

The Role of α -Folate Receptor-Mediated Transport in the Antitumor Activity of Antifolate Drugs

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ABSTRACT

Purpose: Raltitrexed, pemetrexed, lometrexol, and ZD9331 are antifolate drugs transported into cells via the ubiquitously expressed reduced-folate carrier. They display also high affinity for the α -folate receptor (α -FR), a low capacity folate transporter that is highly overexpressed in some epithelial tumors. The role of α -FR in the activity of the antifolates has been evaluated in two α -FR-overexpressing cell lines grown in a physiological concentration of folate (20 nM *R,S*-Leucovorin).

Experimental Design and Results: A431-FBP cells (transfected with the α -FR) were 3–5-fold more sensitive to the antifolates than A431 cells. KB cells (constitutive α -FR overexpression) were less sensitive to the drugs when coexposed to 1 μ M folic acid to competitively inhibit binding to the α -FR. Raltitrexed, pemetrexed, and lometrexol are polyglutamated in cells leading to drug retention, *e.g.*, the raltitrexed 4- and 24-h IC_{50} s in A431 cells were ~ 0.6 and 0.008 μ M, respectively, compared with 0.003 μ M for 72-h continuous exposure. A431-FBP cells were ~ 3 -fold more sensitive to raltitrexed and pemetrexed at all exposure times. ZD9331 is not polyglutamated, and the 4- and 24-h IC_{50} s in A431 cells were >100 and ~ 100 μ M, respectively, reducing to 2 and 0.1 μ M, respectively, in A431-FBP cells. The ZD9331 4- and 24-h IC_{50} s in KB cells were 20 and 1 μ M, respectively, and reversible by coaddition of 1 μ M folic acid. An *in situ* thymidylate synthase assay demonstrated continued thymidylate synthase inhibition after ZD9331-treated A431-FBP and KB, but not A431, cells were placed in drug-free medium for 16 h. A model is proposed in which the antifolates accumulate in the α -FR/endosomal apparatus, leading to slow release into the cytoplasm. In particular, this leads to cellular retention of the nonpolyglutamatable ZD9331.

Conclusions: Antifolate drugs, particularly ZD9331, have the potential for increased efficacy in tumors that highly overexpress the α -FR.

INTRODUCTION

Antifolate drugs have been developed that act on a number of folate-dependent enzymes and include methotrexate (MTX) that primarily targets dihydrofolate reductase; CB3717, raltitrexed (Tomudex; ZD1694), and ZD9331 that target thymidylate synthase (TS); Alimta (pemetrexed; MTA; LY231514) that primarily inhibits TS but may have other relevant targets; and lometrexol (5,8-dideazafolate) that inhibits glycinamide ribonucleotidic formyltransferase (Refs. 1–8; Fig. 1). With the exception of CB3717, these drugs are efficiently transported into cells via the reduced-folate carrier (RFC; Refs. 7 and 9–14). The RFC is a low affinity, high capacity system that bidirectionally transfers folates/antifolates across the plasma membrane via an energy-dependent, carrier-mediated process (15–17). Although the RFC is ubiquitously expressed (18), another folate transporter, the α -folate receptor (α -FR) displays a more restricted range of tissue expression but is nevertheless proposed to function as a high affinity, low capacity folate/antifolate transporter (11, 16, 19, 20). The low capacity is ascribed to the receptor-mediated endocytotic mechanism that requires recycling of the receptor back to the cell surface, a process that has been reported to take between 30 min and 5 h (21–24).

The α -FR is highly overexpressed in some solid epithelial tumors, such as ovarian carcinoma and mesothelioma (25–31). Furthermore, its relatively low expression in most normal tissues (exceptions are placenta, proximal tubule of the kidney, choroid plexus, and glandular epithelia, particularly breast; Refs. 25, 26, and 32) is leading to the development of diagnostic agents and therapy targeted at α -FR-overexpressing tumors (33–36). Some of these therapies are folic acid drug conjugates or novel antifolates that rely on α -FR functional activity to deliver the agent into the cells. Several antifolate drugs have high affinity for the α -FR, similar to that of folic acid or the biologically active reduced folate cofactors (8, 11, 14, 19). Functionality of the receptor in terms of transport of antifolates was demonstrated using cell lines deficient in RFC function but overexpressing the α -FR (11, 37). Uncertainty has surrounded the question of the relevance of α -FR-mediated transport of antifolates when the RFC is coexpressed. This is partly attributable to the fact that *in vitro* models have relied on culturing cells in medium containing a low concentration of folate (≤ 1 nM) either to maintain or increase α -FR expression (11, 37–39). This may result in reduced competition between folates and antifolates for binding to the α -FR or, if intracellular folates are concomitantly low, reduced competition for enzymes relevant to drug action, *e.g.*, folylpolyglutamate synthetase and/or TS. Nevertheless, studies using these model systems demonstrated that raltitrexed, pemetrexed, ZD9331, lometrexol, and particularly CB3717 could be transported into mouse and human cells via the α -FR. However, it was concluded that, possibly with the exception of CB3717, drug uptake was predominantly via the RFC.

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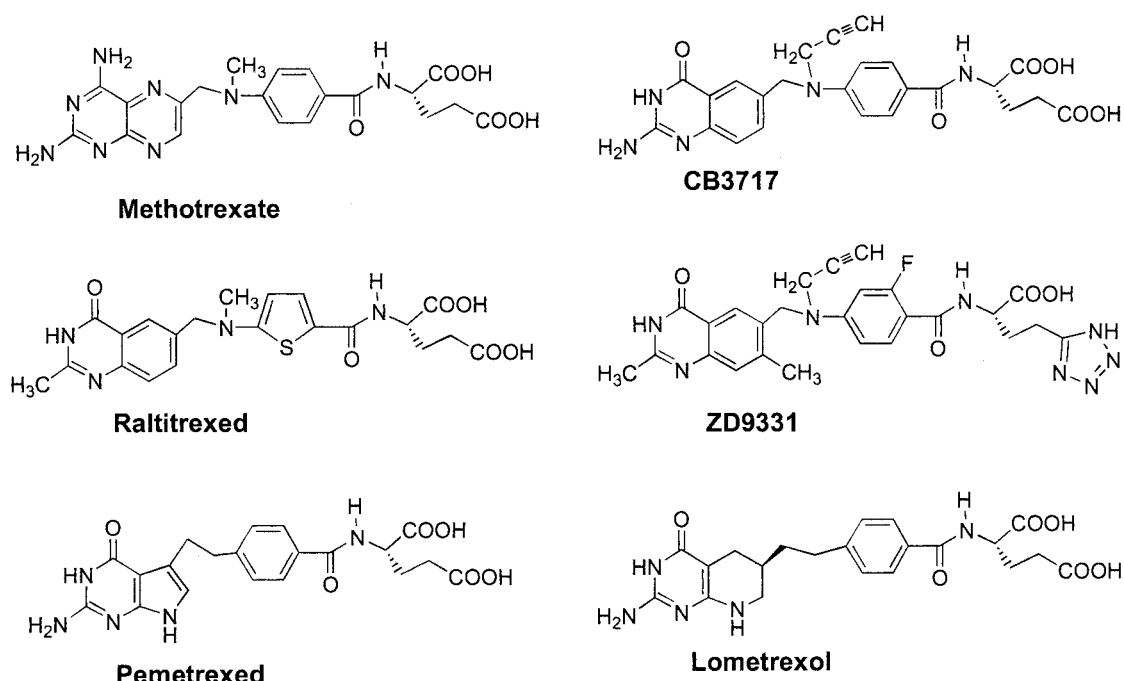


Fig. 1 Structures of antifolates.

Recently, it has been speculated that α -FR overexpression may play a role in the clinical activity of antifolate drugs. This is based partly on the *in vitro* evidence described above but partly on some circumstantial evidence arising from clinical trials with CB3717, raltitrexed, and pemetrexed in α -FR-overexpressing cancers. In Phase I/II clinical studies of CB3717, an \sim 18% response rate was observed in platinum-refractory ovarian cancer (40). Activity of CB3717, raltitrexed, and pemetrexed has been demonstrated in mesothelioma (40–42). A deeper understanding, therefore, of α -FR-mediated uptake may lead to the improved use of antifolate drugs by, *e.g.*, selectively targeting α -FR-overexpressing tumors.

The current study compares several antifolate drugs for their affinity for the human α -FR relative to folic acid and for their activity in two human tumor cell lines (A431-FBP and KB) that highly overexpress the α -FR. Importantly, the cells express functional RFC and can be grown in media containing a physiological folate concentration without down-regulating the α -FR (36). The A431-FBP cell line has been transfected with the α -FR so that direct comparisons can be made with its A431 neo-transfected isogenic partner (43). The KB cell line constitutively overexpresses the α -FR (39, 44). We have shown that A431-FBP tumor cells display a 3–5-fold increased sensitivity, compared with A431 cells, to raltitrexed, ZD9331, pemetrexed, and lometrexol when exposure is continuous and cells are cultured in a physiological concentration of folate. Similar results were obtained with KB tumor cells in which sensitivity was compared with that in the presence of 1 μ M folic acid. This concentration of folic acid competitively inhibits binding to the α -FR but not RFC, because the two transporters display high and very low affinity for folic acid, respectively (16, 17, 36). After short exposure to ZD9331, α -FR-mediated uptake led to

prolonged TS inhibition after extracellular drug removal and a marked increase in the growth inhibitory activity of ZD9331 that was not seen with the other antifolates. This has led to the hypothesis that entrapment of ZD9331 in the endosomal apparatus of α -FR-overexpressing cells leads to the slow but continuous delivery of ZD9331 into the cytosol. The consequences of this effect are less apparent with polyglutamatable drugs that become trapped in cells via polyglutamation, irrespective of the transport route. Thus, it is hypothesized that in a clinical situation, these antifolates, particularly ZD9331, have the potential to localize more highly to tumors overexpressing the α -FR.

MATERIALS AND METHODS

Compounds. Raltitrexed, pemetrexed, and ZD9331 were synthesized at AstraZeneca plc (Alderley Park, Cheshire, United Kingdom). Lometrexol was a kind gift from Eli Lilly Pharmaceuticals (Indianapolis, IN). MTX and folic acid were purchased from Sigma (Poole, Dorset, United Kingdom). The chemical structures are given in Fig. 1. Stock solutions (10 mM; \sim 1 ml) were prepared in 0.15 M NaHCO₃ [one to three drops of 1 M NaOH were added to aid dissolution (pH < 8.0)] and stored at -20° for not >4 months.

Cell Culture. Human A431 epidermoid vulval (neo-transfected) and A431-FBP cells (transfected with the human α -isoform of the FR) were a generous gift from Dr. A. Tomasetti (Istituto Tumori, Milan, Italy). Details of these cell lines and the culture conditions have been published recently (36). Importantly, the medium (DMEM) was purchased without folic acid, because commercial media contain a supra-physiological concentration of folic acid (2–8 μ M). The medium was supplemented before use with 1 or 20 nM *R,S*-leucovorin (LV) and

referred to as low and physiological folate concentrations, respectively. Similarly, the culture conditions for human KB cells (folate-free RPMI medium supplemented with LV) are found in Theti *et al.* (36). Cell surface expression of α -FR, as measured by the surface binding capacity of [3 H]folic acid, was <1 pmol/ 10^7 cells, 171 ± 42 pmol/ 10^7 cells, and 91 ± 17 pmol/ 10^7 cells for A431, A431-FBP, and KB cells, respectively, grown in 20 nM LV. In 1 nM LV, the values were <1 pmol/ 10^7 cells, 211 ± 65 pmol/ 10^7 cells, and 123 ± 43 pmol/ 10^7 cells (36). The population doubling times were ~ 18 , ~ 21 , and ~ 15 h for A431, A431-FBP, and KB cells, respectively, irrespective of the folate concentration.

Affinity of the Antifolates for the α -FR. This method is essentially a whole cell [3 H]folic acid competitive binding assay reported by Westerhof *et al.* (45) adapted for use with adherent cell lines (36). Relative affinities are defined as the inverse molar ratio of compound required to inhibit [3 H]folic acid binding by 50%. The relative affinity of FA is set at 1 (100%). Compounds with a lower or higher affinity have values <1 and >1 , respectively.

Growth Inhibition Studies. Details of the growth inhibition assays are reported in Theti *et al.* (36). Briefly, A431 and A431-FBP cells were incubated for 72 and 96 h, respectively (approximately four control population doublings), with increasing concentrations of the antifolate drugs in 96-well plates before using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as a measure of cell viability. KB cells were incubated for 72 h (approximately four control population doublings) with the drugs in 24-well plates before cell counting. Cell growth in the presence of the drugs is expressed as a percentage of control cell growth and the IC_{50} calculated (concentration of drug to inhibit cell growth by 50%). This was performed in media containing 1 or 20 nM LV as the folate source. In parallel, cells were coexposed to drug and 1 μ M folic acid (added 15 min before drug) to selectively inhibit α -FR- but not RFC-mediated uptake. This, as might be expected, had no effect on the IC_{50} s obtained for any of the antifolates in the α -FR-negative A431 cells.

Short exposure growth inhibition assays refer to experiments in which the exposure time to the antifolates ranges from 1 to 72 h, *i.e.*, the cells were washed, and drug-free medium (DFM) was added for the remaining incubation period as described above, *i.e.*, the end point is the same as for continuous exposure experiments. The IC_{50} s obtained are plotted against exposure time.

In Situ TS Assay. This assay measures the rate of [3 H] release (as [3 H] $_2$ O) from 5-[3 H]dUrd over 1 h and is a semi-quantitative measure of TS inhibition in cells. Details of this method, adapted to the cell lines described herein, have been published recently (36). TS inhibition was measured after 1-, 4-, 8-, and 16-h exposure to increasing concentrations of ZD9331, expressed as a percentage of control and the IC_{50} calculated. In parallel, cells were exposed to ZD9331 and 1 μ M folic acid to assess the contribution of α -FR-mediated drug uptake to TS inhibition.

In some experiments, the exposure to ZD9331 was fixed at 4 h, followed by removal of drug-containing medium and the addition of DFM for 4 or 16 h before the *in situ* TS assay was performed.

RESULTS

Affinity of Antifolates for the α -FR Expressed by L1210-FBP and A431-FBP Cells Relative to Folic Acid. A description of antifolate relative binding affinities for the α -FR expressed by mouse L1210-FBP cells has been published (11, 14), so this cell line was included as a control, and results were compared with A431-FBP α -FR. Raltitrexed, ZD9331, and lometrexol displayed affinities for mouse α -FR that were 61, 54, and 78% that of folic acid, respectively, whereas MTX displayed a considerably lower affinity ($<1\%$; Table 1). Only pemetrexed had a relative affinity higher than that of folic acid (120%). This compares with 150% for CB3717 (data not shown). Some differences in relative affinities were observed when human A431-FBP α -FR-overexpressing cells were used, but generally, the same pattern was observed (Table 1). The published results of raltitrexed and lometrexol for α -FR expressed by human KB cells are also similar, *i.e.*, 31 and 70%, respectively, (11). One study reported that raltitrexed binds to KB α -FR with an ~ 100 -fold lower affinity using a [125 I]-folic acid competitive binding assay (46).

Inhibition of the Growth of the Human A431 and A431-FBP Isogenic Pair of Cell Lines. The sensitivity of the A431 cells to the antifolates in a physiological folate concentration (20 nM LV) ranged from 3 nM IC_{50} (raltitrexed) to 90 nM IC_{50} (ZD9331; Table 2) and is similar to that reported for other non- α -FR-overexpressing tumor cell lines grown in standard commercial media (47). The results were similar in low folate conditions (1 nM LV), except that MTX was 8-fold more active (Table 2). This may be the result of a lower intracellular folate pool decreasing competition for binding to dihydrofolate reductase as reported for other cell lines (48, 49).

In 20 nM LV, A431-FBP cells displayed a 3–5-fold higher sensitivity compared with A431 cells to all of the drugs, except for MTX, consistent with α -FR-mediated transport contributing to drug uptake (Table 2). In 1 nM LV, A431-FBP cells were 14-fold more sensitive than A431 cells to raltitrexed and pemetrexed, suggesting that α -FR-mediated uptake is more efficient in low folate compared with a physiological folate concentration. The similar sensitivity of both cell lines to MTX is consistent with its low affinity for the α -FR relative to that of the

Table 1 Relative affinities of the antifolates for the α -FR expressed by L1210-FBP and A431-FBP cells^a

The α -FR affinity is expressed as the inverse molar ratio of compound required to displace [3 H]-folic acid from the α -FR by 50%. Folic acid is designated as 1 (100%). Results are given as the mean \pm SD of at least three experiments or as individual results. Values for CB3717 are 1.5 and 1.2 for L1210-FBP and A431-FBP, respectively (Ref. 36).

	Affinity relative to folic acid	
	L1210-FBP	A431-FBP
Raltitrexed	0.61 \pm 0.12	0.24 \pm 0.02 ^b
ZD9331	0.54 \pm 0.10	0.30 \pm 0.08 ^b
Pemetrexed	1.2 ^c	1.8 \pm 0.07
Lometrexol	0.78 ^c	0.61 \pm 0.07
Methotrexate	<0.01	<0.01

^a α -FR, α -Folate receptor.

^b Significantly different from L1210-FBP.

^c Pemetrexed = 1.5; Lometrexol = 0.9 (Ref. 11).

Table 2 Inhibition of A431 and A431-FBP cells by antifolate drugs in a low folate (1 nM LV) and physiological folate (20 nM LV) concentration^a

Human A431-FBP cells have been transfected with the α -FR, and A431 cells have been neo-transfected (Ref. 43). Cells were continuously grown in folate-free media supplemented with 1 or 20 nM LV as the folate source. Growth inhibition was measured after 72 and 96 h for A431 and A431-FBP cells, respectively, and an MTT assay was used as a surrogate end point for cell growth. Results are given as mean \pm SD of at least three experiments.

		Inhibition of cell growth, IC ₅₀ , nM		Inhibition of cell growth, IC ₅₀ , nM	
		A431	A431 + 1 μ M FA	A431-FBP (fold increased sensitivity compared with A431)	A431-FBP + 1 μ M FA (fold increased IC ₅₀ in presence of folic acid)
Raltitrexed	1 nM LV	2.3 \pm 1.6	2.2 \pm 1.8	0.17 \pm 0.10 ^b (14)	3.4 \pm 4.7 ^c (20)
	20 nM LV	3.1 \pm 1.4	3.6 \pm 1.6	0.73 \pm 0.31 ^{b,d} (4)	1.3 \pm 0.7 (2)
ZD9331	1 nM LV	61 \pm 14	56 \pm 25	8.8 \pm 5.2 ^b (6)	27 \pm 7.6 ^c (3)
	20 nM LV	86 \pm 36	67 \pm 29	16 \pm 9.7 ^{b,d} (5)	34 \pm 8.7 ^c (2)
Pemetrexed	1 nM LV	32 \pm 13	43 \pm 22	2.3 \pm 0.46 (14)	27 \pm 6.1 ^c (10)
	20 nM LV	40 \pm 9.5	37 \pm 2.9	14 \pm 6.7 ^{b,d} (3)	38 \pm 21 (3)
Lometrexol	1 nM LV	7.4 \pm 0.53	9.1 \pm 0.31	1.7 \pm 0.67 ^b (4)	4.9 \pm 2.3 (3)
	20 nM LV	9.1 \pm 0.8	8.5 \pm 0.42	2.4 \pm 0.21 ^b (4)	8.0 \pm 1.8 ^c (3)
MTX	1 nM LV	4.5 \pm 2.2	5.4 \pm 1.8	4.1 \pm 2.6 (1)	5.2 \pm 3.9 (1)
	20 nM LV	32 \pm 19 ^d	30 \pm 16	27 \pm 18 ^d (1)	25 \pm 11 (1)

^a LV, *R,S*-leucovorin; α -FR, α -folate receptor; MTX, methotrexate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

^b $P < 0.05$ compared with A431.

^c $P < 0.05$ compared with A431-FBP (no folic acid).

^d $P < 0.05$ compared with 1 nM LV.

RFC. The addition of 1 μ M folic acid, to competitively inhibit binding of the antifolates to the α -FR, reduced the activity of all of the drugs, except for MTX, to approximately that seen for A431 cells.

Inhibition of the Growth of Human KB Cells. KB cells displayed a similar or slightly increased sensitivity to the antifolates in a low folate compared within a physiological folate concentration (Table 3). The exceptions were lometrexol and MTX, which were 20- and 9-fold more potent, respectively, in the low folate conditions. For lometrexol, this can be ascribed to increased α -FR-mediated uptake in low folate, because coaddition of 1 μ M folic acid reversed this effect. The IC₅₀s for pemetrexed and ZD9331 increased \sim 8- and 3-fold, respectively, in the presence of folic acid in both folate conditions.

Short Exposure Growth Inhibition Studies with Antifolate TS Inhibitors. These studies were performed in media containing 20 nM LV as the folate source. A431, A431-FBP, and KB cells were incubated with various concentrations of ZD9331, raltitrexed, or pemetrexed for increasing lengths of time before being placed in DFM for the remaining incubation period. Growth inhibition was measured at 96 h for A431-FBP and 72 h for A431 and KB cells (approximately four cell doublings). This is illustrated for ZD9331 in the isogenic A431/A431-FBP cell line pair in Fig. 2.

A431, A431-FBP, and KB cells were sensitive to raltitrexed and pemetrexed when exposure times were short, consistent with the formation of intracellular polyglutamate forms of the drugs. Sixteen to 24-h exposure was sufficient to give growth inhibitory IC₅₀s similar to continuous exposure IC₅₀s for raltitrexed and pemetrexed (Fig. 3). A431-FBP cells were 2–4-fold more sensitive than A431 cells to raltitrexed and pemetrexed when exposure times were \geq 4 h (Fig. 4, A and B). The addition of 1 μ M folic acid decreased the short exposure activity of these drugs 1–3-fold in KB cells (Fig. 3). These data are

consistent with α -FR-mediated uptake, contributing less than RFC-mediated uptake to the activity of these drugs.

As expected, the activity of the nonpolyglutamatable drug, ZD9331, in A431 cells after 1–24 h exposures was very low (IC₅₀s $>$ 100 μ M and \sim 100 μ M, respectively) compared with the polyglutamatable drugs described above. However, the ZD9331 IC₅₀s were two to three orders of magnitude lower for A431-FBP compared with A431 cells after 1–24 h exposure and approximately one order of magnitude after longer exposures (Fig. 3). When A431-FBP cells were coincubated with ZD9331

Table 3 Inhibition of human KB cells by antifolate drugs in low folate (1 nM LV) and physiological folate (20 nM LV) concentrations^a

KB cells were continuously grown in folate-free media supplemented with 1 nM or 20 nM LV as the folate source. Growth inhibition was measured after 72 h by cell counting.

		Inhibition of cell growth, IC ₅₀ nM	
		KB	KB + 1 μ M FA (fold increased IC ₅₀ in presence of folic acid)
Raltitrexed	1 nM LV	0.77 \pm 0.03	1.2 \pm 0.94 (2)
	20 nM LV	1.1 \pm 0.11	1.2 \pm 0.59 (1)
ZD9331	1 nM LV	2.1 \pm 1.3	6.4 \pm 3.5 (3)
	20 nM LV	3.6 \pm 2.2	11 \pm 3.0 ^b (3)
Pemetrexed	1 nM LV	5.9 \pm 0.95	50 \pm 11 ^b (8)
	20 nM LV	6.5 \pm 5.3	55 \pm 5.1 ^b (8)
Lometrexol	1 nM LV	2.2 \pm 0.68	45 \pm 6.1 ^b (20)
	20 nM LV	43 \pm 4.5 ^c	87 \pm 5.8 ^b (2) ^d
MTX	1 nM LV	2.6 \pm 1.3	3.5 \pm 3.2 (1)
	20 nM LV	23 \pm 8.3 ^c	20 \pm 10 (1)

^a LV, *R,S*-leucovorin; MTX, methotrexate.

^b $P < 0.05$ compared with KB (no folic acid).

^c $P < 0.05$ compared with 1 nM LV.

^d $P < 0.05$ compared with 1 nM LV.

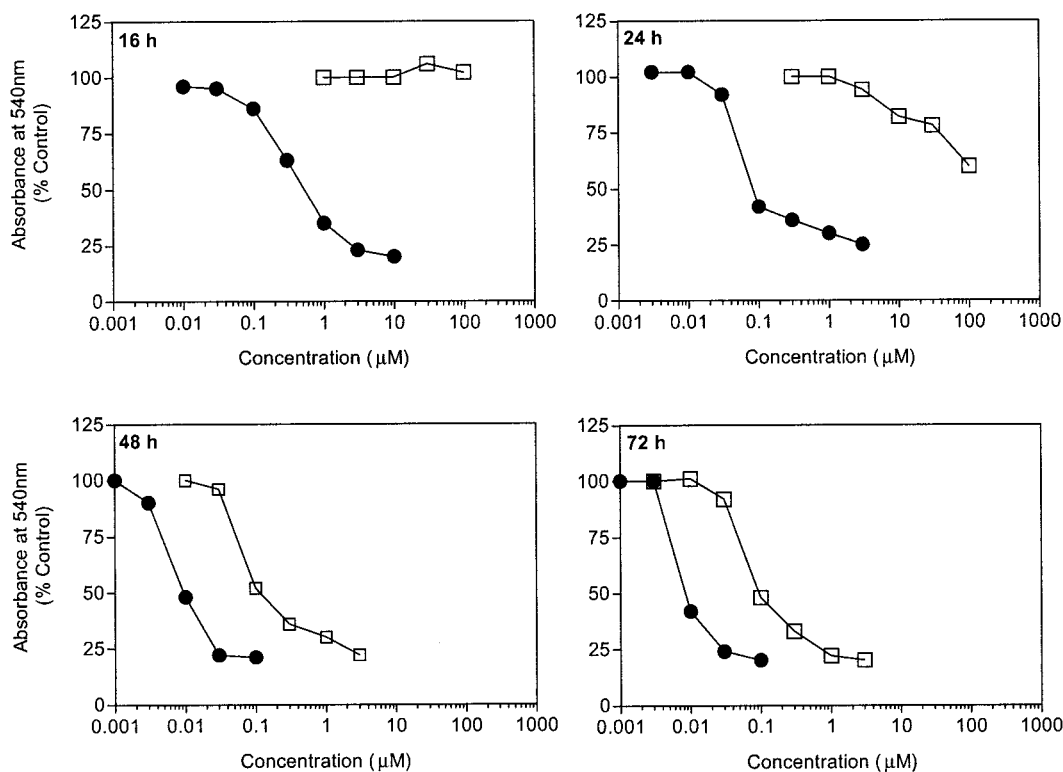


Fig. 2 Inhibition of A431 and A431-FBP cell growth after 16-, 24-, 48-, and 72-h exposure to ZD9331; cells in 20 nM *R,S*-leucovorin were treated with increasing concentrations of ZD9331 and incubated for the times indicated before being placed in drug-free medium for the remainder of the incubation period to allow for approximately four control population doublings (A431 = 72 h; A431-FBP = 96 h). Cell viability was determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and absorbance measured at 540 nm. □, A431; ●, A431-FBP.

and 1 μ M folic acid for 4 or 8 h, the IC_{50} s increased by the same magnitude (IC_{50} s \sim 50% of those for A431 cells). This high level of sensitivity to short exposure ZD9331 is interesting and consistent with ZD9331 being trapped in the α -FR/endosomal apparatus, leading to continuous release of the drug into the cytosol and prolonged inhibition of TS. Although KB cells were \sim 3-fold more sensitive than A431-FBP cells to continuous exposure ZD9331, they were \sim 10-fold less sensitive to short exposure ZD9331 (1–24 h) than A431-FBP cells. Nevertheless, folic acid decreased KB sensitivity to ZD9331 more after short exposures (>6 -fold) than continuous exposure (3-fold; Fig. 3). To exclude the possibility that the different growth media (A431/A431-FBP–DMEM; KB–RPMI 1640) was the reason for the higher activity in A431-FBP cells, the experiment was repeated in RPMI 1640, and similar results were obtained (data not shown). The differences between the two cell lines in sensitivity to short exposure ZD9331 may relate to one or more differences in their capacity for receptor loading, receptor recycling, kinetics of endosomal trafficking and unloading, rate of TS inhibition, drug efflux, or their susceptibility to the downstream effects of TS inhibition.

In Situ TS Inhibition As a Pharmacodynamic End Point of ZD9331 Uptake. The measurement of the flux through TS (rate of [3 H] $_2$ O release from 5- [3 H]deoxyuridine) in intact tumor cells after exposure to ZD9331 was used as a surrogate marker of the rate of accumulation of free drug inside cells to a level

that can inhibit the enzyme. The IC_{50} s for TS inhibition in A431 cells after 1- or 4-h exposure to ZD9331 were 3.6 and 2.3 nM, respectively (Table 4). This is more than four orders of magnitude lower than the growth inhibitory IC_{50} for a 4-h short exposure and consistent with rapid efflux of ZD9331. A431-FBP cells were not significantly more sensitive than A431 cells at 1, 8, and 16 h (1–2-fold) to the TS inhibitory effects of ZD9331. However, a 4-fold lower IC_{50} was observed at 4 h that was reversed by the coaddition of 1 μ M folic acid.

TS was also inhibited rapidly in KB cells, although the IC_{50} s were \sim 2–4-fold lower than in A431-FBP cells, consistent with the \sim 4-fold lower continuous exposure growth inhibition IC_{50} . The coaddition of 1 μ M folic acid increased the IC_{50} s \leq 2-fold. Although these data demonstrate that α -FR-mediated transport can lead to some small increases in TS inhibition, the enzyme is inhibited rapidly in all three cell lines and largely attributable to RFC-mediated uptake of ZD9331. This suggests that the lower growth inhibitory activity of ZD9331 in KB cells compared with A431-FBP cells after short exposure is not caused by a slower onset of TS inhibition.

TS inhibition in non- α -FR-expressing A431 cells exposed to 0.8 μ M ZD9331 (10 \times continuous exposure growth inhibition IC_{50}) for 4 h was reversed when the cells were placed in DFM for 4 h (Fig. 4), consistent with efflux of this nonpolyglutmatable drug. By contrast, TS inhibition in A431-FBP and KB cells exposed to an equitoxic concentration of ZD9331 (0.2 and 0.05

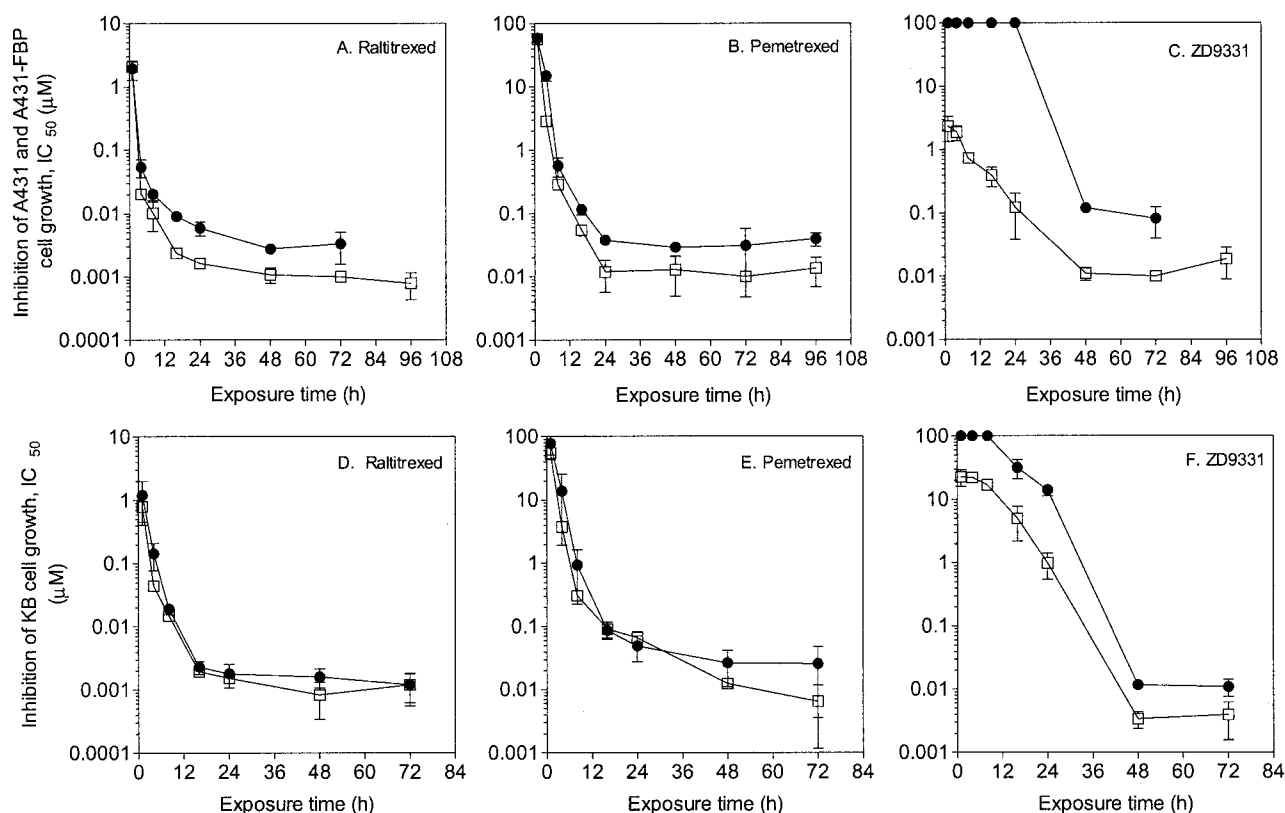


Fig. 3 Short exposure growth inhibitory IC_{50} s for raltitrexed, pemetrexed, and ZD9331 in A431, A431-FBP, and KB cell lines; cells in 20 nM *R,S*-Leucovorin were exposed to increasing concentrations of the drugs for 1–72 h (A431-FBP) and 1–48 h (A431 and KB) and then placed in drug-free medium for the remainder of the incubation period (A431 and KB = 72 h; A431-FBP = 96 h). The activity of the drugs in KB cells was also determined with the coaddition of 1 μ M folic acid. Cell viability was determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and absorbance measured at 540 nm. Results are the mean \pm SD of at least three experiments. IC_{50} s for ZD9331 (1–24 h) and pemetrexed (1 h) in A431 cells were >100 μ M. Similarly, IC_{50} s for ZD9331 (1–8 h) and pemetrexed (1 h) in KB cells (+ folic acid) were >100 μ M. A–C, A431 (●) and A431-FBP (□); D–F, KB cells (□) and KB cells + 1 μ M folic acid (●).

μ M, respectively) was not reversed after 4 or 16 h in DFM (Fig. 4). This has been attributed to α -FR/endosomal trapping of ZD9331 and supported by the observation that TS inhibition was reversible when cells had been coexposed to ZD9331 and 1 μ M folic acid for 4 h. Data are not available for 24 h washout experiments, but it is expected that at least some recovery in TS activity would be seen, consistent with a 4-h exposure to these doses not leading to inhibition of A431-FBP or KB cell growth. Furthermore, the concentrations of ZD9331 used in the *in situ* TS inhibition assay were 400- and 10-fold lower than the 4-h KB and A431-FBP growth inhibitory IC_{50} , respectively.

DISCUSSION

The α -FR is a cell surface protein that has received a significant amount of attention since Antony *et al.* first described this folate-binding protein as a folate transporter on human KB tumor cells and Campbell *et al.* first described its high abundance in human ovarian tumors (30, 50). Since then, several studies have linked α -FR overexpression with other epithelial tumors (25–28). Tomassetti *et al.* (51) recently described the association between the activation of α -FR gene transcription by the transcription factor, vHNF1, and the sus-

ceptibility of ovarian tissue to malignant transformation. The presence of such a tumor-specific protein is leading to its exploitation for cancer diagnosis and treatment, using, *e.g.*, folic acid conjugated to radionuclides or cytotoxic drugs (33–35). The α -FR has been implicated in the transport of some antifolate drugs, but it has never convincingly been established whether or not it is a biologically relevant transporter when the RFC is coexpressed and folate levels are at physiological concentrations.

The data presented in this present study suggest that, although RFC-mediated transport is the major route for antifolate uptake into A431-FBP and KB tumor cells *in vitro*, α -FR-mediated uptake is an additional route relevant to raltitrexed, pemetrexed, lometrexol, and ZD9331 (but not MTX). This was concluded from data demonstrating an \sim 3–5-fold higher activity of the drugs in A431-FBP compared with A431 cells in a physiological concentration of folate (20 nM LV). When extracellular folates were low, this increased to \sim 14-fold for raltitrexed and pemetrexed, and data support this being a result of increased α -FR-mediated uptake. KB cells express high levels of the α -FR (\sim 50% of A431-FBP; Ref. 36) and are highly sensitive to the antifolate drugs. This was ascribed, in part, to

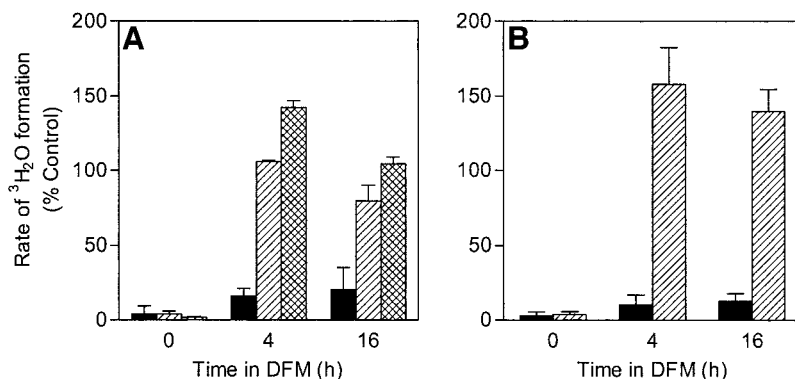


Fig. 4 Inhibition of thymidylate synthase *in situ* in A431 and A431-FBP cells, and KB cells, after 4-h exposure to equitoxic concentrations of ZD9331 followed by placement in drug-free medium for 4 or 16 h; A431, A431-FBP, and KB cells were exposed to equitoxic concentrations ($10 \times$ continuous exposure IC_{50} s; 0.8, 0.2, and 0.05 μM , respectively) of ZD9331 for 4 h. Parallel A431-FBP and KB cells were coexposed to ZD9331 and 1 μM folic acid to inhibit α -FR-mediated uptake. Cells were then resuspended in drug-free medium for 4 or 16 h, and then the rate of [^3H]dUrd was measured over 1 h. Results are given as the mean \pm SD of at least three experiments or mean \pm range of two experiments. Rate of [^3H]dUrd formation for control cells at 4 h, A431 = 4 ± 2.6 pmol/min/ 10^6 cells; A431-FBP = 2.8 ± 0.23 pmol/min/ 10^6 cells; A431-FBP (+ folic acid) = $2.3, 2.4$ pmol/min/ 10^6 cells; KB = 1.6 ± 0.45 pmol/min/ 10^6 cells; KB + folic acid = 1.8 ± 0.4 pmol/min/ 10^6 cells. A, A431-FBP (solid bar); A431-FBP + 1 μM folic acid (hatched bar); A431 (cross-hatched bar). B, KB (solid bar); KB + 1 μM folic acid (hatched bar).

α -FR-mediated uptake of ZD9331, pemetrexed, and lometrexol because the coaddition of 1 μM folic acid reduced the sensitivity of the cells between 3- and 8-fold (in 20 nM LV). Lometrexol was ~ 20 -fold more active in low folate. Thus, there are differences between the two α -FR-overexpressing cell lines in their sensitivity to the antifolates in different folate concentrations. Interestingly, A431-FBP cells were 160- and 5-fold more sensitive than A431 cells to CB3717 in 1 and 20 nM LV, respectively, and KB cells were highly sensitive in either folate concentration (36). Furthermore, the coaddition of 1 μM folic acid decreased KB sensitivity to CB3717 by ~ 100 -fold (36). This demonstrates how a compound, such as CB3717, with relatively low affinity for the RFC can display markedly increased selective activity for α -FR-overexpressing cells. These observations have led to the development of a new class of α -FR-mediated TS inhibitor, exemplified by CB300638, that are exquisitely potent (IC_{50} s ~ 3 nM) and selective for A431-FBP and KB cells (36).

The most interesting discovery was that α -FR-mediated uptake of the nonpolyglutamatable drug ZD9331 impacts significantly on the sensitivity of A431-FBP and KB cells when

drug exposure time is short. Thus, the IC_{50} s for 4- or 24-h exposure to ZD9331 were >50 - and ~ 100 -fold lower, respectively, in A431-FBP compared with A431 cells. KB cells were ~ 10 -fold less sensitive to short exposure ZD9331, but nevertheless, activity was α -FR mediated as shown by the reduction in sensitivity when 1 μM folic acid was added to compete with ZD9331 for binding to the α -FR. TS was inhibited by ZD9331 rapidly in A431 cells, and the slightly greater inhibition seen in A431-FBP and KB cells could not account for the large differences in short exposure growth inhibitory activity. This suggested that TS was inhibited for longer after extracellular drug removal in the α -FR-positive cell lines. Indeed, this was shown to be the case. Consistent with our published observations for α -FR-negative tumor cell lines, TS inhibition was found to be rapidly reversible in A431 cells, because ZD9331 is effluxed (13). Furthermore, because TS requires inhibition for several hours in order for the cell to accumulate DNA damage and elicit the downstream commitment to apoptosis, a short period of TS inhibition and growth arrest does not induce a significant amount of cell death (52). In contrast, because raltitrexed is retained as polyglutamates, the TS inhibition induced is only

Table 4 IC_{50} s for the inhibition of *in situ* TS in A431, A431-FBP, and KB cells after exposure to various concentrations of ZD9331 for 1, 4, 8, and 16 h, in the absence and presence of 1 μM FA^a

Cells were cultured in 20 nM LV, and the values represent individual experiments. The rates of control (pretreatment) flux through TS ($^3\text{H}_2\text{O}$ formed/min/ 10^6 cells) in the cell lines were as follows: A431: 2.6, 2.5 (+1 μM FA = 2.5, 2); A431-FBP: 2.4, 2.5 (+1 μM FA = 2.4, 2.3); and KB: 2.1, 1.9 (+1 μM FA = 2.1, 2.0).

	IC_{50} for inhibition of TS <i>in situ</i> (nM)					
	A431		A431-FBP		KB	
	–	+ 1 μM FA	–	+ 1 μM FA	–	+ 1 μM FA
1 h	3.9, 3.2	3.5, 3.1	2.3, 2.0	4.1, 4.0	0.76, 0.94	0.84, 0.82
4 h	2.4, 2.2	2.6, 2.1	0.62, 0.54	3.2, 3.2	0.2, 0.2	0.47, 0.45
8 h	1.0, 1.2	1.0, 1.1	0.6, 0.54	0.72, 1.0	0.15, 0.15	0.3, 0.3
16 h	1.0, 0.82	1.0, 0.84	0.62, 0.62	0.78, 0.80	0.15, 0.14	0.23, 0.21

^a TS, thymidylate synthase; LV, R,S-leucovorin.

slowly reversible (9, 10), and consequently, the drug continues to exert its effect after extracellular drug removal. Thus, although ZD9331 is not polyglutamated, it induces prolonged TS inhibition in the α -FR-overexpressing A431-FBP and KB tumor cell lines in a similar manner to polyglutamated drugs in α -FR-negative cells.

A model is proposed in which antifolate drugs with high affinity for the α -FR accumulate to high concentrations within the α -FR/endosomal apparatus, leading to continuous unloading into the cytosol. This effect increases the sensitivity of A431-FBP and KB cells to the drugs. This also provides a mechanism by which ZD9331 is retained in cells leading to prolonged TS inhibition after extracellular drug removal. It is interesting to speculate the clinical relevance of this model. ZD9331 is administered generally as a short 30-min infusion on days 1 and 8 in a three weekly cycle (5, 53). Clearance is slow, leading to prolonged TS inhibition but with some recovery between doses, at least in normal proliferating tissues, as evidenced by plasma dUrd measurements (54). ZD9331 could be predicted to localize more highly to, and for longer in, α -FR-overexpressing tumors compared with normal proliferating tissues by the mechanism suggested above. ZD9331 displayed some activity in Phase I and II trials in ovarian cancer (53, 55–57), a tumor type in which it is reported that \sim 90% of cases overexpress the α -FR. Response rates (Phase II) were \sim 10% in refractory disease, including some heavily pretreated patients (a complete response was seen in a patient receiving 8th line therapy), but the α -FR expression status of the tumors is not known. Raltitrexed displayed similar activity in ovarian cancer, although low folylpolyglutamate synthetase expression in ovarian tumors may be a factor contributing to this low response rate (47, 58). The activity of raltitrexed and pemetrexed in mesothelioma has been speculated to be attributable, at least in part, to α -FR-mediated uptake (42). Recently, Wang *et al.* (59) have described a novel transporter of pemetrexed in mesothelioma cell lines that may contribute to the activity of this drug in this tumor type. MTX has relatively low affinity for the α -FR, and it has been reported that this pathway probably does not contribute significantly to the uptake of MTX in human mesothelioma cell lines (60). The impact of α -FR-mediated uptake on efficacy of the antifolates may be limited by the doses that can be administered, *i.e.*, RFC-mediated, dose-limiting toxicities. Possibly, α -FR overexpression is a determinant of antitumor activity only when expression levels are very high. Therefore, to exploit α -FR-mediated drug uptake and retention, it may be necessary first to describe the relationship between α -FR expression levels in tumor material and response.

In summary, we have described experiments that suggest that raltitrexed, pemetrexed, lometrexol, and particularly ZD9331 may display increased activity in α -FR highly overexpressing tumor cell lines that express functional RFC and that are exposed to a physiological concentration of folate. Whether or not this is a relevant mechanism in patients treated with these drugs is not clear at present. These data do, however, provide a rationale for the development of new agents targeted selectively at α -FR-overexpressing tumors without the dose restrictions imposed by RFC-mediated uptake in normal proliferating tissues. We have described recently the discovery of such a class of compound (36, 61).

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