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Carrier- and Receptor-Mediated Transport of Folate Antagonists Targeting Folate-Dependent Enzymes: Correlates of Molecular-Structure and Biological Activity

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SUMMARY

The transport properties and growth-inhibitory potential of 37 classic and novel antifolate compounds have been tested *in vitro* against human and murine cell lines expressing different levels of the reduced folate carrier (RFC), the membrane-associated folate binding protein (mFBP), or both. The intracellular targets of these drugs were dihydrofolate reductase (DHFR), glycinamide ribonucleotide transformylase (GARTF), folylpolyglutamate synthetase (FPGS), and thymidylate synthase (TS). Parameters that were investigated included the affinity of both folate-transport systems for the antifolate drugs, their growth-inhibitory potential as a function of cellular RFC/mFBP expression, and the protective effect of either FA or leucovorin against growth inhibition. Methotrexate, aminopterin, N^{10}-propargyl-5,8-dideazafolic acid (CB3717), ZD1694, 5,8-dideazaisofolic acid (IAHQ), 5,10-dideazatetrahydrofolic acid (DDATHF), and 5-deazafolic acid (efficient substrate for FPGS) were used as the basic structures in the present study, from which modifications were introduced in the pteridine/quinazoline ring, the C^9-N^{10} bridge, the benzoyl ring, and the glutamate side chain. It was observed that RFC exhibited an efficient substrate affinity for all analogues except CB3717, 2-NH_2-ZD1694, and glutamate side-chain-modified FPGS inhibitors. Substitutions at the 2-position (e.g., 2-CH_3) improved the RFC substrate affinity for methotrexate and aminopterin. Other good substrates included PT523 (N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine), 10-ethyl-10-deazaaminopterin, and DDATHF. With respect to mFBP, modifications at the N-3 and 4-oxo positions resulted in a substantial loss of binding affinity. Modifications at other sites of the molecule were well tolerated. Growth-inhibition studies identified a series of drugs that were preferentially transported via RFC (2,4-diamino structures) or mFBP (CB3717, 2-NH_2-ZD1694, or 5,8-dideazaisofolic acid), whereas other drugs were efficiently transported via both transport pathways (e.g., DDATHF, ZD1694, BW1843U89, or LY231514). Given the fact that for an increasing number of normal and neoplastic cells and tissues, different expression levels of RFC and mFBP are being recognized, this folate antagonist structure-activity relationship can be of value for predicting drug sensitivity and resistance of tumor cells or drug-related toxicity to normal cells and for the rational design and development of novel antifolates.

For more than 40 years, the folate antagonist MTX has had an established role in cancer chemotherapy, both as a single agent and in combination regimens (1). Knowledge of the factors that contribute to the preclinical and clinical activity of MTX, i.e., membrane transport, intracellular retention, and inhibition of the target enzyme DHFR, has provided a solid basis for the design and synthesis of novel antifolates that are either transported more efficiently, have a prolonged

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ABBREVIATIONS: RFC, reduced folate/methotrexate carrier; mFBP, membrane-associated folate binding protein; FCS, fetal calf serum; HBSS, HEPES balanced salt solution; LV, L-leucovorin (L-5-formyltetrahydrofolate); 5-CH_3-THF, 5-methyltetrahydrofolate; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; GARTF, glycinamide ribonucleotide transformylase; TS, thymidylate synthase; FA, folic acid; PteGlu, pteroyl glutamate (FA); MTX, methotrexate; 2-dMTX, 2-desamino-MTX; 2-CH_3-MTX, 2-desamino-2-methyl-MTX; AMT, aminopterin; 2-dAMT, 2-desamino-AMT; 2-CH_3-AMT, 2-desamino-2-methyl-AMT; 10-EdAM, 10-ethyl-10-deazaaminopterin; PT523, N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-(hemiphthaloyl)-L-ornithine; DDATHF, 5,10-dideaza-5,6,7,8-tetrahydrofolic acid; 5-d(i)H_4PteGlu, 5-deaza-5,6,7,8-tetrahydroisofolic acid; N^9-CH_3-5-d(i)H_4PteGlu, N^9-methyl-5-deaza-5,6,7,8-tetrahydroisofolic acid; 5-dPteHCysA, N^{α}-(5-deazapteroyl)-L-homocysteic acid; 5-dPteAPBA, N^{α}-(5-deazapteroyl)-DL-2-amino-4-phosphonobutanoic acid; 5-dPteOrn, N^{α}-(5-deazapteroyl)-L-ornithine; 5-dH_4PteHCysA, N^{α}-(5-deaza-5,6,7,8-tetrahydropteroyl)-L-homocysteic acid; 5-dH_4PteAPBA, N^{α}-(5-deaza-5,6,7,8-tetrahydropteroyl)-DL-2-amino-4-phosphobutanoic acid; 5-dH_4PteOrn, N^{α}-(5-deaza-5,6,7,8-tetrahydropteroyl)-L-ornithine; CB3717, N^{10}-propargyl-5,8-dideazafolic acid; ICI-198,583, 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid; 4-H-ICI-198,583, 4-deoxy-ICI-198,583; 4-OCH_3-ICI-198,583, 4-methoxy-ICI-198,583; Glu→Val-ICI-198,583, valine-ICI-198,583; Glu→Sub-ICI-198,583, 2-amino-suberate-ICI-198,583; 7-CH_3-ICI-198,583, 7-methyl-ICI-198,583; ZD1694, *N*-[5(*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-yl-methyl)amino)2-thenyl)]-L-glutamic acid; 2-NH_2-ZD1694, 2-amino-ZD1694; BW1843U89, (*S*)-2-[5-(((1,2-Dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-1-oxo-2-isoindolinyl]-glutaric acid; LY231514, *N*-(4-(2-(2-amino-4,7-dihydro-4-oxo-^{3}H-pyrrolo[2,3-D]pyrimidin-5-yl)ethyl)benzoyl]-L-glutamic acid; IAHQ, 5,8-dideazaisofolic acid; 2-d-IAHQ, 2-desamino-IAHQ; 2-CH_3-dIAHQ, 2-desamino-2-methyl-IAHQ; 5-d(i)PteGlu, 5-deazaisofolic acid; N^9-CH_3-5-d(i)PteGlu, N^9-methyl-5-deazaisofolic acid; N^9-CHO-5-d(i)PteGlu, N^9-formyl-5-deazaisofolic acid; AG337, 3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)quinazoline; AG377, 2,4-diamino-6-[*N*-(4-(phenylsulfonyl)benzyl)ethyl)amino]quinazoline.

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intracellular retention, or are more potent inhibitors of the target enzyme (2–13). Other than DHFR, key enzymes in folate metabolism, such as TS, GARTF, and FPGS, have been recognized as potential targets for folate-based chemotherapeutic agents (14–19).

Traditionally, the biological activity of novel antifolates is evaluated with *in vitro* model systems (usually leukemia cell lines) that express the RFC as the major transport route for natural reduced folate cofactors (e.g., LV and 5-CH_3-THF) and classical antifolate compounds (6, 20–22). Although the role of the RFC in antifolate transport is undisputed, an increasing number of reports suggest that at least one other folate transport protein, an mFBP, may have an additional role in antifolate uptake (23–28). A functional role of mFBP in folate uptake has been demonstrated in a number of *in vitro* (28–32) and *in vivo* (33) studies in which mFBP-mediated transport of natural reduced folate cofactors supported cellular growth at nanomolar extracellular concentrations. Other reports have demonstrated mFBP-mediated transport of antifolates, particularly if the mFBP exhibits a high binding affinity for these drugs (23, 34–36). Because the mFBPs appear to be expressed in a range of normal and neoplastic cells and tissues (37–42), further analysis of the potential role of this protein in antifolate drug uptake is warranted.

The RFC and mFBP are structurally and functionally unrelated proteins. The RFC is an integral membrane protein with a molecular weight ranging from 43–48 kDa in rodent cells (43, 44) to 80–120 kDa in human cells (42, 45, 46). The mFBP is a 38–40-kDa protein that is linked to the plasma membrane via a glycosylphosphatidylinositol anchor (47, 48). The mechanism of RFC-mediated folate and antifolate uptake has been the subject of extensive studies, which have demonstrated the uptake process is both temperature and energy dependent and able to be inhibited by structurally unrelated anions (6, 49–52). These observations support the hypothesis that an anion exchange mechanism may be involved in carrier-mediated antifolate uptake (6). At least two mechanisms have been described for mFBP-mediated folate uptake: one by the classic receptor-mediated endocytosis pathway (53) and the other, most extensively studied in monkey kidney MA-104 cells, via a novel mechanism called potocytosis. Potocytosis (27) comprises a series of events that involve clustering of folate-bound mFBP molecules in membrane invaginations (caveolae), followed by transient closure of caveolae from the extracellular medium and acidification of their lumen, after which the folate molecule is dissociated from the mFBP and translocated across the plasma membrane via a specific carrier protein (27, 54). Whether the latter protein is the RFC is not clear.

The kinetic properties of the RFC and mFBP for transport also differ significantly. The RFC exhibits a high affinity (K_m, 1–10 μM) for natural reduced folate cofactors and antifolates such as MTX (6), which can be internalized at a maximal rate of 10–20 molecules per minute per transport molecule (20). A characteristic feature of the RFC is its poor affinity for FA (K_m, 200–400 μM), which is in contrast to that of mFBP. mFBP has a high affinity (K_d, 1–10 nM) for FA and reduced folate cofactors, which may favor uptake at a physiological folate concentration of 5–50 nM (26). On the other hand, the recycling rate of mFBP is much slower than that for the RFC: from ~30 min in MA104 cells (27, 55) to 5 hr in L1210 cells (56, 57).

We report on a comprehensive study of the functional aspects of both the RFC and mFBP in antifolate drug transport, which allowed us to directly compare the efficiency of these transport proteins. As models, three CCRF-CEM human leukemia cell lines were used, which are characterized by normal, upregulated, or defective RFC transport (22, 58). For comparison, three (variant) L1210 murine leukemia cells were included that express the RFC (50), coexpress the mFBP and the RFC (59), or lack functional RFC activity while retaining mFBP (60). In the present study, we used a series of folate-based inhibitors of DHFR (MTX and AMT) (61), GARTF (DDATHF and 5-d(i)H_4PteGlu) (15, 62), FPGS (glutamate side chain-modified 5-deazafolic, 5-deazatetrahydrofolic acid structures) (63), and TS (CB3717, ZD1694, and IAHQ) (64–66). These compounds and their analogues with structural alterations in the pteridine/quinazoline ring (substitutions at C-2, C-7, N-3, and C-4), *p*-aminobenzoate ring (thiophene for benzoyl), the C^9-N^{10} bridge, and the glutamate side chain (replacement by 2-aminosuberate, valine, and ornithine) were used in the study. All of these compounds were evaluated for changes in the substrate specificity for the RFC and mFBP; the growth-inhibitory effects against cell lines with different expression levels of the RFC, mFBP, or both; and the protective effect of LV, FA, or both.

The structure-activity relationships described in the present study for antifolate transport via the RFC, mFBP, or both may be of predictive value in assessing the preclinical and clinical activity of antifolate drugs and can also be used for the rational future design of new antifolates.

Experimental Procedures

Materials. RPMI 1640, with and without FA, and FCS, dialysed and nondialysed, were obtained from GIBCO (Grand Island, NY). FA and DL-5-CH_3-THF were purchased from Sigma Chemical Co. (St. Louis, MO). LV (L-stereoisomer) was a gift from Lederle (Pearl River, NY). [3′,5′,7,9-^{3}H]FA (35 Ci/mmol), [3′,5′,7-^{3}H]MTX (20 Ci/mmol), and [3,4-^{3}H]glutamic acid (56.6 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). [^{3}H]FA and [^{3}H]MTX were purified as described previously (20, 59, 60). All other chemicals were of the highest purity available.

Drugs. The chemical structures of the antifolate compounds described in the study are depicted in Fig. 1. The inhibitory potential of these compounds against their target enzyme, as well as their substrate affinity for FPGS, are described in Tables 1 (DHFR, GARTF, and FPGS inhibitors) and 2 (TS inhibitors). It should be noted that these data were taken from the indicated references and that the experimental conditions were not identical. Nevertheless, these data were included to explain possible growth-inhibitory effects on the basis of poor enzyme inhibition or substrate affinity for FPGS. Further details regarding chemical synthesis of the drugs are given in references as indicated in Tables 1 and 2.

DHFR inhibitors. MTX was a gift from Pharmachemie (Haarlem, The Netherlands). AMT was purchased from Sigma Chemical Co. 2-dMTX, 2-CH_3-MTX, 2-dAMT, and 2-CH_3-AMT were synthesized as previously described (61). 10-EdAM was a gift from CIBA-GEIGY (Basel, Switzerland). PT523 (67, 68) was a gift from Dr. W. T. McCulloch (Sparta Pharmaceuticals, Research Triangle Park, NC).

GARTF inhibitors. The synthesis of 5-d(i)H_4PteGlu and N^9-CH_3-5-d(i)H_4PteGlu has been described previously (62). DDATHF was a gift from the late Dr. G. B. Grindey (Lilly Research Labs., Indianapolis, IN).

FPGS inhibitors. The 5-deazafolate analogues 5-dPteHCysA, 5-dPteAPBA, 5-dPteOrn, 5-dH_4PteHCysA, 5-dH_4PteAPBA, and 5-dH_4PteOrn were synthesized by Rosowsky *et al.* (63).

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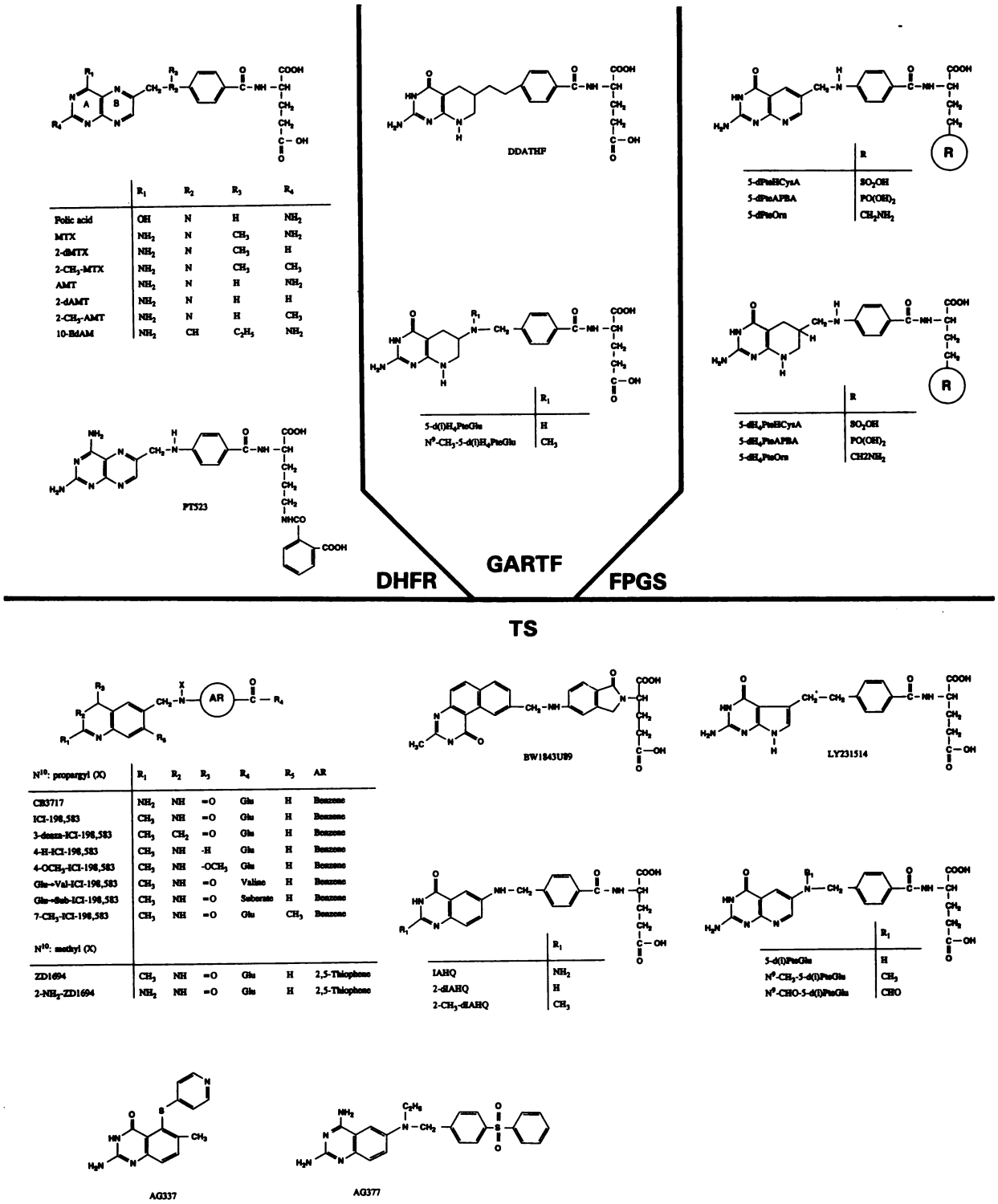

Fig. 1. Molecular structure of folate-based inhibitors of DHFR, GARTF, FPGS, and TS.

TS inhibitors. CB3717 (64, 69) and ICI-198,583 (70) served as basic structures for the following analogues: 3-deaza-ICI-198,583, 4-H-ICI-198,583, 4-OCH_3-ICI-198,583, Glu→Val-ICI-198,583 (71, 72), 7-CH_3-ICI-198,583 (73, 74), Glu→Sub-ICI-198,583 (72), ZD1694 (65), and 2-NH_2-ZD1694 (75, 76). The isofolic analogues IAHQ, 2-desamino-IAHQ, and 2-desamino-2-methyl-IAHQ and the 5-deazaisofolates 5-d(i)PteGlu, N^9-CH_3-5-d(i)PteGlu, and N^9-CHO-5d(i)PteGlu were synthesized as described previously (62, 66, 77, 78). LY231514 (79) was a gift from the late Dr. G. B. Grindey. BW1843U89 (80) was provided by R. Ferone (Burroughs Wellcome Co., Research Triangle Park, NC). AG337 (81, 82) and AG377 (83) were gifts from Agouron Pharmaceuticals, Inc. (San Diego, CA).

Cell culture. The cell lines used in the present study include RFC-expressing CCRF-CEM human leukemic lymphoblasts (20–22); a variant of this line, CEM/MTX, lacking functional RFC (58); and another variant (CEM-7A), characterized by a 30-fold overexpression of the RFC compared with CCRF-CEM cells (22). In addition, the following murine cell lines were used: wild-type RFC expressing

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TABLE 1

Characteristics of folate-based inhibitors of DHFR, GARTF, and FPGS with respect to enzyme inhibition and substrate activity for FPGS[a]

Compound	Main target	$K_{i_{app}}$			FPGS substrate activity[b]	Ref.
		DHFR	TS	GARTF		
			μM			
MTX	DHFR	0.024			+	61,110
2-dMTX		6			NR	61
2-CH_3-MTX		>20			NR	61
AMT		0.025			+++	61, 87
2-dAMT		19			NR	61
2-CH_3-AMT		>50	29	>10	NR	12, 61
10-EdAM		2.3 pM			+++	111
PT523		0.052			NS	68
DDATHF	GARTF	>100	>100	0.12	+++	112,113
5-d(i)H_4PteGlu		>1000	>100	5.3	++	62
N^9-CH_3-5-d(i)H_4PteGlu		>1000	>100	26	+++	62
					$K_{i_{app}}$ μM	
5-dPteHCysA	FPGS	0.4	55		570	63
5-dPteAPBA		4.7	300		9.0	63
5-dPteOrn		8.3			5.7	63
5-dH_4PteHCysA		12	2100	0.19	22	63
5-dH_4PteAPBA		220	4200	0.047	3.2	63
5-dH_4PteOrn				3.7	0.027	63

[a] The data in this table are obtained from the indicated references. Please note that $K_{i_{app}}$ determinations of enzyme inhibition and substrate activity determination for FPGS may not have been carried out under identical conditions with respect to substrate, inhibitor, and cofactor concentration. For experimental details, see the indicated references.

[b] FPGS substrate activity (K_m) is defined as follows: K_m 5–25 μM: (+++); K_m 25–100 μM: (++); K_m > 100 μM: (+). NR, not reported; NS, nonsubstrate.

TABLE 2

Characteristics of folate-based inhibitors of TS with respect to enzyme inhibition and substrate activity for FPGS[a]

Compound	Main target	$K_{i_{app}}$			FPGS substrate activity[b]	Ref.
		DHFR	TS	GARTF		
			μM			
MTX	DHFR	0.024			+	110
CB3717	TS	0.91	0.02		++	70, 110
ICI-198,583		5.5	0.046	>17	++	69, 70
3-deaza-ICI-198,583			6.3		+	72
4-H-ICI-198,583			7.9			114
4-OCH_3-ICI-198,583			6.0			114
Glu→Val-ICI-198,583			0.042			71
Glu→Sub-ICI-198,583			0.018			72
7-CH_3-ICI-198,583			0.028		NS	73
ZD1694		12	0.65		++++	65
2-NH_2-ZD1694			0.44			76
BW1843U89			0.00009		++++	80
LY231514			0.44		++++	79
IAHQ		0.11	1.3		+++	78
2-dIAHQ		0.32	25		+++	78
2-CH_3-IAHQ		3.1	17		+++	78
5-d(i)PteGlu		0.21	7.1	85.9	+	62
N^9-CH_3-5-d(i)PteGlu		0.25	0.48	>100	++	62
N^9-CHO-5-d(i)PteGlu		1.95	28.8	>100	+	62
AG337			0.011		NS	81
AG377			0.033		NS	83

[a] The data in this table are depicted from the indicated references. Please note that $K_{i_{app}}$ determinations of enzyme inhibition and substrate activity determination for FPGS may not have been carried out under identical conditions with respect to substrate, inhibitor, and cofactor concentration. For experimental details, see the indicated references. Moreover, it should be noted that the K_i for the compounds listed may not reflect the increased potency of inhibiting TS when polyglutamate derivatives are formed.

[b] FPGS substrate activity (K_m) is defined as follows: K_m < 5 μM: (++++); K_m 5–25 μM: (+++); K_m 25–100 μM: (++); K_m > 100 μM: (+). NS, nonsubstrate.

L1210 leukemia cells, one variant (L1210-B73) expressing both the RFC and mFBP (59), and another variant (L1210-FBP) in which mFBP is the only functional transport protein (60). (This cell line was erroneously reported to be derived from CEM/MTX cells, but on karyotype and restriction polymorphism analysis it appeared to be a subline of L1210-B73 cells lacking functional RFC activity (60a).

Parental cells (CCRF-CEM and L1210) and CEM/MTX cells were grown in RPMI-1640 medium containing 2 μM FA supplemented with 10% FCS, 2 mM L-glutamine, and 100 units/ml each of penicillin and streptomycin. CEM-7A, L1210-FBP, and L1210-B73 cells were grown in folate-free RPMI 1640 supplemented with 10% dialysed FCS, antibiotics, and glutamine as described. LV was added as folate source at final concentrations of 0.2, 1.0, and 1.0 nM, respectively (unless otherwise indicated). The cell cultures of L1210, L1210-FBP,

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and L1210-B73 cells also contained 50 μM β-mercaptoethanol. Cells were kept at 37° in a humidified atmosphere with 5% CO_2.

DHFR and FPGS levels in cell lines. The DHFR concentration of the cell lines used in the present study as assessed by [^{3}H]MTX-binding (84) was comparable in CEM, CEM/MTX, and CEM-7A cells (1.3 pmol/mg protein). Likewise, DHFR levels in L1210 cells and L1210-FBP cells were similar (2.6 pmol/mg protein). The enzyme level in L1210-B73 cells was 2.8-fold higher (7 pmol/mg) than in L1210 cells. FPGS activity was measured in all cell lines as described by Jansen *et al.* (85) with 250 μM MTX as a substrate. Enzyme activities expressed as picomoles of MTX-[^{3}H]glutamate formed per hour per milligram of protein were as follows: 402 (L1210-FBP), 618 (L1210-B73), 829 (CEM), 992 (CEM-7A), 1168 (L1210), and 1200 (CEM/MTX) (results not shown).).

RFC substrate specificity. CEM-7A cells (3×10^6) in the logarithmic phase of growth were washed with 10 ml HBSS buffer (107 mM NaCl, 20 mM HEPES, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 7.0 mM D-glucose adjusted to pH 7.4 with NaOH), centrifuged, and suspended in 1 ml HBSS-buffer at 37°. Influx of [^{3}H]MTX (0.5 Ci/mmol) was measured over a period of 1.5 min at 37° at an extracellular concentration of 5 μM in the absence or presence of increasing concentrations of unlabeled folate or antifolate compound. Uptake was terminated by the addition of 9 ml of ice-cold HBSS buffer, centrifugation at 800 × *g* for 5 min at 4°, and a second wash with 10 ml ice-cold buffer. Cell pellets were resuspended in water and analyzed for radioactivity with Ultima-Gold scintillation fluid and a scintillation counter (both from Packard, Brussels, Belgium) with a counting efficiency for [^{3}H] of approximately 55%. The drug concentration required to inhibited [^{3}H]MTX influx by 50% of control values (221 pmol/min/10^7 cells) was taken to be a measure of relative affinity for the RFC.

mFBP substrate specificity. An intact cell binding assay for competitive binding of [^{3}H]FA was performed as described previously (86). Briefly, L1210-FBP cells were washed twice with ice-cold HBSS buffer. One milliliter of L1210-FBP cell suspension (3×10^6 cells) was incubated with 100 pmol [^{3}H]FA (specific activity, 0.5 Ci/mmol) in the presence or absence of increasing concentrations of unlabeled folate or antifolate compound. After 10 min, the cells were collected by centrifugation (for 5 min at 800 × *g* at 4°), after which the supernatant was removed. Pellets were resuspended in water and analyzed for radioactivity as described. Relative affinities are defined as the inverse molar ratio of compound required to displace 50% of [^{3}H]FA from mFBP on L1210-FBP cells. The relative affinity of mFBP for FA is set at 1.

Growth-inhibition studies. All cell lines were plated at an initial density of 7.5×10^4 cells/ml in individual wells of a 24-well tissue culture plate. The growth medium for CCRF-CEM, CEM/MTX, and L1210-cells was RPMI 1640 with 10% FCS, supplemented as described. CEM-7A cells were grown in folate-free RPMI 1640 with 10% dialysed FCS and 1.0 nM LV as the sole folate source; L1210-FBP and L1210-B73 cells were grown in the same medium but with 1 nM LV, 20 nM LV, or 20 nM FA as folate source. Drugs were added at the time of plating. After 72 hr of exposure, cell counts and viability were determined with a hemocytometer by trypan blue exclusion. IC_{50} values are given as the concentration of drug at which the growth is inhibited by 50% compared with controls.

Results

RFC substrate specificity. The affinity of the RFC for the series of antifolate drugs, expressed as the drug concentration required to inhibit [^{3}H]MTX influx by 50%, is shown in Fig. 2. MTX (represented by a black bar and vertical line) was used as a reference compound. Compounds on the left side of this line were better substrates for the RFC than MTX, whereas compounds on the right side were poorer substrates. All DHFR inhibitors included in the study appeared to be better substrates by RFC than MTX. Replacement of the 2-amino group by 2-methyl in MTX and AMT led to an enhanced affinity. For example, 2-CH_3-dAMT was a 10-fold better substrate for RFC than MTX.

In the series of GARTF inhibitors, only DDATHF is a more efficient substrate for RFC than MTX. The group of FPGS inhibitors [5-deazatetrahydrofolic acid analogues with the glutamate side chain replaced by other charged residues (homocysteic acid, 2-amino-4-phosphonobutanoic acid, and ornithine)] are very poor substrates for RFC-mediated transport.

Within the group of TS inhibitors, the 2-amino-based structures CB3717, 2-NH_2-ZD1694, and IAHQ are low affinity substrates for the RFC. However, their 2-methyl analogues (ICI-198,583, ZD1694, and 2-CH_3-IAHQ) are excellent substrates for the RFC. Modification at the 4-oxo position of ICI-198,583 did not alter the substrate affinity for the RFC. A modification at the N-3 position led to a 2-fold better affinity for the RFC. Replacement of the glutamate side chain by valine and 2-aminosuberate or a 7-CH_3 substitution resulted in a 4–5-fold and a 3-fold lower affinity for the RFC than for ICI-198,583, respectively. The RFC had the highest affinity for BW1843U89. LY231514 was a 2-fold better substrate for the RFC than MTX. The affinity of the RFC for 5-d(i)PteGlu and its N^9-substituted analogues was more than 10-fold lower compared with MTX. As might be expected, the lipophilic antifolates AG337 and AG377 were poor substrates for the RFC.

mFBP substrate specificity. Relative affinities of the mFBP for the series of antifolate drugs are presented in Fig. 3. FA (represented by the black bar and vertical line), for which mFBP has a binding affinity constant (K_d) of 0.5–1 nM (not shown), was used as the reference compound. Compared with FA, the mFBP had a lower affinity for all of the DHFR, GARTF, and FPGS inhibitors, especially the 4-amino-based compounds, e.g., MTX, AMT, and their 2-desamino/2-methyl analogues 10-EdAM and PT523. Modifications at sites other than the pteridine ring, such as the C^9-N^{10} bridge and the glutamate side chain, were better tolerated with respect to efficient binding by mFBP. For example, mFBP demonstrated good binding affinity for DDATHF and 5-dPteHCysA. In contrast to 5-dPteHCysA, the tetrahydrofolate forms of 5-dPteAPBA and 5-dPteOrn were better substrates for mFBP than were their nonreduced counterparts.

From the group of TS inhibitors, four compounds were identified for which mFBP has a higher affinity than for FA: LY213514, CB3717, IAHQ, and 2-NH_2-ZD1694. Unlike observations for the RFC, the 2-methyl analogues of the latter three compounds are characterized by significantly decreased binding affinity to mFBP. Most dramatic in terms of abrogation of binding affinity are modifications at the N-3 position (3-deaza-ICI-198,583) and structural alterations at the 4-oxo position (4-deoxy-ICI-198,583), although the 4-methoxy analogue of ICI-198,583 retained the same binding affinity as the parent compound. Modifications at other sites of the molecule (the quinazoline ring [substitution at the 7-position], the C^9-N^{10} bridge, the *p*-aminobenzoyl, and the glutamate side chain) did not dramatically influence the binding affinity with respect to binding by mFBP. mFBP exhibits a moderately high binding affinity for BW1843U89. Similar to the RFC, the mFBP has a poor affinity for the lipophilic drugs AG337 and AG377.

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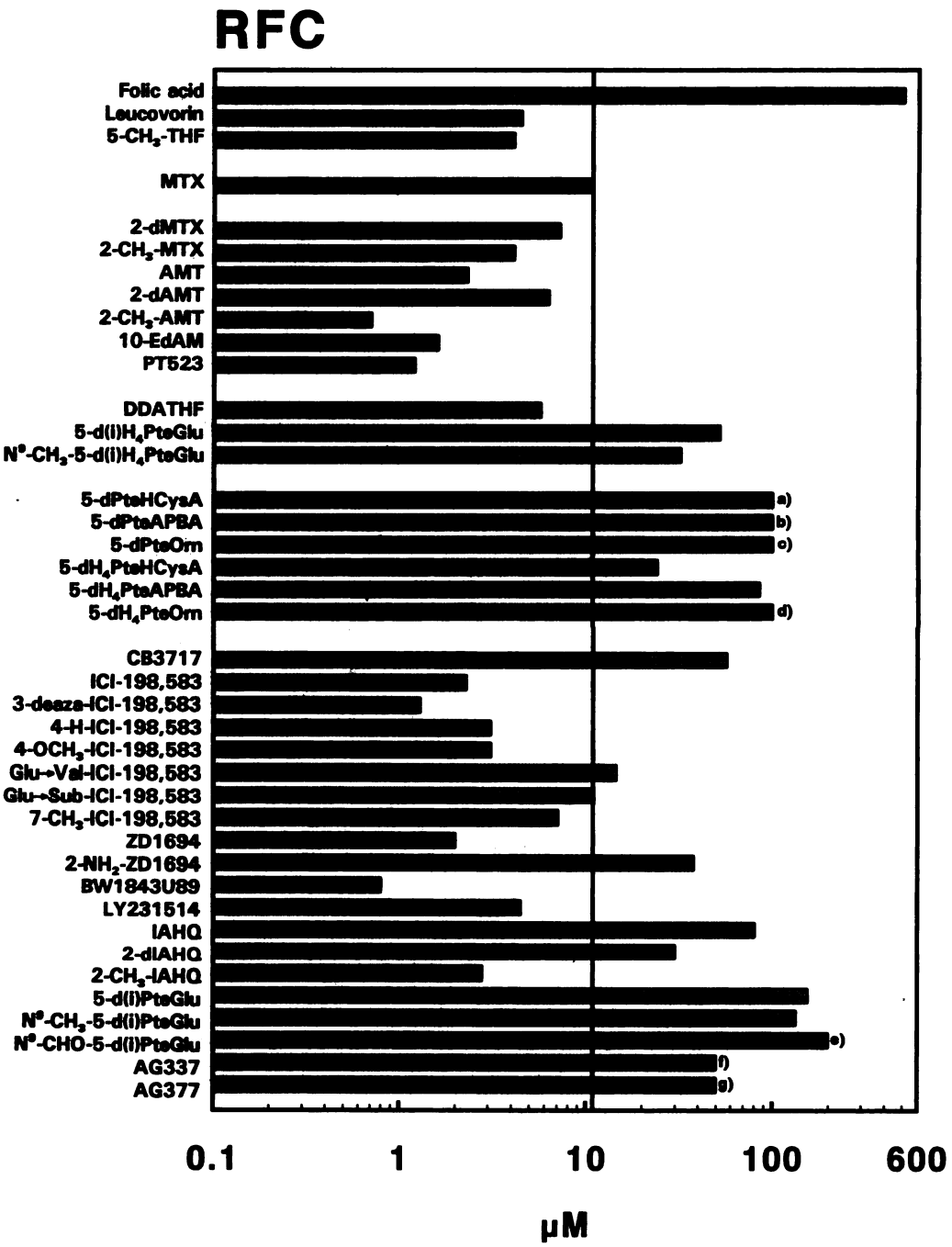

Fig. 2. Concentrations for 50% inhibition of RFC-mediated influx of [^{3}H]MTX. Values represent the concentrations of folate or antifolate compound necessary to inhibit RFC-mediated [^{3}H]MTX influx in CEM-7A cells by 50% at 5 μM extracellular concentration. *Vertical line* and *black bar*, concentration of unlabeled MTX necessary to inhibit [^{3}H]MTX uptake by 50%. For further details, see Experimental Procedures. *Bars* labeled with *a* through *g*, actual value exceeds the indicated value. Residual uptake values as percentage of control at the indicated drug concentration were as follows: *a*, 57% at 100 μM; *b*, >90% at 100 μM; *c*, >90% at 100 μM; *d*, 57% at 100 μM; *e*, >90% at 200 μM; *f*, >90% at 50 μM; *g*, >90% at 50 μM.

Growth-inhibition studies. The role of the RFC in drug transport and growth inhibition was analyzed by using three cell lines that differ in RFC expression and MTX transport (22) (Tables 3 and 4): CEM-7A cells are characterized by a 30-fold overexpression of RFC compared with parental CCRF-CEM cells, whereas CEM/MTX cells exhibit a MTX transport defect. In general, compounds that are good substrates for the RFC (e.g., MTX and AMT with their 2-desamino and 2-methyl analogues ICI-198,583, ZD1694, LY231514, BW1843U89, and DDATHF) were potent growth inhibitors provided that the compounds were efficiently polyglutamated, are potent inhibitors of their target enzymes, or both (Tables 1 and 2). For example, drugs like 2-dMTX, 2-CH_3-dAMT, 3-deaza-ICI-198,583, and 4-H-ICI-198,583 appeared to be efficient substrates for the RFC, but because they are poor inhibitors of both DHFR and TS, their growth-inhibitory activity was low. Nevertheless, the role of the RFC in transport of these compounds and of most of the TS inhibitors is illustrated by the fact that RFC-overproducing CEM-7A cells are more sensitive than parental cells, whereas MTX transport-defective CEM/MTX cells are cross-resistant to these drugs. In this respect, it is of interest to note that cross-resistant factors for DDATHF and PT523 were significantly lower than for MTX, AMT, and 10-EdAM. Poor growth inhibition associated with inefficient RFC transport was observed for CB3717, 2-NH_2-ZD1694, IAHQ, and the series of glutamate side chain-modified FPGS inhibitors. In addition, for 5-deazaisofolic acid, 5-deazatetrahydroisofolic acid and their N^9-substituted analogues, the poor growth-inhibitory effect correlates with a low transport efficiency and target

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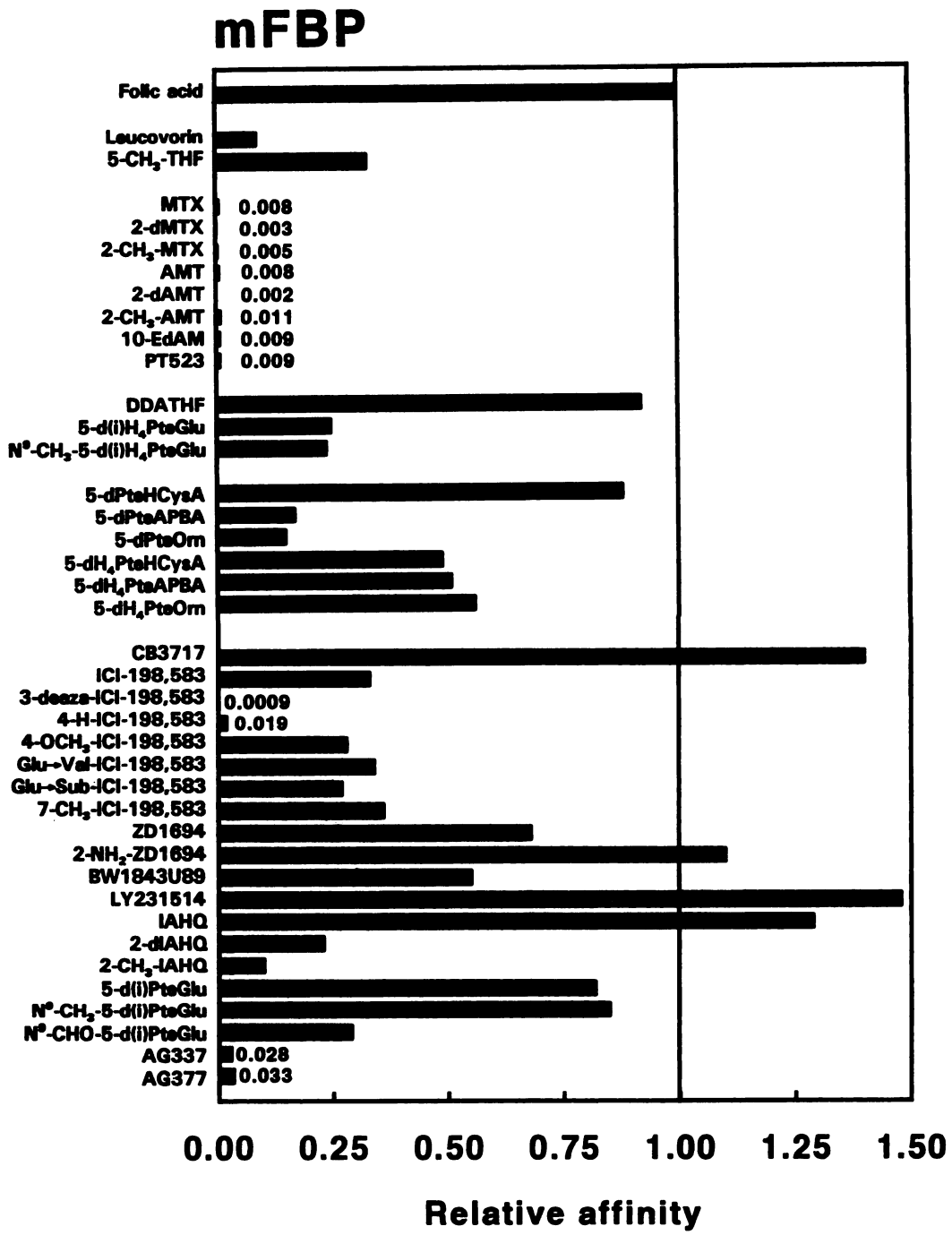

Fig. 3. Relative affinity of mFBP for folate and antifolate compounds. Values indicate relative affinities defined as the inverse molar ratio of compound required to displace 50% of [^{3}H]FA from mFBP in L1210-FBP cells. Relative affinity of FA is set at 1 (*black bar* and *vertical line*).

enzyme inhibition. The lack of correlation between RFC expression and growth-inhibitory effect of the lipophilic drugs AG337 and AG377 is indicative of an RFC-independent process of drug uptake.

The drug sensitivity profiles of human CCRF-CEM cells (Tables 3 and 4) and murine L1210 cells (Tables 5 and 6) were in large part similar. Nevertheless, absolute IC_{50} values for the individual drugs were most often lower for L1210 cells than for CCRF-CEM cells, which may be consistent with a 3-fold higher level of RFC expression in L1210 cells versus CCRF-CEM cells, whereas other transport kinetics properties are similar (20). The one exception is the uptake of BW1843U89 (80), which is a 17-fold poorer substrate for the murine RFC (K_i, 14 μM) compared with human RFC (K_i, 0.8 μM; see Fig. 2). This difference in transport characteristics is reflected in a 10-fold lower growth-inhibitory activity of BW1843U89 against L1210 versus CCRF-CEM cells (80).

In addition to binding of antifolates, the results given in Tables 7 and 8 support a functional role for mFBP in the uptake process of antifolates. In the absence of competing folate cofactors in the growth medium, most of the compounds tested demonstrated a growth-inhibitory effect against (RFC−/mFBP+++) L1210-FBP cells, provided they were efficiently polyglutamated or were potent inhibitors of their target enzyme (Tables 1 and 2). Compounds for which the RFC has poor affinity (e.g., CB3717, IAHQ, and 2-NH_2-ZD1694) appeared to be good growth inhibitors, consistent with the high binding affinity of mFBP for these compounds.

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TABLE 3

Growth-inhibitory effects (IC_{50} values in nM[a]) of antifolate compounds against human CEM cells with upregulated and downregulated RFC-mediated transport (RFC expression in parentheses)

Compound	Cell line (RFC expression)[b]		
	CCRF-CEM (+)	CEM/MTX (−)	CEM-7A (++++)
MTX	8.1	1,950	1.2
2-dMTX	1,870	40,000	49
2-CH_3-MTX	837	41,300	31
AMT	2.0	1,010	0.70
2-dAMT	500	12,600	14.6
2-CH_3-AMT	1,157	10,000	42
10-EdAM	1.3	533	0.3
PT523	1.1	33	0.7
DDATHF	11	80	2.2
5-d(i)H_4PteGlu	37,020	36,025	164
N^9-CH_3-5-d(i)H_4PteGlu	42,670	>100,000	1,740
5-dPteHCysA	>25,000	>50,000	4,500
5-dPteAPBA	>25,000	>50,000	>25,000
5-dPteOrn	>50,000	>50,000	31,000
5-dH_4PteHCysA	>25,000	>50,000	13,000
5-dH_4PteAPBA	6,100	>50,000	1,400
5-dH_4PteOrn	>50,000	>50,000	3,200

[a] IC_{50} values are given as the concentration of drug at which growth is inhibited by 50% compared with controls. Values are the mean of at least four separate experiments (SD ≤ 15%).
[b] RFC expression levels: CCRF-CEM, CEM-MTX, and CEM-7A-cells, 0.23, <0.02, and 8.1 pmol/mg protein, respectively (22).

TABLE 4

Growth-inhibitory effects (IC_{50} values in nM[a]) of antifolate compounds against human CEM cells with upregulated and downregulated RFC-mediated transport (RFC expression in parentheses)

Compound	Cell line (RFC expression)[b]		
	CCRF-CEM (+)	CEM/MTX (−)	CEM-7A (++++)
MTX	8.1	1,950	1.2
CB3717	705	7,750	36
ICI-198,583	17	605	3.2
3-deaza-ICI-198,583	>100,000	>100,000	1,940
4-H-ICI-198,583	680	80,000	74
4-OCH_3-ICI-198,583	46	3,300	32
Glu→Val-ICI-198,583	4,400	35,000	380
Glu→Sub-ICI-198,583	910	48,800	220
7-CH_3-ICI-198,583	243	6,480	82
ZD1694	3.5	444	0.65
2-NH_2-ZD1694	115	4,000	48
BW1843U89	2.4	520	1.1
LY231514	23	470	5.2
IAHQ	1,090	17,075	71
2-dIAHQ	690	19,865	23
2-CH_3-IAHQ	41	1,680	3.4
5-d(i)PteGlu	17,465	>100,000	1,540
N^9-CH_3-5-d(i)PteGlu	72,340	>100,000	7,450
N^9-CHO-5-d(i)PteGlu	>100,000	>100,000	69,750
AG337	1,800	1,590	1,640
AG377	28	10	10.7

[a] IC_{50} values are given as the concentration of drug at which growth is inhibited by 50% compared with controls. Values are the mean of at least four separate experiments (SD ≤ 15%).
[b] RFC expression levels: CCRF-CEM, CEM-MTX, and CEM-7A-cells, 0.23, <0.02, and 8.1 pmol/mg protein, respectively (22).

TABLE 5

Growth-inhibitory effects (IC_{50} values in nM[a]) of antifolate compounds against murine L1210- (RFC+)[b] and L1210-B73 (RFC+/mFBP+++)[b] cells grown in different folate concentrations in the medium

Compound	L1210	L1210-B73		
		1 nM LV	20 nM LV	20 nM FA + 1 nM LV
MTX	2.2	3.4	15	10
2-dMTX	169	1,095	20,300	2,460
2-CH_3-MTX	119	622	14,800	2,600
AMT	2.8	3.7	21	5.0
2-dAMT	91.5	612	33,500	1,670
2-CH_3-AMT	97	578	16,300	1,750
10-EdAM	1.6	2.9	11	4.3
PT523	0.7	2.7	4.5	3.0
DDATHF	9.2	4.1	42	88
5-d(i)H_4PteGlu	4,490	24	4,200	473
N^9-CH_3-5-d(i)H_4PteGlu	24,300	34	6,635	2,735
5-dPteHCysA	27,000	20,000	>25,000	>25,000
5-dPteAPBA	>50,000	19,000	>25,000	>25,000
5-dPteOrn	>50,000	>25,000	>25,000	>25,000
5-dH_4PteHCysA	30,000	9,400	>25,000	23,000
5-dH_4PteAPBA	3,300	1,600	8,300	4,200
5-dH_4PteOrn	16,500	9,600	>25,000	21,000

[a] IC_{50} values are given as the concentration of drug at which growth is inhibited by 50% compared with controls. Values are the mean of at least four separate experiments (SD ≤ 15%).
[b] RFC expression levels: L1210 and L1210-B73, 0.9 and 0.8 pmol/10^7 cells; mFBP expression level L1210-B73, 50–100 pmol/10^7 cells (59).

TABLE 6

Growth-inhibitory effects (IC_{50} values in nM[a]) of antifolate compounds against murine L1210- (RFC+)[b] and L1210-B73 (RFC+/mFBP+++)[b] cells grown in different folate concentrations in the medium

Compound	L1210	L1210-B73		
		1 nM LV[c]	20 nM LV	20 nM FA + 1 nM LV
MTX	2.2	3.4	15	10
CB3717	395	2.8	4.0	29
ICI-198,583	9.1	0.2	5.9	57
3-deaza-ICI-198,583	1,917	16,300	>50,000	>50,000
4-H-ICI-198,583	357	29	443	2,117
4-OCH_3-ICI-198,583	41	3	31	57
Glu→Val-ICI-198,583	1,740	1,080	6,733	7,730
Glu→Sub-ICI-198,583	660	45	308	537
7-CH_3-ICI-198,583	105	6.0	41	115
ZD1694	3	0.5	21	23
2-NH_2-ZD1694	190	1.2	3.2	14
BW1843U89	23	1.9	2.8	12
LY231514	14	4	15	16
IAHQ	705	2.1	6.6	30
2-desamino-IAHQ	271	3.1	562	1,745
2-CH_3-IAHQ	32	1.6	158	246
5-d(i)PteGlu	9,365	52	15,935	4,335
N^9-CH_3-5-d(i)PteGlu	60,700	19,500	>100,000	43,000
N^9-CHO-5-d(i)PteGlu	85,665	11,325	>100,000	55,000
AG337	737	1,470	3,500	1,890
AG377	6	26	204	43

[a] IC_{50} values are given as the concentration of drug at which growth is inhibited by 50% compared with controls. Values are the mean of at least four separate experiments (SD ≤ 15%).
[b] RFC expression levels: L1210 and L1210-B73, 0.9 and 0.8 pmol/10^7 cells; mFBP expression level L1210-B73, 50–100 pmol/10^7 cells (59).

The possible role of mFBP-mediated transport in the growth inhibition of antifolates was also studied in L1210-B73 cells (Tables 5 and 6), which coexpress mFBP (to the same level as L1210-FBP cells) and the RFC (to approximately the same level as parental L1210) (86). Data given in Tables 5 and 6 show that drugs that were growth inhibitory

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TABLE 7

Growth-inhibitory effects (IC_{50} values in nM[a]) of antifolate compounds against L1210-FBP cells grown in different folate concentrations in the medium

Compound	L1210-FBP (RFC−/mFBP+++)[b]		
	1 nM LV	20 nM LV	20 nM FA + 1 nM LV
MTX	19.4	67	1,400
2-dMTX	1,880	14,700	13,500
2-CH_3-MTX	717	12,500	>50,000
AMT	8.1	49	1,497
2-dAMT	1,320	10,100	19,500
2-CH_3-AMT	638	6,300	>25,000
10-EdAM	24.5	208	703
PT523	54	118	2,504
DDATHF	1.6	5.1	93
5-d(i)H_4PteGlu	12	367	6,735
N^9-CH_3-5-d(i)H_4PteGlu	27	433	42,000
5-dPteHCysA	12	30	555
5-dPteAPBA	71	210	3,400
5-dPteOrn	325	2,900	>50,000
5-dH_4PteHCysA	26	170	>25,000
5-dH_4PteAPBA	11	44	11,600
5-dH_4PteOrn	370	2,400	>50,000

[a] IC_{50} values are given as the concentration of drug at which growth is inhibited by 50% compared with controls. Values are the mean of at least four separate experiments (SD ≤ 15%).
[b] mFBP expression level: 50–100 pmol/mg protein (60).

TABLE 8

Growth-inhibitory effects (IC_{50} values in nM[a]) of antifolate compounds against L1210-FBP cells grown in different folate concentrations in the medium

Compound	L1210-FBP (RFC−/mFBP+++)[b]		
	1 nM LV	20 nM LV	20 nM FA + 1 nM LV
MTX	19.4	67	1,400
CB3717	1.1	1.9	24
ICI-198,583	0.28	1.6	74
3-deaza-ICI-198,583	39,000	>50,000	>50,000
4-H-ICI-198,583	42	128	7,700
4-OCH_3-ICI-198,583	1.6	11.7	660
Glu→Val-ICI-198,583	435	3,020	25,000
Glu→Sub-ICI-198,583	38	72	5,050
7-CH_3-ICI-198,583	3.8	8.2	1,865
ZD1694	0.24	2.5	117
2-NH_2-ZD1694	1.2	2.9	17.4
BW1843U89	0.8	1.0	12.3
LY231514	1.6	5.6	162
IAHQ	0.94	3.4	39
2-dIAHQ	2.4	226	9,760
2-CH_3-IAHQ	1.2	109	10,100
5-d(i)PteGlu	35	613	12,660
N^9-CH_3-5-d(i)PteGlu	8,475	46,340	78,670
N^9-CHO-5-d(i)PteGlu	2,100	45,670	>100,000
AG337	350	777	775
AG377	14.2	66	9.9

[a] IC_{50} values are given as the concentration of drug at which growth is inhibited by 50% compared with controls. Values are the mean of at least four separate experiments (SD ≤ 15%).
[b] mFBP expression level: 50–100 pmol/mg protein (60).

to L1210 cells were also effective growth inhibitors against L1210-B73 cells grown in 1 nM LV as the sole folate source (Tables 5 and 6, second column), suggesting that the RFC is the main transport route for these drugs in L1210-B73 cells. On the other hand, growth inhibition by drugs for which mFBP has a high affinity (e.g., CB3717, ICI-198,583, 2-NH_2-ZD1694, IAHQ, and 5-d(i)PteGlu) was markedly enhanced in L1210-B73 cells compared with L1210 cells. In addition, mFBP appears to contribute in the uptake of BW1843U89, which is poorly transported via murine RFC. In contrast to RFC-mediated transport, transport of this drug via mFBP does not appear to be species dependent. In contrast to the L1210-FBP cells, L1210-B73 cells were relatively resistant to growth inhibition by the series of FPGS inhibitors. Their sensitivity profile for these drugs was comparable with that of L1210 cells.

Protection of cytotoxicity by LV and FA. The growth-inhibition studies were extended to conditions in which 20 nM FA or 20 nM LV was added to the medium (Tables 3–8). These conditions may be more representative of physiological levels of the predominant serum folate 5-CH_3-THF (5–50 nM). Based on the relative affinities for the RFC and mFBP (Figs. 2 and 3), it can be anticipated that LV will compete for RFC-mediated drug uptake, whereas FA will be more effective in competing for mFBP binding and drug uptake. The relative affinity of mFBP for LV is 3-fold lower than that for 5-CH_3-THF, whereas the affinity for FA is 3-fold higher than that for 5-CH_3-THF (Fig. 3). Consistent with the difference in relative affinity of mFBP for FA and LV, FA provided much better protection against mFBP-mediated drug uptake and growth inhibition in L1210-FBP cells than did LV. In these cells, the protective effect of FA was most pronounced against antifolate drugs for which mFBP has a low affinity (e.g., MTX, AMT, 10-EdAM, PT523, 2-d-IAHQ, and 2-CH_3-IAHQ). The correlation between the affinity of mFBP and the degree of protection by FA was further illustrated by the fact that in the presence of 20 nM FA, compounds with high affinity such as CB3717, ZD1694, 2-NH_2-ZD1694, IAHQ, LY231514, and BW1843U89 retained a considerable growth-inhibitory effect against L1210-FBP cells. It should be noted that for compounds for which mFBP has a moderately high affinity (e.g., 5-dH_4PteAPBA, 2-d-IAHQ, and 2-CH_3-IAHQ), FA provided a protective effect that was substantially higher (1000-, 4000-, and 8000-fold, respectively) than could be predicted from the difference in relative affinity alone. This suggests that other factors (e.g., polyglutamylation) may have an additional role in this process. For L1210-B73 cells (Tables 5 and 6), LV was a more effective protective agent than FA against growth inhibition by the group of DHFR inhibitors. This demonstrates that despite a 100-fold higher absolute expression level of mFBP over the RFC, transport of these compounds proceeds more efficiently via the RFC than mFBP. For each of the GARTF and FPGS inhibitors, it was difficult to assess the exact relative contribution of mFBP- or RFC-mediated uptake in L1210-B73 cells on the basis of LV/FA protection studies. For DDATHF only, which has good substrate affinity for both the RFC and mFBP, a protective effect from growth inhibition by LV as well as FA suggests that both transporters are active in drug uptake in L1210-B73 cells.

For the TS inhibitors, we observed that in L1210-B73 cells (Table 6), FA and LV can act as protective agents against growth inhibition. Due to the multiplicity of entry routes (RFC and mFBP), the level of protection will depend on how efficiently the TS inhibitor uses either one or both transport systems at different extracellular concentrations. As with L1210-FBP cells, the transport of compounds characterized by a high binding affinity for mFBP and a poor substrate affinity for the RFC (e.g., CB3717, 2-NH_2-ZD1694, and IAHQ) appeared to be mediated mainly via mFBP, as illus-

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trated by significant protection by FA and less protection by LV. On the other hand, for compounds that can also use the RFC (e.g., ZD1694, 4-H-ICI-198,583, Glu→Sub-ICI-198,583, LY231514, and 2-CH_3-IAHQ), the substantial protection by LV indicates that part of the uptake of these compounds is mediated by the RFC.

Discussion

In the present study, we demonstrated the differential role of RFC- and mFBP-mediated transport of a series of antifolates targeted to the folate-dependent enzymes DHFR, GARTF, FPGS, and TS. Correlates were established between growth-inhibitory effects and parameters such as affinity of the RFC and mFBP for the antifolate drugs, the levels of expression of the RFC and mFBP, and the protective effects of concentrations of natural folates that mimic physiological concentrations. Along with the functional aspects of the RFC and mFBP individually, the effects of RFC and mFBP coexpressed in the same cell were studied.

Although the majority of compounds included in the study have been previously evaluated for their growth-inhibitory activity against RFC-expressing cell lines (22, 61–63, 65, 70, 78, 87–90), the present study enabled us to correlate these results with the affinity of the RFC for the antifolate compound. For example, the poor growth-inhibitory effects of two potent folate-based inhibitors of TS, CB3717 (64, 69) and IAHQ (66, 77), can now be ascribed to their poor substrate affinity for the RFC (Fig. 2). Also, for other compounds, especially the series of glutamate side chain-modified analogues of 5-dPte(H_4)Glu, inefficient transport via the RFC probably accounts for their weak growth-inhibitory effect (17, 63, 91, 92). On the other hand, modifications of the glutamate side chain should not necessarily lead to loss of substrate affinity for the RFC. This is illustrated by the data from compounds in which replacement of the glutamate moiety in ICI-198,583 by valine or 2-aminosuberate does not significantly alter transport efficiency via the RFC. In overview, structural features that may be relevant to the affinity of the RFC for an antifolate include the following: (a) poor affinity for the RFC is noted for 2-NH_2/4-oxo-oxidized structures (CB3717, IAHQ, 5-d(i)PteGlu, and 2NH_2-ZD1694) and analogues with major modifications in the glutamate side chain (5-dPteCysA, 5-dPteAPBA, and 5-dPteOrn); (b) replacement of 2-NH_2 for 2-CH_3 in ring A of the pteridine/quinazoline (Fig. 1) markedly improves the affinity for the RFC, as was shown for five parent compounds (MTX, AMT, CB3717, IAHQ, and 2-NH_2-ZD1694); (c) other modifications in ring A do not dramatically influence the affinity for the RFC, except for the 3-deaza modification of ICI-198,583, which enhanced the affinity by 2-fold; (d) modifications in the B-ring, e.g., 5-deaza, 5,8-dideaza, 7-CH_3 addition (7-CH_3-ICI-198,583) and LY231514 (pyrrolopyrimidine), are well tolerated; and (e) modifications and/or substitutions at the C^9-N^{10} bridge, benzoyl ring, and glutamate side chain do not cause a significant loss or improvement of affinity for the RFC. It should be realized that all of these considerations do not take into account whether the antifolate compounds can be polyglutamated or are good inhibitors of their target enzyme (Tables 1 and 2). For example, even though 2-CH_3-dAMT, 3-deaza-ICI-198,583, and 7-CH_3-ICI-198,583 are good substrates for the RFC, their diminished inhibition of DHFR (2-CH_3-dAMT) or TS (3-deaza-ICI-198,583) and impaired polyglutamylation (7-CH_3-ICI-198,583) greatly reduced their growth-inhibitory activity.

For mFBP as well, a number of structural features can be identified that determine its binding affinity for folate analogues: (a) a high binding affinity is seen for 2-NH_2/4-oxo-oxidized strutures (e.g., FA, CB3717, 2-NH_2-ZD1694, and IAHQ), whereas (b) a poor affinity is observed for 2,4-diamino structures (e.g., MTX, AMT, 10-EdAM, and PT523), suggesting that modifications at the 4-oxo position result in a substantial loss of binding affinity. Also, (c) 3-deaza and 4-deoxy modifications result in substantial abrogation of binding affinity, and (d) modifications at the C^9-N^{10} bridge, *p*-aminobenzoyl ring, or glutamate side chain have only minor effects on binding affinity. These results suggest that the N-3 and the 4-oxo parts of the pteridine/quinazoline ring are most important for high binding affinity to the binding protein and may be important in H-bond interactions.

Growth-inhibition studies were designed to obtain information about the effectiveness of antifolates as a function of their RFC- and mFBP-mediated transport, the expression levels of the RFC and mFBP, and the folate concentrations in the extracellular medium. These parameters will also be relevant in determining the successful application of these antifolates in a clinical setting. Based on the binding affinity, in combination with effective polyglutamylation and target enzyme inhibition, a number of drugs were identified that preferentially use either the RFC (2,4-diaminofolates) or mFBP (5-dPteHCysA, CB3717, 2-NH_2-ZD1694, and IAHQ) or can efficiently use both transporters (DDATHF, ZD1694, BW1843U89, and LY231514). The latter group of compounds demonstrated a potent growth-inhibitory effect against RFC or mFBP cell lines, both under low folate (1 nM LV) conditions and in folate-conditioned medium (20 nM FA/LV).

To put these *in vitro* results into a possible *in vivo* perspective, it is imperative to recognize that there may be differences in the expression levels of RFC and mFBP in neoplastic cells and normal tissues compared with the leukemia cell lines used as model systems in the present study. The availability of molecular and immunological probes for the RFC and mFBP has recently enabled researchers to determine the tissue distribution of these proteins (37–42, 93, 94). mFBP expression is constitutively high in ovarian carcinoma (95, 96), where expression levels may approximate those in L1210-FBP or L1210-B73 cells (50–100 pmol/10^7 cells) (59, 60). Variable mFBP expression levels were measured in other neoplastic and normal cells but in general were significantly lower than for the leukemia cell lines used in the present study. On the other hand, RFC expression in CCRF-CEM, L1210, and L1210-B73 cells may be more representative for expression levels identified in neoplastic cells or normal cells (6, 42, 97–99).

Rather than considering one antifolate transport system (either the RFC or mFBP) in malignant and nonmalignant cells, it may be reasonable to speculate that the two transport systems may be coexpressed in one cell. This phenomenon was described initially for L1210 cells adapted to grow under low folate conditions (56, 59, 86, 100) but was recently also described for KB human nasopharyngeal carcinoma cells, monkey kidney MA-104 cells, and IGROV-1 ovarian carcinoma cells (101, 102). In the case of L1210-B73 cells, we demonstrated that RFC and mFBP appear to function inde-

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pendent of each other and follow independent kinetics (86), which was confirmed in a recent study by Spinella *et al.* (57). In the present study, an interesting observation suggests that RFC expression may influence mFBP transport activity. This is indicated by the fact that the group of FPGS inhibitors inhibited the growth of (RFC−/mFBP+++) L1210-FBP cells (Table 7) but not that of (RFC+/mFBP+++) L1210-B73 cells (Table 5). Whether this observation points to a coupling of folate and antifolate transport and polyglutamylation (103) is unknown.

A number of the folate antagonists included in the present study (DDATHF, ZD1694, BW1843U89, and LY231514) are being evaluted in phase I through III clinical trials (104–106). Given the fact that these drugs may be efficiently transported not only via the RFC but also potentially via mFBP, it is possible that mFBP-mediated drug uptake can contribute to their antitumor toxicity or toxic effects, depending on mFBP expression levels in neoplastic and normal cells, respectively. To avoid possible mFBP-associated drug-related toxicity, alternative strategies may have to be applied that include FA rather than LV to competitively inhibit binding to mFBP. Recent reports (107, 108) that FA was effective in preventing delayed toxicities by DDATHF and BW1843U89 may be compatible with this concept.

In summary, we have demonstrated the relative importance of the RFC- and mFBP-mediated membrane transport for a large series of antifolate drugs. This information is of potential relevance in assessing the preclinical activity of these drugs and should lead to a more rational design of novel antifolates in the future. Possible clinical implications of the present study can be put into a proper perspective only after detailed analyses are made of the tissue distribution of the RFC and mFBP. These analyses are now possible due to the availability of immunological and molecular probes for both proteins (38–40, 42, 93, 109).

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