vesicles were documented for their effectiveness as permeants for ATP-dependent efflux in intact L1210 cells.

Key words: folate compounds; ATP-dependent efflux; L1210 cells

Previous studies from our laboratory [1, 2] have provided evidence for the notion that net intracellular accumulation of 4-amino folate analogues, such as MTX†, in L1210 cells reflects the operation of at least two separate systems that mediate their inward and outward flux. Although the one-carbon, reduced folate transport system, which mediates [3–5] influx of these agents, is also capable of mediating their bidirectional flux [3–5], under physiological conditions efflux is mediated predominantly [2, 6] by a specific outwardly directed ATP-dependent system. Although some difference of opinion has arisen with regard to the conclusions of these studies [1, 2–6] and of others reported elsewhere [7, 8], concerning the multiplicity of separate efflux routes in these cells, the results from both laboratories are in agreement as to the predominance of an ATP-dependent, BSP-sensitive route in the mediation of MTX efflux in L1210 cells. The BSP-sensitive system has been identified recently in our laboratory [9] as a translocating ATPase in studies utilizing inside-out plasma membrane vesicles derived from these cells.

Since a number of new folate analogues are under development [10–12] as therapeutic agents in patients presenting with neoplastic disease, it was of interest to examine some of these agents, as well as natural folate compounds, as permeants for this translocating ATPase. Also, a comparison among selected folate compounds and their analogues identifying structural preferences as permeants may provide additional insights as to the interaction of this class of small molecule permeants with this ATPase, which may also be useful in the design of more efficacious folate analogues. For these studies, we utilized the same inside-out plasma membrane vesicle technology that was described in our original report [9]. To ascertain preferences among these structurally related compounds as permeants for this system, we applied kinetic approaches to a determination of their effectiveness as inhibitors of mediated efflux of [^{3}H]MTX on the assumption that such data would be predictive of their effectiveness as permeants. Our results, which also provide further evidence for the identity of this ATPase as the BSP-sensitive, ATP-dependent route in intact L1210 cells [6, 9] effluxing folate analogues, are presented below.

MATERIALS AND METHODS

The cells used as a source of membrane vesicles were maintained [4] in culture in RPMI-1640 medium

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† Abbreviations: MTX, methotrexate; BSP, bromosulfophthalein; and DDATHF, 5,10-dideaza-folateH_4.

supplemented with 10% fetal bovine serum. L1210/R24 cells for membrane vesicle preparations were obtained from $BD2F_1$ ($C57BL/DBA/2F_1$) mice following transplantation of cells in culture. Egress of folate analogues from L1210 cells was measured [4] in cells preloaded with each compound (5 μM) during incubation in the absence of glutamine in buffered-salts transport medium (107 mM NaCl, 10 mM Tris–HCl, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1 mM $MgCl_2$ and 7 mM D-glucose, pH 7.4) for 10 min at 37°. After preloading, cells were washed once with cold (0–4°) buffered, isotonic saline (0.14 M NaCl + 0.02 M sodium phosphate, pH 7.4) and resuspended in transport medium plus 7 mM D-glucose. Aliquots of cells were removed after various periods of incubation at 37° [1, 2, 6] and washed by centrifugation and resuspension in buffered isotonic saline. Cellular content of radioactive folate analogues was determined by processing [1, 2, 6] in scintillation fluid and scintillation counting. Cellular content of nonradioactive folate analogues was determined by a titration assay with dihydrofolate reductase [4].

The preparation of inside-out plasma membrane vesicles fromL1210/R24 cells has been described in detail in our earlier report [9]. Since these preparations were only 77% inside-out, the complication of influx in the right-side-out fraction was avoided by using the influx defective [9] L1210/R24 cells as a source of vesicles. Approximately 1 mg of inside-out vesicles was obtained from 2.2×10^9 cells. Vesicle sidedness and contamination by membranes from intracellular organelles were monitored by marker enzyme analysis described in detail in our earlier report [9]. Vesicle preparations were 94 ± 6% pure plasma membrane. Measurements of ATP-dependent intravesicular accumulation of [^{3}H]MTX were carried out with a modification [9] of the procedure of Horio *et al.* [13]. The procedure utilizes the ATP-regenerating system described earlier by Kamimoto *et al.* [14]. Usually, a 20-μL aliquot of vesicles (50–70 μg of membrane protein) was incubated at 37° in the presence and absence of 5 mM ATP and regenerating system. Intravesicular accumulation of [^{3}H]MTX was initiated by the addition of the permeant. The final buffer content of the transport medium (100 μL) was 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.1, with MgO and 225 mM sucrose. The pH used (6.1) was the measured optimum for folate compound efflux [9] in this vesicle system. Incubation was stopped at various times by rapid dilution with 9 mL of cold (0–4°) Tris–HCl–sucrose (20 mM Tris–HCl, pH 7.4, and 250 mM sucrose), and the vesicles were collected by filtration and dissolved in scintillation fluid for radioactive counting. Absorption of radiolabeled permeant to the vesicle surface was determined by a 5-sec exposure to permeant at 0–4°. Alternatively, surface binding was determined by back-extrapolation of the 37° time–course to the origin. Each time point was performed in duplicate, and the data represent an average of at least three separate experiments. Kinetic constants (K_m, V_{max} and K_i) for initial intravesicular accumulation were determined by Lineweaver–Burk [15] or Dixon [16] plots of the time–course data at various external concentrations with the application of linear regression analysis.

3′,5′,9-[^{3}H]MTX (sp. act. = 12–20 Ci/mmol) and 3′,5,9-[^{3}H]aminopterin (sp. act. = 10–15 Ci/mmol) were purchased from Moravek Biochemicals, City of Industry, CA. MTX and aminopterin were provided by the Division of Cancer Treatment, National Cancer Institute, and ZEN D1694 was a

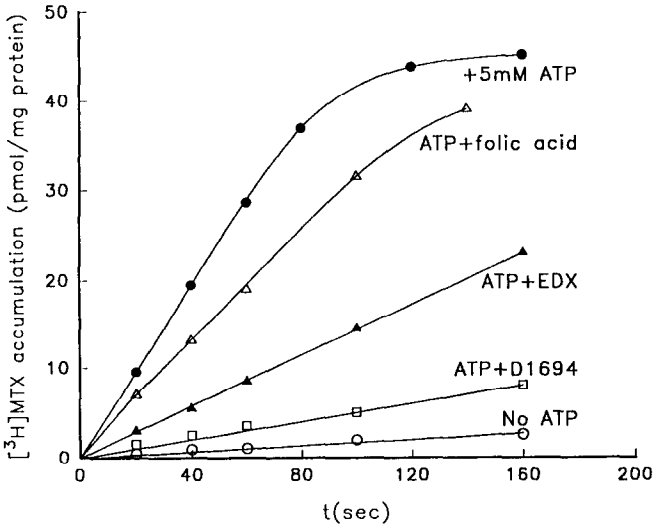

Fig. 1. Inhibition by folate analogues of ATP-dependent internalization at 37° of [^{3}H]MTX by inside-out plasma membrane vesicles from L1210/R24 cells. The concentration of each analogue employed was 100 μM with 20 μM [^{3}H]MTX. EDX = edatrexate (10-ethyl-10-deaza-aminopterin). The data represent an average of 3 separate experiments. Additional details are provided in the text. SEM = < ± 14%.

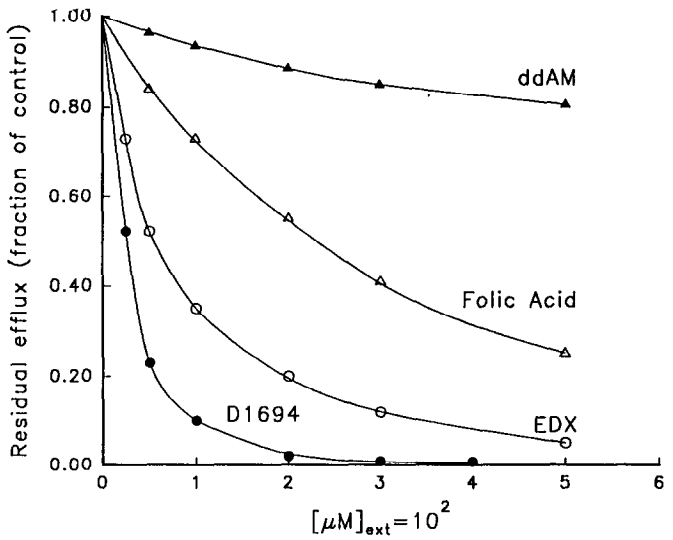

Fig. 2. Concentration-dependence for the inhibition by folate analogues of ATP-dependent internalization of [^{3}H]MTX by inside-out plasma membrane vesicles from L1210/R24 cells. The rate of internalization of [^{3}H]MTX at 20 μM was determined by sampling after 15, 30 and 45 sec of incubation at 37°. Abbreviations: EDX, edatrexate; and ddAM, 5,8-dideaza-aminopterin. Additional experimental details are provided in the text. SEM = < ± 13%.

gift of Zeneca Pharmaceuticals, Surrey, U.K. Lometrexol (DDATHF) was provided by Dr. J. R. Piper of the Southern Research Institute, and edatrexate was provided by the Ciba Geigy Corp., Summit, NJ. Dideaza-aminopterin was provided by Dr. L. Werbel of the Parks–Davis Corp., Summit, NJ. IL5-CH_3-folateH_4 was purchased from Dr. B. Schirks Laboratories, Jona, Switzerland. All folate analogues were shown by HPLC [17] to be greater than 97% pure. All other chemicals were reagent grade.

RESULTS AND DISCUSSION

The results in Fig. 1 show a typical time–course for intravesicular accumulation of [^{3}H]MTX at an external concentration of 20 μM by inside-out plasma membrane vesicles from L1210/R24 cells. The initial rate of accumulation was 20- to 25-fold more rapid in the presence of 5 mM ATP than in its absence. Our prior studies [9] of ATP-dependent efflux in this system documented rapid, saturable (K_m = 47 ± 8), and concentrative internalization of [^{3}H]-MTX that was dependent upon ATP hydrolysis. As inhibitors of ATP-dependent efflux of [^{3}H]MTX, the folate compounds and their analogues that we examined varied considerably in their potency. For example (Fig. 1), at a concentration of 100 μM, folic acid was minimally effective (30% inhibition); edatrexate, the 4-amino folate analogue, was substantially more effective (68% inhibition); and D1694, the 4-oxa quinazoline analogue, was the most effective (89% inhibition). Efflux of [^{3}H]MTX in the presence of various amounts of some of these inhibitors is shown in Fig. 2. Data in this figure focus on the effects on ATP-dependent efflux of [^{3}H]MTX only after they were corrected for ATP-independent efflux of [^{3}H]MTX (Fig. 1), which had been shown earlier [9] to represent simple diffusion into these vesicles. Again, D1694 and edatrexate were among the most potent inhibitors. In the concentration range shown, they achieved complete or nearly complete inhibition of ATP-dependent efflux of [^{3}H]MTX consistent with the notion that such flux is mediated by a single ATPase or more than one ATPase with similar sensitivity to these inhibitors. By comparison, folic acid was a modest inhibitor and the 4-NH_2-quinazoline analogue dideaza-aminopterin was extremely ineffective as an inhibitor.

Data similar to that shown in Fig. 2 were generated (not shown) for the remainder of the compounds selected for use in these studies. All of the data was then analyzed by a Dixon plot [16] to obtain individual values for K_i. Some of these plots are shown in Fig. 3. In every case, a single inhibition component, as shown by a linear plot of inhibitor concentration versus the reciprocal of efflux velocity, was obtained regardless of the extent of the inhibition observed in each case. A summary of these values and the most relevant structural details for all of the compounds examined are given in Table 1. The actual structures are given in Fig. 4. Substantial diversity in K_i values was shown for this group of compounds. Among the pteridinyl and pyridopyrimidinyl compounds studied, folic acid was the least effective as an inhibitor of ATP-dependent [^{3}H]MTX efflux. The largest value for K_i was obtained with this compound, while the value for the natural diastereoisomer of 5-CH_3-folateH_4 was 4-fold lower; the values for all of the other pteridinyl compounds and the 4-amino analogues, including aminopterin, MTX and edatrexate, were comparable to IL5-CH_3-folateH_4. Likewise, a similar value for K_i was obtained with the pyridopyrimidinyl analogue of folate H_4, lometrexol. These results appear to document an equivalence in structural preference by

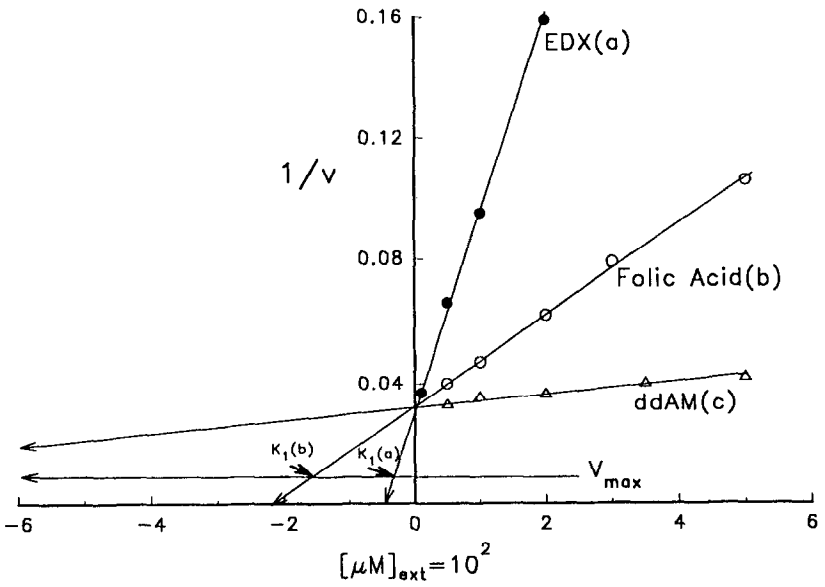

Fig. 3. Dixon plot of data generated for the inhibition by folate analogues of ATP-dependent internalization of [^{3}H]MTX by inside-out plasma membrane vesicles from L1210/R24 cells. See the legend of Fig. 2 and the text for additional details. SEM = < ± 13%.

this ATPase for 5-substituted reduced folates, unsubstituted reduced folate analogues as well as 4-amino substituted analogues of folic acid when compared with folic acid itself. In sharp contrast, two of the quinazoline analogues examined were extremely poor inhibitors of ATP-dependent [^{3}H]-MTX efflux. K_i values for both the 4-oxa and 4-amino quinazolinyl analogues dideaza-aminopterin and CB3717, were extremely high (15- to 18-fold greater than those of all the folate compounds except folic acid). Most interestingly, the third quinazolinyl analogue examined, D1694, was the most effective inhibitor of ATP-dependent [^{3}H]MTX efflux overall. This 2-desamino-4-oxa-quinazoline has CH_3 at the 2 position and a thiophene in place of benzene on the side-chain, in addition to CH_3 at N10.

We also found during these studies that the same differences seen among some of these analogues as inhibitors of [^{3}H]MTX efflux in inside-out vesicles were documented for their effectiveness as permeants for ATP-dependent efflux in intact L1210 cells. Some data pertaining to the latter studies are shown in Fig. 5. These data show that efflux of all three of the 4-amino folate analogues from ATP-replete L1210 cells was extremely rapid ($k_{eff} = 0.24$ to $0.27\ min^{-1}$), whereas efflux of the quinazoline dideaza-aminopterin was 14- to 17-fold lower ($k_{eff} = 0.017\ min^{-1}$). These values are consistent with the corresponding values for K_i obtained with these compounds (Table 1) as inhibitors of ATPase-mediated efflux of [^{3}H]MTX in the vesicle system. A value for decay-time for folic acid could not be

Table 1. Folate compounds and their analogues as inhibitors of ATP-dependent efflux of [^{3}H]MTX by inside-out plasma membrane vesicles from L1210/R24 cells

Compound	Basic ring structure*	Substituents 2	4	5	R = 10	K_i (μM)
Folic acid	Pteridinyl	NH_2	OH		NH	216 ± 28
lL5-CH_3-folateH_4	PteridinylH_4	NH_2	OH	CH_3	NH	54 ± 7
Aminopterin	Pteridinyl	NH_2	NH_2		NH	49 ± 8
Methotrexate	Pteridinyl	NH_2	NH_2		NCH_3	53 ± 8
Edatrexate	Pteridinyl	NH_2	NH_2		CHC_2H_5	59 ± 9
Dideaza-aminopterin	Quinazolinyl	NH_2	NH_2	H	NH	920 ± 130
Lometrexol	PyridopyrimidinylH_4	NH_2	OH	H	NH	57 ± 5
CB3717	Quinazolinyl	NH_2	OH	H	NC—C≡CH	880 ± 99
ZEN D1694†	Quinazolinyl	CH_3	OH	H	NCH_3	19 ± 3

Experimental details are provided in Materials and Methods. The data are means ± SEM of 3–5 separate experiments.

* Pyridopyrimidinyl = 5 deazapteridinyl; quinazolinyl = 5,8-dideazapteridinyl.

† 2-Benzyl replaced by thiophene.

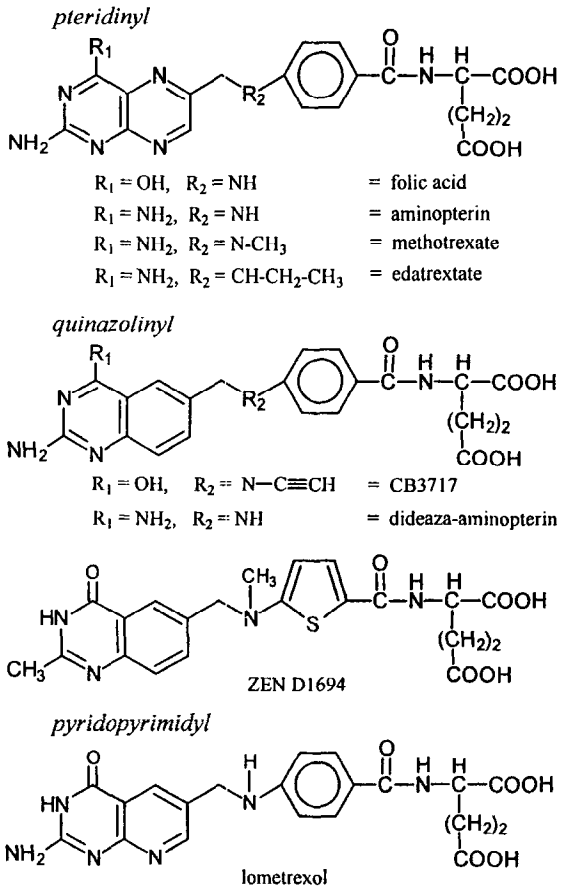

Fig. 4. Structural properties of the various folate compounds used in these studies.

derived because of the rapid intracellular metabolism [17] of this compound. Such data strongly suggest that the potency of these compounds as inhibitors of this process reflects the relative preference for them by this ATPase as permeants and support the conclusion of our earlier studies [9] that the ATP-dependent process under study is the same in each case.

Although a relatively small group of structurally related compounds were examined in these initial studies, a number of conclusions can be derived from these results. Clearly, the folate compounds and their 4-amino-pteridine and pyridopyrimidine analogues examined are preferred over the simple quinazolines as permeants for this translocating ATPase and lL5-CH_3-folateH_4, and probably other 5-substituted reduced folates are preferred over folic acid. In the latter regard, the equivalence in preference apparent for lometrexol is most likely related to the fact that this fully reduced, 4-oxa-pyridopyrimidine is a close structural analogue of folateH_4. The results also suggest that the 4-position in the case of the folate analogues, but not in the case of the quinazoline analogues, is an important determinant, with 4-amino preferred over 4-oxa. They also suggest that the bridge region in both series of compounds and probably in the pyridopyrimidine, specifically the N10 position, is not an important determinant. This is concluded from the fact that both the folate and quinazoline compounds, as a group, exhibit considerable diversity at this region (see Fig. 4 and Table 1), but compounds within each series were essentially equivalent as inhibitors of ATP-dependent efflux of [^{3}H]MTX. The third quinazoline compound was the most unusual overall. Its marked effectiveness as an

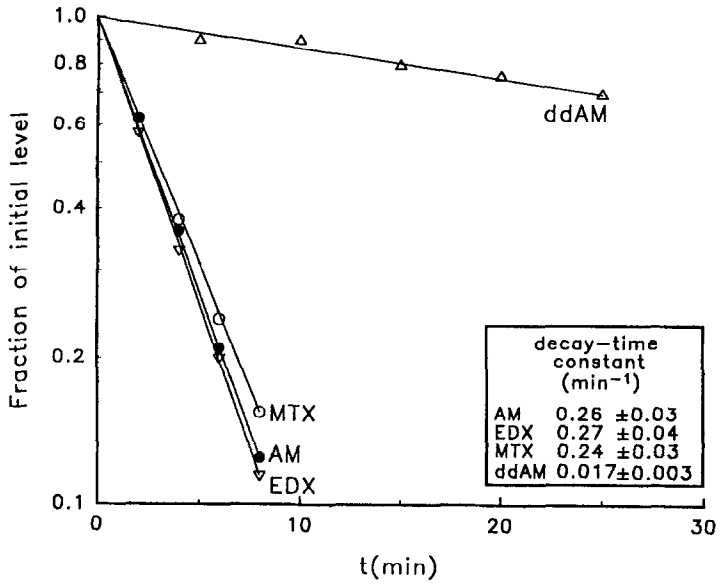

Fig. 5. Time-course for efflux at 37° of folate analogues from L1210 cells. After a 10-min incubation of cells in transport medium at 37° in the presence of a 5 μM concentration of each analogue, cells were cooled to 0–4°, centrifuged and resuspended in analogue-free transport medium at 37°. The cell suspension was sampled at various intervals after resuspension. Abbreviations: AM, aminopterin; ddAM, 5,8-dideaza-aminopterin; EDX, edatrexate; and MTX, methotrexate. Additional details are provided in the text. The data are an average of 3 separate experiments done on separate dates. SEM = < ± 12%.

inhibitor, particularly when compared with the other quinazolines, most likely relates to the 2-CH_3 desamino modification on the pyrimidine ring. However, we have no data that formally exclude an effect of the benzene to thiophene modification on the side chain. Under the likely assumption that all of these structurally related compounds are, themselves, permeants for this translocating ATPase, the data presented above document differences in preferences among these compounds as permeants that is characteristic of a specific binding interaction with a site on the ATPase.

Net intracellular accumulation of folate compounds in L1210 cells under physiological (ATP-replete) conditions should reflect the effectiveness of each compound as a permeant for this outwardly directed translocating ATPase as well as the one-carbon, reduced folate transport system that mediates [1–8] their influx. Therefore, it was of interest to note that the relative preferences among this group of compounds for ATP-dependent efflux were different from those shown [4, 11, 12, 18–22] for the influx route. In the latter case, overall saturability among these compounds was greater, and folic acid was relatively less effective as a permeant compared with the reduced folate analogue. However, both systems exhibited similar saturability for folic acid. Although there were some differences among the pteridine derivatives, these as well as dideaza-aminopterin, lometrexol and D1694 were similar overall as permeants for this influx route. Interestingly, in contrast to dideaza-aminopterin, the quinazoline analogue CB3717 was a relatively poor permeant for influx as in the case of ATP-dependent efflux. Intracellular accumulation of these analogues would also be influenced by their polyglutamylation once inside the cell. However, internalization of many of these analogues [11, 12, 18] is 10- to 20-fold more rapid than their polyglutamylation. Also, we have shown [23] that polyglutamates of at least the pteridine analogues, if not the other compounds, appear to be equivalent as permeants for this translocating ATPase.

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