

Characterization of Binding of Folates and Antifolates to Brush-Border Membrane Vesicles Isolated from Human Kidney

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ABSTRACT

Antifolates such as methotrexate, raltitrexed, and pemetrexed are among the most effective and widely used anticancer drugs. The antifolates are also among the most unpredictable of anticancer drugs with respect to pharmacokinetics and toxicity. In this study, we assessed the binding of folates and antifolates to the folate receptors (FRs) of human proximal tubules and the effects of pH on binding. Binding of [³H]folic acid was pH-dependent, with maximal binding seen at pH 6. Equilibrium binding experiments with [³H]folic acid showed that K_d values were unaffected, and B_{max} values increased as the pH was decreased from 8.0 to 6.0. Increasing the osmolarity at pH 6.0 had no effect on intravesicular content, confirming that increased site-specific binding caused the observed changes in B_{max} values. Enzymatic cleavage of glycosyl-phosphatidyl-

inositol linkages abolished binding of [³H]folic acid to brush-border membrane vesicles, suggesting that [³H]folic acid was bound to FRs. In concentration-effect experiments conducted at different pH values, the antifolates raltitrexed and (2S)-2-[o-fluoro-*p*-[*N*-(2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-ylmethyl)-*N*-(prop-2-ynyl)amino]benzamido]-4-(tetrazol-5-yl) butyric acid (ZD9331) bound more tightly as pH increased from 6.0 to 8.0, whereas binding of 10-propargyl-5,8-dideazafolic acid (CB3717) was unchanged. The results obtained when K_i values were converted to binding energies suggested that binding of some, but not all, antifolates and folates to FRs was pH-dependent, further indicating roles of luminal pH in renal reabsorption or secretion processes.

Methotrexate and other antifolates are among the most effective and widely used drugs for the treatment of cancer, and methotrexate remains the cornerstone of curative treatment for childhood leukemia (Pui and Evans, 1998). Novel antifolates, including raltitrexed, ZD9331, and pemetrexed, have shown promise in treating a variety of cancers (Cocconi et al., 1998; Rusthoven et al., 1999; Goh et al., 2001). Even after 50 years of use, questions remain regarding methotrexate's nephrotoxicity and renal elimination. Some studies have shown that methotrexate is secreted by the kidney (Monjanel et al., 1979), and other studies have shown methotrexate to be reabsorbed (Huffman et al., 1972; Calvert et al., 1977). Studies of the novel antifolate ZD9331 showed that

ZD9331 exhibits saturable renal reabsorption (Goh et al., 2001; Sawyer et al., 2003). In contrast to the antifolates, renal handling of folic acid has been well described. Although antifolates and folate share a common pathway of renal elimination, the role of renal folate receptors (FRs) has yet to be studied with respect to the antifolates.

Early studies showed that folic acid undergoes saturable renal reabsorption. Goresky et al. (1963) showed that as the plasma levels of folic acid increase, the renal clearance of folic acid also increases. Studies of renal reabsorption of folic acid have identified the presence of tight-binding proteins, the FRs, in renal proximal tubule brush-border membranes (Selhub and Rosenberg, 1978; Corrocher et al., 1985). Renal clearance of folate derivatives was inversely related to the affinity of FRs for folates (McMartin et al., 1981), suggesting involvement of FRs in renal reabsorption of folates by proximal tubules. Data from primary cultures of human proximal tubule cells (Morshed et al., 1997) demonstrated involvement of both FRs and reduced folate carriers (RFCs) in apical

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ABBREVIATIONS: ZD9331, (2S)-2-[o-fluoro-*p*-[*N*-(2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-ylmethyl)-*N*-(prop-2-ynyl)amino]benzamido]-4-(tetrazol-5-yl) butyric acid; CB3717, 10-propargyl-5,8-dideazafolic acid; BBMVs, brush-border membrane vesicle; ΔG^0 , Gibbs free energy; FR, folate receptor; RFC, reduced folate carrier; GPI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol phospholipase C; 5-CH₃-THF, 5-methyl tetrahydrofolate.

uptake of folates, whereas basolateral-mediated uptake was primarily by RFCs. Folate reabsorption was shown to be pH-dependent with a marked reduction at alkaline pH (McMartin et al., 1992).

The ability of human kidney to reabsorb antifolates via the FRs has not been studied, and the effect of changes of pH on antifolate binding to kidney FRs is unknown. We therefore undertook studies of the interactions of folates and antifolates with FRs on human kidney brush-border membranes to better understand their role in renal handling of antifolates. We hypothesized that antifolates would bind to brush-border membrane vesicles and that this binding would be consistent with involvement of the FRs in their reabsorption. In contrast to the rest of the human body, the renal tubular fluid is acidic, with an average pH of 6.0. To approximate normal renal conditions, our studies of antifolate binding to brush-border membrane vesicles were carried out at pH 6.0. We expected that the FRs of the proximal tubule would exhibit the highest affinity for the most nephrotoxic antifolate (i.e., CB3717) and the least affinity for the least nephrotoxic antifolate (i.e., methotrexate).

Materials and Methods

Materials. [^3H]Folic acid (250 $\mu\text{Ci}/\text{mmol}$ at 98% purity) was purchased from Moravsek Biochemicals (Brea, CA) and was used without further purification. Protein was determined with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO) and were of analytical grade. GF/B filters were obtained from Fisher Scientific Canada (Nepean, ON, Canada). Ecolite was purchased from MP Biomedicals (Irvine, CA). The antifolates raltitrexed and ZD9331 were kindly provided by AstraZeneca Pharmaceuticals LP (Wilmington, DE). CB3717 was a gift from Dr. A. Jackman (Institute of Cancer Research, Sutton, Surrey, England).

Tissue Source. Normal human kidney cortex from tumor-free regions was obtained from patients with renal cell cancer after nephrectomy. The outer capsule, fat, and medulla were removed; tissue was cut into small pieces, washed twice with ice-cold solution of phosphate buffer solution to remove blood, and snap-frozen in liquid nitrogen for storage at -80°C until use.

Isolation of Brush-Border Membrane Vesicles. Brush-border membrane vesicles (BBMVs) from human kidney cortex were prepared as described earlier (Booth and Kenny, 1974). The final pellet of each preparation of BBMVs was resuspended in an appropriate volume of 300 mM mannitol and 5 mM Tris-HCl buffer, pH 7.4, and passed through a fine needle to produce uniform BBMVs. All procedures were carried out at 4°C . Alkaline phosphatase (EC 3.1.3.1) activity was monitored using the Sigma alkaline phosphatase activity kit as an indicator of enrichment of brush-border membranes, and protein content was determined with the Bio-Rad protein assay kit. Alkaline phosphatase activity was enriched 8- to 12-fold with respect to starting homogenates. BBMVs were snap-frozen in liquid nitrogen and stored at -80°C until their use for experiments (10–80 μg per assay). Initial studies compared experimental results obtained with BBMVs from a single source (patient) versus pooled sources (i.e., from different patients), and similar results were obtained. The experiments reported below were conducted using BBMVs prepared from pooled sources.

Determination of pH Optimum for Folic Acid Binding. Binding of [^3H]folic acid to kidney BBMVs was assessed using a filtration assay described previously for the collection of radiolabeled membrane vesicles (Agbanyo et al., 1988). The pH optimum for binding of folic acid was determined at room temperature by incubating BBMVs for 45 min in triplicate (20–30 μg protein per assay) in binding buffer (1.0 ml) consisting of 200 mM mannitol, 20 mM

phosphate-citrate buffers (used to enable studies over a wide pH range with a single buffer) at pH values of 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0 that contained 10 nM [^3H]folic acid in the presence or absence of 10 μM nonradioactive folic acid. At the end of incubations, ice-cold binding buffer of the appropriate pH was added to stop binding reactions, and the resulting mixtures were immediately filtered through Whatman GF/B filters (Whatman, Springfield Mill, KY) under vacuum. Filters were washed twice with 3 ml each of ice-cold binding buffer to remove unbound [^3H]folic acid, and radioactivity was measured by scintillation counting. Specifically bound [^3H]folic acid was calculated as the difference between the amount of total [^3H]folic acid bound in the absence of 10 μM folic acid and the amount that bound in its presence.

Determination of Dissociation Constants for Folic Acid Binding. K_d values for folic acid binding were calculated from equilibrium binding data that were subjected to mass law analysis. BBMVs (20–30 μg protein per assay) were incubated in duplicate at room temperature for 45 min with graded concentrations (0.12–12 nM) of [^3H]folic acid in the presence or absence of excess (10 μM) unlabeled folic acid in 40 mM phosphate buffer at pH 6.0, 7.0, or 8.0 containing 150 mM NaCl. This buffer is used for all of the experiments reported in this article (except the pH optimum experiment) because it approximates the conditions seen in lumen of proximal tubules of human kidney. At the end of the incubations, BBMVs were collected by filtration, washed with ice-cold buffer, and the filter-bound [^3H]folic acid was quantified by scintillation counting. Specifically bound folic acid was determined as described above.

Involvement of FRs in Binding. To determine whether glycosyl-phosphatidylinositol (GPI)-anchored FRs were involved in the binding of folic acid to BBMVs from human kidney cortex, BBMVs (80 μg per tube) were treated at 37°C at pH 6.0 for 30 min with increasing concentrations (0–0.2 units/ml) of phosphatidylinositol phospholipase C (PI-PLC), (1-phosphatidyl-D-myo-inositol phosphohydrolase, cyclic-phosphate forming; EC 3.1.4.10; Sigma-Aldrich). After treatment with PI-PLC, the BBMVs (20 μg per assay) were tested for binding with 15 nM [^3H]folic acid at pH 6.0. As controls, BBMVs were incubated under identical conditions in the presence (nonspecific) or absence (total) of excess 10 μM unlabeled folic acid but without PI-PLC.

Determination of IC_{50} Values for Inhibition of Binding of [^3H]Folic Acid by Folates and Antifolates. Inhibition of [^3H]folic acid binding to BBMVs by folates and antifolates was investigated in 40 mM phosphate buffer at pH 6.0, 7.0, or 8.0 containing 150 mM NaCl (a more physiological buffer), and incubations of BBMVs with 1 nM [^3H]folic acid were carried out in the absence or the presence of graded concentrations of the test analogs. The resulting concentration-effect curves were analyzed by nonlinear regression using GraphPad Prism software version 3.0 (GraphPad Software Inc., San Diego, CA) to obtain IC_{50} values for compounds that inhibited binding of [^3H]folic acid. K_i values were calculated using the Cheng and Prusoff equation (Cheng and Prusoff, 1973). Thermodynamic stability of the receptor-ligand complexes was estimated from ΔG° as described elsewhere (de Koning and Jarvis, 2001).

Results

Effects of pH on [^3H]Folic Acid Association with BBMVs. Because variations in pH are common in renal proximal tubules, we investigated the effects of pH on folic acid binding. Binding of 10 nM [^3H]folic acid to human kidney cortical BBMVs was measured in the presence or absence of 10 μM excess unlabeled folic acid at different pH values (Fig. 1). There was a clear dependence of specifically bound folic acid on pH, with maximum binding observed at pH 6.0. The observed binding was 3-fold higher at pH 6.0 than at 7.0.

Relationship between [^3H]Folic Acid Binding and BBMV Abundance. To evaluate dependence of folic acid

binding on the quantity of BBMVs, binding assays were conducted at pH 6.0 with graded quantities of BBMVs (10–80 μg protein per assay). BBMVs were incubated with 10 nM of [^3H]folic acid in the presence or absence of excess (10 μM) unlabeled folic acid (Fig. 2). Binding was linear up to 80 μg of protein, indicating that the concentrations of folic acid used in the binding assays were not limiting. For all subsequent experiments, the amount of protein in BBMVs was kept within 10 to 80 μg per assay.

Effects of Osmolarity on Binding of [^3H]Folic Acid to BBMVs. The higher binding of [^3H]folic acid to human kidney cortical BBMVs seen at pH 6.0 could have resulted if there was higher uptake of [^3H]folic acid into vesicles at pH 6.0 than at either pH 7.0 or 8.0. A time-course experiment conducted at pH 6.0 and 325 mOsm showed that binding of [^3H]folic acid to BBMVs plateaus after approximately 30 min, indicating equilibration with the binding sites (Fig. 3A). To distinguish FR binding from intravesicular uptake, the effects of varying osmolarity of the assay medium on association of [^3H]folic acid with BBMVs at pH 6.0 were examined. Osmotic swelling or shrinkage of membrane vesicles caused by lower or higher external osmolarity, respectively, will change the intravesicular volume. BBMVs were first incubated for 30 min with binding buffer, pH 6.0, at different osmolarities, followed by the addition of 15 nM [^3H]folic acid, after which the mixtures were incubated for an additional 45 min. The binding activity observed (Fig. 3B) was independent of external osmotic pressure, indicating that vesicle-associated folic acid was a measure of binding to vesicles rather than accumulation in the intravesicular space.

Equilibrium Binding of Folic Acid to FRs as a Function of pH. Although many folates exist in different protonated states at different pH values, changes in pH could also change the charge on the amino acid residues of FRs, thereby altering the binding of folates and antifolates to BBMVs. To better understand the effects of pH on binding of folic acid to BBMVs, equilibrium binding of folic acid was determined at different pH values. BBMVs were incubated with [^3H]folic acid for a minimum of 45 min to ensure that equilibrium between free and bound ligand was reached. In the experiment shown in Fig. 4, BBMVs were subjected to analysis of

[^3H]folic acid binding at graded concentrations of [^3H]folic acid at pH 6.0, 7.0, and 8.0 to quantify changes in the number of binding sites and the relative affinities of FRs for [^3H]folic acid. Binding of [^3H]folic acid to BBMVs was saturable at all

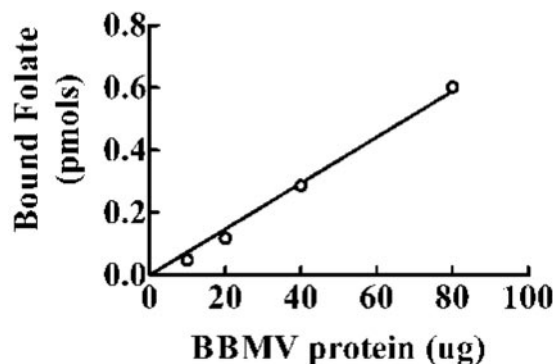


Fig. 2. Influence of abundance of human kidney BBMVs on binding of [^3H]folate. The binding of 10 nM [^3H]folate to BBMVs (10–80 μg) at pH 6.0 was measured as described under *Materials and Methods*. Specifically bound folate (as defined in Fig. 1) was plotted as a function of BBMVs protein (in micrograms) concentration. The standard deviation at each of the data points is indicated ($n = 3$), and where S.D. values are not shown, values were smaller than the symbols.

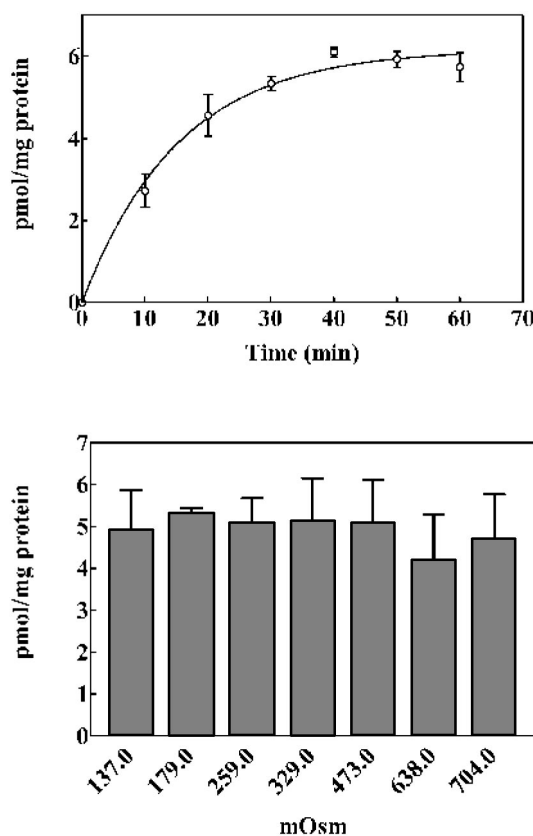


Fig. 3. Effects of osmolarity on association of [^3H]folate with human kidney BBMVs. BBMVs were incubated with 40 mM phosphate buffer containing increasing amounts of mannitol at pH 6.0 for 30 min at room temperature. Binding was initiated by the addition of 15 nM [^3H]folate in the appropriate buffers to BBMVs suspensions, and the mixtures were further incubated at room temperature for 45 min. Top, the time course of binding of [^3H]folate to BBMVs at 325 mOsm. Bottom, specifically bound folate (obtained as described in Fig. 1) was plotted as a function of medium osmolarity (mOsm). Means and standard deviations were derived from assay triplicates and are representative of two independent experiments.

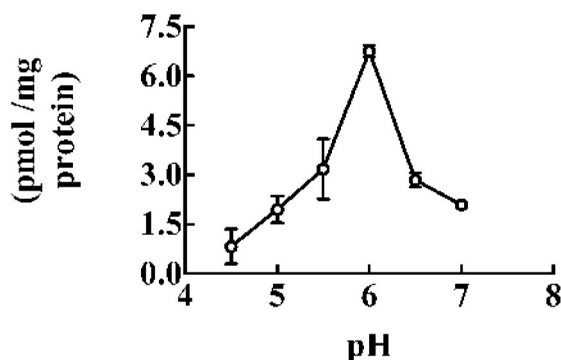


Fig. 1. Effects of pH on association of [^3H]folate to human kidney BBMVs. The effects of different pH values on binding of folate to BBMVs were investigated as described under *Materials and Methods*. Binding of 10 nM [^3H]folate to BBMVs was measured in the presence or absence of 10 μM unlabeled folate in 20 mM phosphate-citrate buffer containing 200 mM mannitol at the pH values indicated. Specific binding (defined as total binding minus the nonspecific binding) was plotted as a function of pH. The results are representative of two independent experiments, and the standard deviation at each of the data points is indicated ($n = 3$).

three pH values tested (A) and, because the Scatchard plots were linear (B), a one-site binding model was used to estimate B_{\max} and K_d values for binding at different pH values (Table 1). B_{\max} values were highest at pH 6.0, and K_d values were ≤ 1 nM at all three pH values tested.

Involvement of FRs in Binding of [3 H]Folic Acid to BBMVs. The C termini of FRs are anchored to membranes via GPI linkers, and treatment of BBMVs with PI-PLC, which is known to cleave GPI linker arms and free membrane-bound receptors (Verma et al., 1992), provides a diagnostic assay for the involvement of FRs in folate binding. The experiments shown in Fig. 5 investigated the effects of PI-PLC on binding of [3 H]folic acid to BBMVs. At the highest concentration of PI-PLC tested, specific binding decreased to almost zero relative to that observed with untreated BBMVs samples. These results indicated that binding of [3 H]folic acid to human kidney cortical BBMVs was caused by GPI-anchored proteins, most likely FRs.

Interaction of FRs with Folate Analogs and Antifolates. The ability of various folate and antifolate derivatives to interact with FRs was assessed by evaluating the inhibition of binding of 1 nM [3 H]folic acid to BBMVs in the presence of increasing concentrations of folates and antifolates.

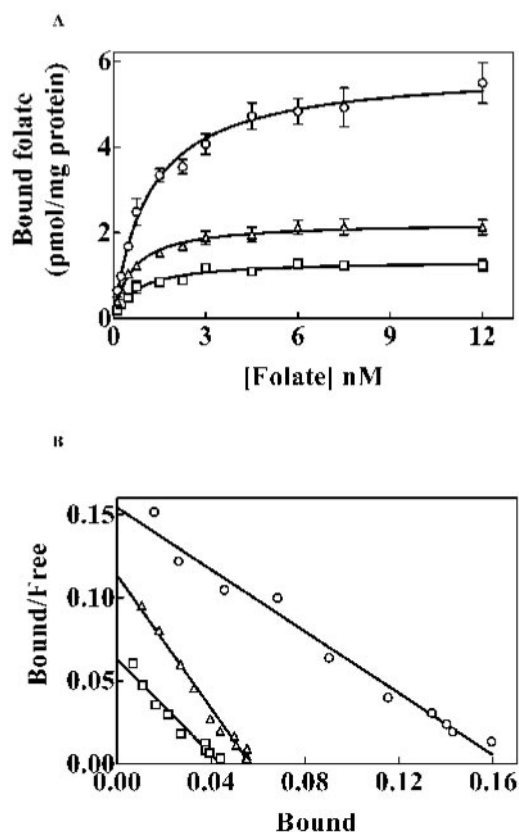


Fig. 4. Equilibrium binding of [3 H]folate to human kidney BBMVs. Binding of graded concentrations (0.12–12 nM) of [3 H]folate to human kidney BBMVs at pH 6.0 (\circ), 7.0 (Δ), and 8.0 (\square) was measured as described under *Materials and Methods*. A, specific binding (obtained as described in Fig. 1) plotted as a function of free folate concentrations at equilibrium. B, the mass law analysis (Scatchard plot) of relationships between specific binding of [3 H]folate and the equilibrium concentrations of free [3 H]folate. The K_d and B_{\max} values were calculated by nonlinear regression analysis and are summarized in Table 1. Each data point (mean \pm S.E.) was derived from three independent experiments, and where S.D. values are not shown, values were smaller than the symbols.

Concentration-effect (IC_{50}) experiments were conducted at pH 6.0, 7.0, and 8.0 to examine the effects of changes in pH on site-specific binding because pH-induced changes in protonation states of folate derivatives and/or amino acid residues of the FRs may influence receptor-ligand interactions. In all cases, Hill coefficients were close to -1 , consistent with competitive inhibition of binding of folic acid by the test compounds. IC_{50} values obtained from concentration-effect relationships yielded K_i values, which provided an indication of the affinity of the FRs for the analogs tested. Figure 6A summarizes results from experiments with folate analogs at pH 6.0. Compared with folic acid itself, the concentrations required to displace bound [3 H]folic acid with folinic acid and 5-methyl tetrahydrofolate (5- CH_3 -THF) were considerably higher, indicating lower receptor affinities for these analogs. Similar experiments at pH 6.0 with several antifolates (Fig. 6B) demonstrated differences ($CB3717 < ZD9331 <$ raltitrexed) in IC_{50} values for inhibition of binding of [3 H]folic acid to FRs.

A summary of K_i and ΔG^0 values for folates and antifolates tested at pH 6.0, 7.0, and 8.0 is given in Table 2. Among the folates tested, 5- CH_3 -THF showed significant differences in the K_i value at pH 6.0 (62.4 nM) compared with those obtained at pH 7.0 and 8.0 (7.6 and 3.8 nM, respectively). Among the antifolates tested, ZD9331 and raltitrexed showed marked differences in their IC_{50} values at different pH values, whereas the pH-dependence of aminopterin and CB3717 was less significant. CB3717 exhibited the lowest K_i value of all the compounds tested.

TABLE 1

B_{\max} and K_d values for binding of [3 H]folate to human BBMVs. Values, presented as mean \pm S.E. of three independent experiments, were calculated by non-linear regression analysis of the data from Fig. 4.

pH	B_{\max} pmol/mg protein	K_d nM
6.0	5.9 ± 0.12	1.1 ± 0.102
7.0	2.2 ± 0.04	0.60 ± 0.04
8.0	1.2 ± 0.03	0.76 ± 0.071

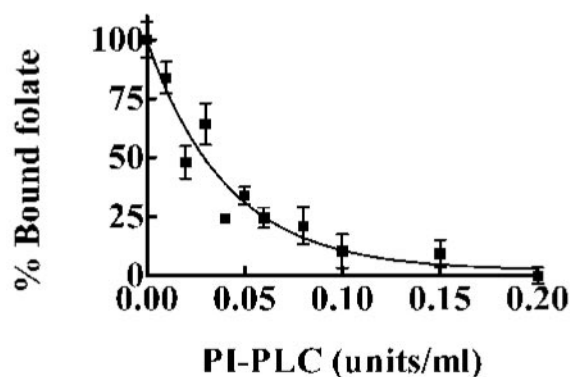


Fig. 5. Effects of PI-PLC on binding of [3 H]folate to human kidney BBMVs. Binding of [3 H]folate to BBMVs treated with the enzyme PI-PLC was measured at pH 6.0, as described under *Materials and Methods*. Values expressed as the percentage of folate bound are plotted as a function of increasing concentrations (units) of PI-PLC. Data points (mean and standard deviations) are means (\pm S.D.) of triplicate assays and are representative of two independent experiments.

Discussion

The kidney has a major role in folate homeostasis. Goresky et al. (1963) first showed that folate had nonlinear pharmacokinetics caused by active renal reabsorption. Subsequently Selhub and Rosenberg (1978) showed that kidney proximal tubules possess high-affinity binding proteins for folate on

their luminal borders. Ross et al. (1994) showed that these proteins were the α FRs. Human kidney cells transport folate from apical to basolateral surfaces (McMartin et al., 1992), and transport is pH-dependent, with a maximum at 6.0.

The antifolates, like natural folates, are eliminated via the kidney (Azarnoff et al., 1974; Sessa et al., 1988; Beale et al., 1998; Rinaldi et al., 1999). Despite the dominant role of FRs in renal elimination of natural folates, their involvement in the renal elimination of antifolates has not been studied. A new antifolate, ZD9331, which cannot be polyglutamated (Jackman et al., 1994), has raised questions about renal elimination of antifolates (Sawyer et al., 2000; Goh et al., 2001), because ZD9331 had nonlinear pharmacokinetics that seemed to be caused by saturable renal reabsorption.

The ZD9331 studies led to the hypothesis that antifolates are being reabsorbed by the FRs that reabsorb natural folates. If renal reabsorption of antifolates is involved in antifolate nephrotoxicity, the hypothesis predicts that affinity of FRs for antifolates will correlate with that tendency of antifolates to cause nephrotoxicity. Our initial experiments were undertaken to optimize conditions for binding of folic acid to BBMVs prepared from human kidney cortex. Binding of folic acid to BBMVs was maximal at an acidic pH, and changes in medium osmolarity indicated that the observed association of folic acid with BBMVs was caused by specific binding and not by accumulation mediated by a transport process. Similar results were reported in human cultured proximal tubule cells (McMartin et al., 1992). We demonstrated specific and saturable binding of [3 H]folic acid to BBMVs with K_d and B_{max} values, respectively, of 1.1 ± 0.102 nM and 5.9 ± 0.12 pmol/mg protein. B_{max} values for interaction of folic acid with BBMVs decreased as the pH of the incubation medium was increased from 6.0 to 8.0. The very low K_d value was consistent with folic acid binding to FRs and not to RFCs. Physiologically, a pH-dependent decrease in B_{max} value could result in lowered reabsorption of folates and antifolates during urine alkalinization. Using PI-PLC, we demonstrated that the binding of folic acid was caused by proteins anchored to plasma membranes via GPI linkers, which further supports the idea that binding was to FRs and not RFCs.

Having optimized conditions for binding of folic acid to BBMVs, we examined binding of folates and antifolates to BBMVs in concentration-effect experiments that assessed their abilities to inhibit binding of [3 H]folic acid. K_i values

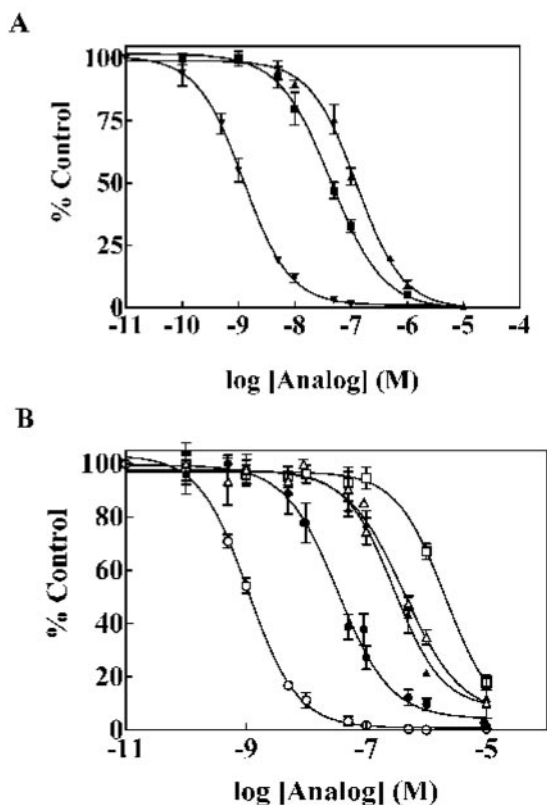


Fig. 6. Inhibition of site-specific binding of [3 H]folate to BBMVs of human kidney by folates and antifolates. The BBMVs were incubated with 1 nM [3 H]folate in pH 6.0 buffer with increasing concentrations of folates (A) folate (●), folinic acid (■), and 5-CH₃-THF (▲) or with antifolates (B) ZD1694 (△), ZD9331 (●), CB3717 (○), aminopterin (▲), and methotrexate (□). Specifically bound folate (calculated as total binding minus the nonspecific binding) was plotted as a function of log[test compound] concentration (molar concentration). Data points (mean and standard deviations) indicated are derived from three independent experiments, and where S.D. values are not shown, values were smaller than the symbols. The calculated IC₅₀ values (mean \pm S.E.) at pH 6.0, 7.0, and 8.0 buffers are summarized in Table 2.

TABLE 2

K_i and Gibbs free-energy values for inhibition of binding of [3 H]folate to BBMVs by folates and antifolates

IC₅₀ values (defined as the concentrations of test compounds that inhibited 50% of specific binding of [3 H]folate) were determined in concentration-effect experiments as described in the legend to Fig. 6. IC₅₀ values (mean \pm S.E.) at pH 6.0 to 8.0 were converted to K_i values using the Cheng-Prusoff equation. ΔG^0 values were calculated from $\Delta G^0 = -RT \ln(K_i)$ and are shown for each of the compounds tested.

	pH 6.0		pH 7.0		pH 8.0	
	K_i	ΔG^0	K_i	ΔG^0	K_i	ΔG^0
	nM	kJ/mol	nM	kJ/mol	nM	kJ/mol
Folates						
Folic acid	0.609	52.6	0.44	53.4	0.65	52.5
Folinic acid	22.2	43.7	7.9	46.3	15.3	44.6
5-CH ₃ -THF	62.4	41.1	7.6	46.3	3.8	48.1
Antifolates						
CB3717	0.53	52.9	0.28	54.6	0.31	54.2
ZD9331	16.5	44.4	2	49.7	5.2	47.3
ZD1694	198	38.3	29	43	21.4	43.8
Aminopterin	204	38.2	109	39.7	180	38.5
Methotrexate	1290	33.6	361	36.8	447.5	36.3

generated from IC_{50} values calculated from competitive binding experiments provided a measure of affinity of FRs for folates and antifolates. A systematic examination of binding of folates and antifolates to kidney BBMVs demonstrated binding to FRs on BBMVs. These results suggest that FRs may have a role in the renal elimination of antifolates and that kidney cells have the potential to reabsorb antifolates. Results of studies of renal elimination of antifolates are contradictory. In one study (Monjanel et al., 1979), 12 patients treated with methotrexate were hydrated and their urine alkalinized. Renal elimination of methotrexate exceeded creatinine clearance, suggesting renal secretion. In contrast, Calvert et al. (1977) studied renal elimination of methotrexate in 18 patients and found that renal clearance of methotrexate was substantially less than creatinine clearance, suggesting renal reabsorption of methotrexate. Likewise, Huffman et al. (1972), who studied renal elimination of methotrexate in 22 patients, found that methotrexate clearance was less than creatinine clearance, suggesting renal reabsorption. In the studies that showed methotrexate secretion, patients were hydrated and had high urine-flow rates, whereas in the studies that showed methotrexate reabsorption, patients were not hydrated. Active reabsorption by FRs could explain the contradiction between the studies. In hydrated patients, urine flow rates would be too high to allow FRs in the proximal tubules time to reabsorb methotrexate, whereas in nonhydrated patients, urine flow rates would be low enough to allow methotrexate reabsorption.

In addition to urine flow rates, changes in proximal tubular pH (e.g., as a result of hydration versus no hydration), as well as variable folate levels (which in turn compete for the binding sites on FRs), could explain the contradictions in literature on methotrexate clearance.

There were substantial effects of pH on FR affinities for various folates and antifolates, as judged from their K_i values. Interaction of 5- CH_3 -THF with FRs showed significant pH-dependent alterations in K_i and ΔG^0 values. K_i values differed by more than 15-fold between pH 6.0 and 8.0. The free-energy ΔG^0 values for the interaction of 5- CH_3 -THF with the FRs increased from 41.1 kJ/mol at pH 6.0 to 48.1 kJ/mol at pH 8.0. The increase in free energy could have resulted from formation of new hydrogen bonds at pH 8.0 or increased van der Waals interactions with the FRs. Folic acid exhibited a ΔG^0 value of 52.6 kJ/mol, compared with 33.6, 38.2, 38.3, 44.4, and 52.9 kJ/mol, respectively, for methotrexate, aminopterin, raltitrexed, ZD9331, and CB3717. Raltitrexed and ZD9331 demonstrated significant pH-dependence with ZD9331 going from a K_i value of 16.5 ± 2.9 nM at pH 6.0 to 5.2 ± 0.35 nM at pH 8.0, and raltitrexed from a K_i value of 198 ± 48 nM at pH 6.0 to 21.4 ± 2.9 nM at a pH 8.0. Changes in methotrexate K_i values were much smaller, and CB3717 K_i values did not change over the pH range tested. We hypothesize that some antifolates have variation in their K_i value with pH because of the presence of pH-sensitive groups on some antifolates. We speculate that the antifolates that exhibited increased binding to FRs with alkalinization have pH-sensitive groups that either become protonated at low pH and are repulsed out of the binding pocket or become deprotonated at high pH and have enhanced ability to interact with the binding pocket of FRs.

Methotrexate is widely used, and its nephrotoxicity is uncommon with current hydration and alkalinization strate-

gies. In contrast, CB3717, despite showing promising antitumor activity, was abandoned because of its unpredictable and frequent nephrotoxicity. We predicted that FRs in the kidney would have the highest and lowest affinities, respectively, for CB3717 and methotrexate of the antifolates tested. We found that the K_i value for methotrexate at pH 6.0 was 1290 nM, whereas the K_i values for CB3717 and folic acid at pH 6.0 were 0.53 and 0.61 nM, respectively. The K_i value for CB3717 was lower than those of any of the naturally occurring folates for FRs in kidney.

The pharmacology of aminopterin raises questions about decreased solubility and precipitation as a cause of antifolate nephrotoxicity. Aminopterin is more soluble than methotrexate in urine at 37°C and 10-fold more potent than methotrexate in inhibiting dihydrofolate reductase. On the basis of the precipitation theory of antifolate nephrotoxicity, aminopterin should be less toxic than methotrexate. Although Glode et al. (1979) predicted that aminopterin would be more efficacious and less nephrotoxic than methotrexate, they found that 50% of patients who received aminopterin without hydration developed dose-limiting nephrotoxicity. Two patients who died from renal failure lacked aminopterin precipitates in the kidney at autopsy, and these authors suggested that antifolates may cause nephrotoxicity independent of renal precipitation. In contrast, our theory of renal antifolate elimination predicts that aminopterin would be more nephrotoxic than methotrexate. Renal FRs had higher affinities for aminopterin than for methotrexate at every pH tested. Although hydration and alkalinization decreased aminopterin toxicity (Glode et al., 1979), the efficacy of hydration alone in decreasing nephrotoxicity was not tested.

Our data on binding of methotrexate to FRs suggest that alkalinization of urine would increase reabsorption of methotrexate if it were not for the increased tubular fluid flow rates associated with hydration. No study has studied the effects of hydration and folic acid supplementation without alkalinizing the urine. Urine alkalinization may have an additional beneficial effect in that alkalinization would improve the solubility of methotrexate and thus prevents its precipitation in renal tubules.

In conclusion, we report pH-dependent interaction of folates and antifolates with FRs on brush-border membranes of human kidney proximal tubule cells. The pH-dependent changes in binding affinities and binding-site abundance could contribute to the tendency of antifolates to cause nephrotoxicity. For some antifolates, changes in pH have large effects on binding to FRs which, we hypothesize, are caused by changing charges on the FRs and/or antifolates. The reabsorption of antifolates may explain the unexpected delayed elimination of antifolates seen in some patients. Our results, which showed that FRs exhibit high affinities for many antifolates, support the hypothesis that antifolates are reabsorbed by the same process that conserves folic acid in the kidney. We are now studying the interaction of antifolates with FRs of primary cultures of human renal proximal tubule cells.

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