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Review paper

Folate-based thymidylate synthase inhibitors in cancer chemotherapy

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Understanding the relationship between chemical structure and biological properties of folate analogs, particularly their interactions with the target enzymes, transport proteins and folate-metabolizing enzyme, folylpolyglutamate synthetase (FPGS), has enabled the rational design and development of the selective thymidylate synthase (TS) inhibitors with folate-based structures for clinical uses. These compounds specifically inhibit TS devoid of concomitant effects at other loci, unlike 5-fluorouracil (5-FU). ZD1694 ('Tomudex') was designed as a non-nephrotoxic and highly active analog of N^{10}-propargyl-5,8-dideazafolic acid (CB3717), which is a potent TS inhibitor but had unacceptable nephrotoxicity caused by its poor water solubility. The potent cytotoxic activity of ZD1694 is dependent upon active uptake into cells via the reduced folate carrier (RFC), and subsequent rapid and extensive metabolism to polyglutamate forms inside cells. Marked enhancement of the TS inhibitory activity has been noted as the glutamate chain is elongated. Polyglutamation is critical to the biological activity of ZD1694 against tumor and normal proliferating tissues. The retentive property of ZD1694 polyglutamates inside cells led to a single, infrequent administration schedule in clinical studies. ZD1694 has completed phase I and phase II evaluation with activity observed in several tumor types, particularly in colorectal cancer with a 26% objective response rate. A recent European phase III study of ZD1694, randomized against a 5-FU plus leucovorin regimen, demonstrated an equivalent response rate for advanced colorectal cancer (complete or partial responses; 20 versus 17%) and less toxicity than seen with the latter regimen. The newer selective TS inhibitors, which retain potency for TS inhibition but are not substrates for RFC and/or FPGS, are currently under clinical evaluation. These classes of compound may have benefits for circumvention of resistance by virtue of alterations in these protein functions and for the management of toxicity.

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Introduction

Thymidylate synthase (TS) is a critical enzyme for the *de novo* synthesis of thymidine-5′-monophosphate (thymidylate; TMP) from deoxyuridine-5′-monophosphate (dUMP), the former being essential for DNA synthesis and repair after metabolism to thymidine-5′-triphosphate (TTP) (Figure 1). The activity of TS is dependent upon a cofactor, 5,10-methylene tetrahydrofolate (5,10-CH_2FH_4), which forms a ternary complex with TS and dUMP. The reductive methylation of dUMP to TMP results in the oxidization of 5,10-CH_2FH_4 to dihydrofolate (FH_2), an inactive folate cofactor until reduced back to FH_4 through the activity of dihydrofolate reductase (DHFR). Thus these two enzymes (TS and DHFR) participate in a 'thymidylate cycle' and both have been considered attractive targets for the development of anticancer chemotherapeutic agents. Methotrexate (MTX) is a potent DHFR inhibitor active against tumor cells of various origins in both the laboratory and the clinic. However, prolonged or repeated treatment of tumor cells growing *in vitro* with this drug causes the emergence of resistant clones by virtue of: (i) an increase in DHFR activity due to gene amplification, (ii) alteration of the reduced-folate carrier (RFC) which is responsible for the cellular uptake of physiological reduced folates and some antifolate drugs such as MTX, (iii) induction of mutated DHFR with low affinity for MTX and (iv) defective drug polyglutamation which results in reduced drug retention inside cells.[1,2] At least some of these acquired resistance mechanisms have been document to occur in the clinic.[1,2]

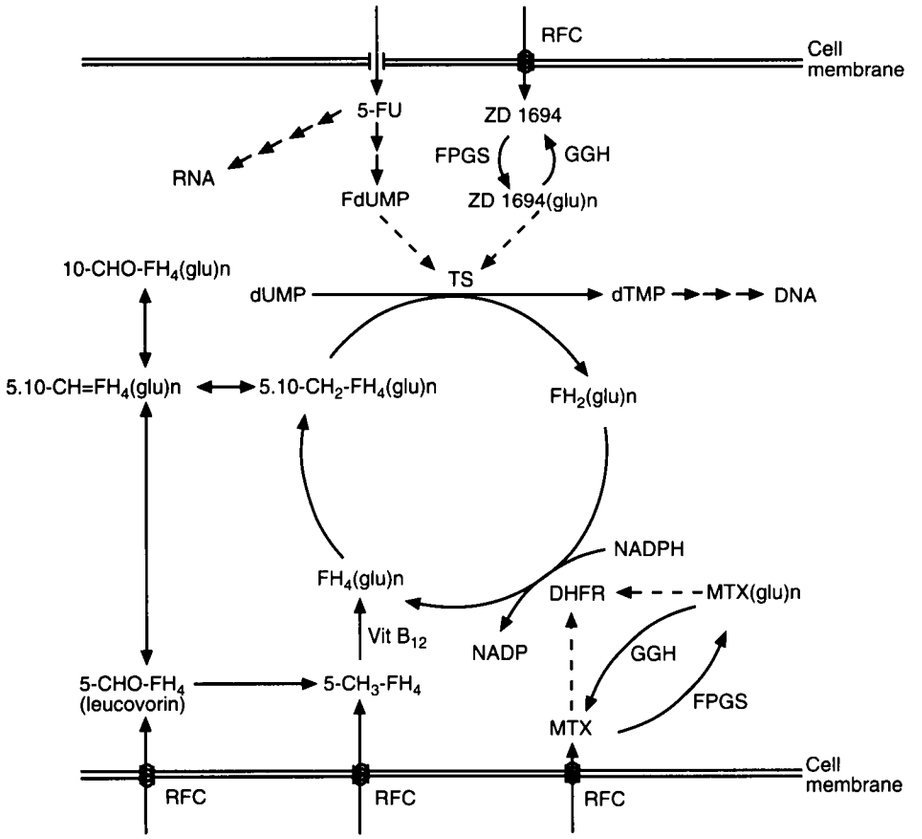

Figure 1. Enzymes of the thymidylate cycle as targets of antifolate drugs and folate metabolism.

The metabolic consequences of DHFR inhibition by MTX is indirect inhibition of *de novo* thymidylate and purine biosynthesis (Figure 1). MTX polyglutamates may also have some direct inhibitory effects on TS and aminoimidazole carboxamide ribonucleotide transformylase (AICART). There is evidence to suggest that the antipurine effect of MTX antagonizes the TS-mediated cytotoxic effects of the drug.[3] Direct inhibition of TS may be achieved by an active metabolite of the anticancer drug, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine monophosphate (FdUMP). However, 5-FU resistance is frequently accompanied by deletion or diminished activity of various activating enzymes in the multistep activation process.[4–8] Furthermore, 5-FU is extensively metabolized in cells to several anabolites other than FdUMP so that their incorporation into RNA and possibly DNA may be important additional determinants of the antitumor activity and toxicities induced by 5-FU.[9]

As the TS-mediated effects of both MTX and 5-FU are considered important determinants of their antitumor activity, it was realized that a more selective TS inhibitor would be highly desirable. The problems associated with incorporation into nucleic acids could be overcome by the design of analogs of the folate cofactor rather than the pyrimidine substrate.[9] Furthermore, the rise in dUMP that follows TS inhibition, and which can prevent stable ternary complex formation with FdUMP, may even enhance a folate-based inhibitor binding. A highly specific TS inhibitor may be expected to have activity equal to, or superior to, MTX not only in MTX-sensitive tumors, but also in those resistant by virtue of increased activity of DHFR. Moreover, such a compound may be less toxic to the gut since the synthesis of purines would not be affected. In a search for novel folate-based TS inhibitors, modifications were made to the quinazoline analog of folic acid. Introduction of a propargyl group at the N^{10} position of 5,8-dideazafolic acid was shown to enhance greatly TS inhibitory action,[10] leading to the development of the first folate-based TS inhibitor for clinical use (CB3717; N^{10}-propargyl-5,8-dideazafolic acid). Clinical trials of CB3717 demonstrated

significant activity in a number of solid tumors but sporadic, life-threatening nephrotoxicity, a result of the poor aqueous solubility of the drug at urinary pH,[11,12] led to withdrawal of the drug from further study. A non-dose-related and self-limiting hepatotoxicity was also noted. The unacceptable nephrotoxicity of this prototype TS inhibitor urged further modification of the folate structure in the search for water-soluble, non-nephrotoxic analogs of CB3717. Removal of the amino group at the 2-position of CB3717 was shown to improve its solubility (more than 340 times at pH 7.4) and a series of 2-desamino-2-methyl-N^{10}-substituted-5,8-dideazafolate analogs followed, which were new highly water soluble and non-nephrotoxic (in mice) experimental agents.[13–18] Structurally diverse compounds had different affinities for TS, folate transport proteins and the folate-metabolizing enzyme, folylpolyglutamate synthetase (FPGS).[19,20] Among these water-soluble compounds synthesized, a number had a heterocyclic ring replacing the benzoyl ring. From these, the N^{10}-methyl thiophene substitute compound (ZD1694; raltitrexed; Figure 2) was identified as the most promising compound *in vivo* in terms of potency and spectrum of activity against a range of tumor models, including human tumor xenografts.[21–24] ZD1694 entered phase I clinical study in Europe in 1991, and was soon followed by a US phase I trial and several multi-center worldwide studies. The results of phase II evaluation demonstrated antitumor activity in several tumor types, particularly in advanced colorectal and breast cancer with a 26% objective response rate.[25] Recent results of a multicenter phase III study of ZD1694 ('Tomudex') in 439 patients with advanced colorectal cancer, randomized against a 5-FU plus low-dose leucovorin regimen, showed an equivalent or higher response rate and reduced toxicities such as leucopenia and mucositis compared with the latter regimen.[26,27]

In this review, we will concentrate on the integration of biochemical and pharmacological properties with *in vitro* and *in vivo* activity, and with clinical efficacy and toxicity of folate-based, selective TS inhibitors. Particular attention is paid to the critical role of polyglutamation of ZD1694 on biological and toxicological aspects of drug action. Resistance mechanisms to the folate-based TS inhibitors will also be discussed.

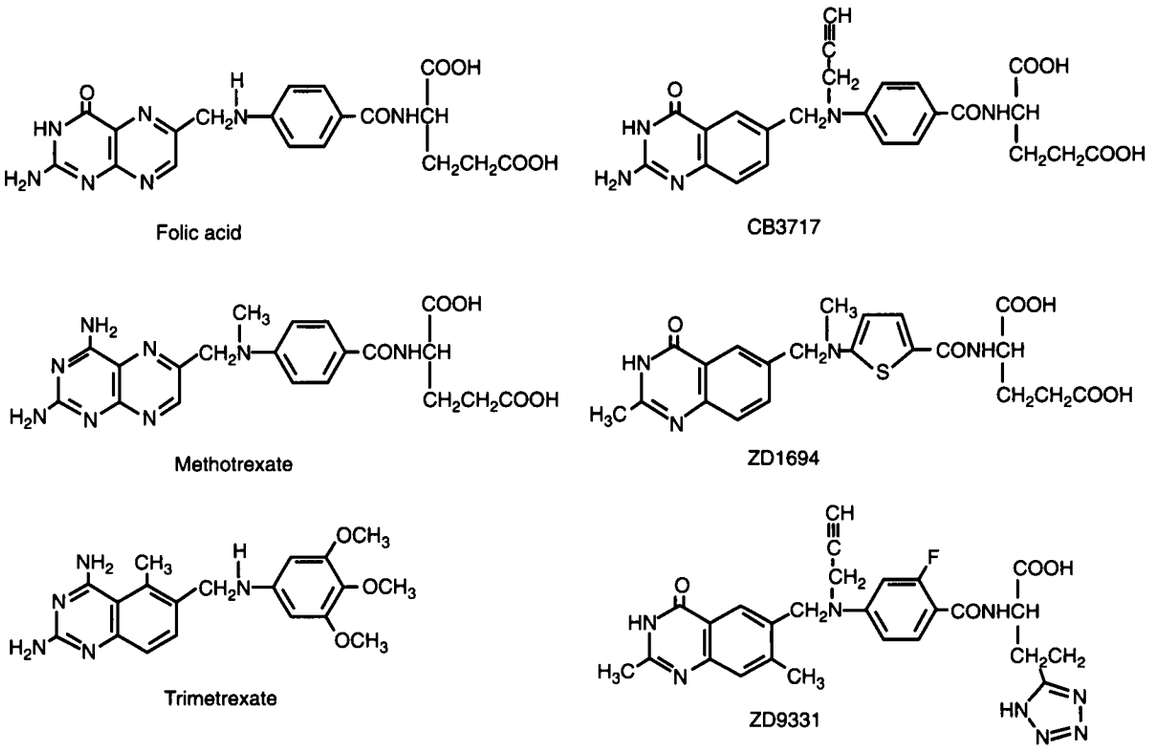

Figure 2. Chemical structures of the folate-based TS inhibitors.

Biochemical properties and cellular pharmacokinetics

CB3717 is a potent inhibitor of isolated TS ($K_i \sim 3$ nM), but with only moderate potency as an inhibitor of tumor cell growth *in vitro* and *in vivo*.[9,10,15,28–31] As shown in Table 1, ZD1694 is approximately 500-fold more active as an inhibitor of cell growth (murine and human cell lines) than CB3717 despite being 20-fold less effective as an inhibitor of isolated TS ($K_i \sim 62$ nM).[24] The facts that the cytotoxic effect of ZD1694 was prevented by co-incubation with thymidine alone[24] and ZD1694 is not active against TS overproducing cell lines[32,33] but has good activity against DHFR overproducing cells[34,35] support TS but not DHFR as the cytotoxic locus of action. For the folate-based TS inhibitors, potent inhibition of isolated TS does not necessarily translate into biological activity in whole cell systems or *in vivo* and this discrepancy suggests the existence of other factors which influence the cytotoxic potency of TS inhibitors. Major factors include cell membrane transport and polyglutamation.

It has been shown that there are at least two cellular membrane transport pathways for folate analogs; folic acid enters cells predominantly via a membrane-associated folate-binding protein (FBP) system, whereas reduced folates and MTX utilize the RFC as the cellular entry route. The observation that mutant cell lines, with impaired RFC, did not show marked cross-resistance to CB3717[28,36] suggested that the RFC was not the predominant cell uptake mechanism for this compound. CB3717 does have a high-affinity for the FBP[37] and cells overexpressing this protein are very sensitive to CB3717. CB3717-resistant leukemia cells displayed a decreased uptake for MTX.[34,38] Thus, multiple membrane transport systems may operate for CB3717.[37] On the other hand, ZD1694 is actively transported via the RFC (K_m ZD1694 ~ 2.5 μM; K_m CB3717 ~ 40 μM);[24,35] therefore, the MTX-resistant cells (impaired RFC) demonstrated a high level of cross-resistance to ZD1694. In fact, [^{3}H]ZD1694 uptake was markedly decreased in these cells.[39]

After uptake into cells, CB3717 and ZD1694 are metabolized to polyglutamate forms by FPGS, which catalyzes the addition of glutamate residues to folates and some glutamate-containing 'classical' antifolates. Polyglutamation of these folate-based TS inhibitors is considered to be significant for two reasons: (i) drug retention in the cells and (ii) increased potency of the drugs towards TS. MTX is also a polyglutamatable antifolate drug; MTX-polyglutamates are retained inside cells, however, unlike these TS inhibitors, are not significantly more potent as inhibitors of the target enzyme, DHFR, than the parent monoglutamate,[40] and the importance of their formation lies in their drug-retentive properties.[41] In contrast, the tetraglutamate derivative of ZD1694 (the predominant metabolite in most cultured cells), for example, has been shown to have 60-fold higher affinity for TS than the parent drug.[42] Much *in vitro* data suggests that the formation of polyglutamates and their accumulation and retention inside cells seem to be responsible for the enzyme inhibition and the biological efficacy of the drug. A difference in substrate activity for FPGS has been demonstrated among physiological folates and antifolate drugs.[43–47] ZD1694 is an excellent substrate for isolated FPGS (mouse liver $K_m = 1.3$ and 40 μM for ZD1694 and CB3717, respectively) which suggested that, at low intracellular concentrations, ZD1694 would be metabolized to at least the diglutamate 100-fold faster than CB3717.[43] This is supported by experiments that actually measured CB3717 and ZD1694 polyglutamation in L1210 cells. ZD1694 at a considerably lower concentration (0.1 μM) formed polyglutamates more rapidly and to a greater extent than CB3717 (50 μM).[48,49] The higher rate of polyglutamation, in addition to the effective utilization of the RFC for cellular uptake, seems to account for the two or three orders of magnitude greater potency of ZD1694 against various tumor cell lines compared with CB3717 (Table 1). The difference in polyglutamation rate among antifolate drugs may relate not only to biological activity of, but also to the emergence of resistance mechanisms to, an antifolate. Indeed, L1210 murine leukemia cells with acquired ZD1694 resistance exhibited greatly diminished polyglutamation of ZD1694 and were cross-resistant to antifolate active through polyglutamation.[50] However, L1210 cells acquired CB3717 resistance through TS overproduction in the same drug exposure conditions (continuous exposure).[51]

Taking into account the critical role of polyglutamation on the TS inhibitory effect of these drugs, the intrinsic biological expression of enzymes such as FPGS in tumor cells must be responsible for antitumor efficacy (and toxicity). For example, a 3-fold higher rate of polyglutamation in K562 leukemia cells compared with MOLT-3 cells seemed to account for the higher sensitivity of the former cells to ZD1694.[52] A decreased cellular content of ZD1694 polyglutamates was associated with cross-resistance to this drug in various antifolate-resistant leukemia sublines of MOLT-3.[34] Furthermore, ZD1694 polyglu-

Table 1. Biological activities of the folate-based TS inhibitors against the sensitive or resistant tumor cell lines to various folate analogs

Cell line	IC_{50} values (μM) (relative resistance)				Mechanisms of resistance to folate analogs
	CB3717	ZD1694	MTX	TMQ	
MOLT-3 (human, leukemia)[a]	1.1	0.0038	0.0090	0.0040	
MOLT-3/MTX_{10000}[a]	30 (27)	5.5 (1400)	70 (7800)	0.0035 (0.88)	impaired RFC; increase in DHFR activity
MOLT-3/MTX · P-9[a]	5.0 (4.5)	0.020 (5.3)	0.014 (1.6)[c]	0.00068 (0.17)	defective polyglutamation for MTX
MOLT-3/TMQ_{800}[a]	0.88 (0.80)	0.0046 (1.2)	0.063 (7.0)	1.2 (300)	impaired transport for TMQ; increase in DHFR activity
MOLT-3/TMQ_{800}-MTX_{10000}[a]	2.0 (1.9)	0.026 (6.8)	100 (11000)	>10 (>2500)	amplification of mutated DHFR with low affinity for MTX
L1210 (murine, leukemia)[b]	5.0	0.0088	0.011	0.018	
L1210:1565[b]	3.8 (0.76)	0.76 (86)	0.94 (85)		impaired RFC
L1210:R7A[b]	41 (8.2)	0.028 (3.2)	7.1 (640)		increase in DHFR activity
K562 (human, leukemia)[a]	0.90	0.0015	0.032	0.0028	
W1L2 (human, lymphoblastoid)[b]	2.6	0.0046	0.0094	0.014	
CH1 (human, ovarian)[b]	7.0	0.025	0.018		
41M (human, ovarian)[b]	5.6	0.013	0.025	0.082	

[a]Determined by the MTT assay.[34,39,52]
[b]Determined by the cell growth inhibition assay.[35,50]
[c]Relative resistance is more than 250-fold when the cells were exposed to MTX for 24 h and then cultured in drug-free medium for 72 h.

tamation in normal proliferating tissues may relate to the toxic effects of ZD1694; the high rate of polyglutamation in normal gut mucosal cells in the mouse is believed to be responsible for the intestinal toxicity of this drug.[53] The intracellular content of polyglutamate derivatives is probably determined not only by FPGS activity but also γ-glutamyl hydrolase (GGH) activity, the latter being responsible for the hydrolysis of the poly-γ-glutamyl chain and the degradation of polyglutamates back to the monoglutamate. Therefore, a low FPGS activity or high GGH activity may result in poor accumulation and retention of intracellular polyglutamates and contribute to resistance to polyglutamatable antifolate drugs.[50,54–58]

Leucovorin (folinic acid, 5-formyl-tetrahydrofolate; LV) prevented the cytotoxic effects of the folate-based TS inhibitors[24,59] through competition for cellular uptake (RFC) and FPGS. LV reversed the cytotoxicity of ZD1694 more effectively than that of CB3717. In contrast, it enhances the TS inhibitory effect produced by 5-FU in many colon tumors through stabilizing the enzyme-FdUMP-5,10-CH_2FH_4 ternary inhibitory complex.[60]

In vitro activity against resistant tumor cell lines and acquired resistance mechanisms to the folate-based TS inhibitors

The antifolate-resistant cell lines with impaired RFC function (MOLT-3-MTX_{10000} and L1210:1565) showed a marked cross-resistance to ZD1694 (Table 1). Polyglutamation-defective cell lines showed a remarkable cross-resistance to ZD1694 and to some extent to CB3717, but not to MTX in the continuous drug exposure conditions.[39] Similarly the L1210:R^{D1694} cell line (acquired resistance to ZD1694) was only cross-resistant to MTX under short-exposure conditions. Since potency of MTX-polyglutamates towards DHFR inhibition is not significantly higher than the parent monoglutamate, continuous exposure of cells to MTX overcomes defective polyglutamation.[39,61] Overexpression of DHFR had little influence on the cytotoxic efficacy of the TS inhibitors irrespective of normal (L1210:R7A) or mutated gene expression (MOLT-3-TMQ_{800}-MTX_{10000}).

There have been a number of experimental studies relating to the cellular and molecular mechanisms of acquired resistance to MTX *in vitro* and *in vivo*. Resistance to 5-FU, a pyrimidine-based drug, may be mediated by multiple mechanisms related to the complexity of fluoropyrimidine metabolism and the multiple sites of biochemical action of 5-FU. In addition to its metabolism to FdUMP, a potent inhibitor of TS, 5-FU is also converted to several ribo- and deoxyribopyrimidine intermediates,[4] thus, deletion of the activating enzymes is shown to be a common cause of resistance.[4–8] An increase in TS activity concomitant with gene amplification and/or overexpression has been reported as a mechanism of experimental resistance to pyrimidine-based TS inhibitors[62–66] and may occur in the clinical situation in response to treatment with 5-FU.[67]

It is of interest to document the resistance mechanisms developed in tumor cells following exposure to highly specific TS inhibitors of the quinazoline antifolate class of compound. Table 2 summarizes cross-resistance patterns and resistance mechanisms in tumor cell lines made resistant to various folate-based TS inhibitors. Three major mechanisms of resistance have been recognized: (i) overproduction of the target enzyme, TS, (ii) impaired RFC-mediated membrane drug transport and (iii) diminished polyglutamation. It is expected that the development of a particular resistance phenotype is affected by factors such as the biochemical properties of the antifolate drugs, the intrinsic cellular characteristics and the manner in which drug resistance is raised. For example, the finding that L1210 cells developed TS overproduction or defective polyglutamation as resistance mechanisms following continuous exposure to CB3717 (L1210:C15)[51] or to ZD1694 (L1210:R^{D1694}),[50] respectively, may relate to relatively greater importance of polyglutamation to the activity of the ZD1694. However, it is possible that for a given cell line, all potential mechanisms of resistance may be individually displayed in resistant clones obtained on different occasions or under different culture conditions. K562 cells, with an intrinsically high rate of polyglutamation of ZD1694, acquired resistance to ZD1694 by under-expressing (or altering) the RFC. On the other hand, MOLT-3 cells developed resistance by reducing the rate of ZD1694 polyglutamation. Although MOLT-3 cells developed different resistance mechanisms to MTX in cells made resistant using different drug exposure schedules (impaired RFC plus DHFR overproduction versus diminished polyglutamation for continuous or high-dose, short-term drug exposure, respectively),[39,61,68] the exposure of MOLT-3 cells to ZD1694 resulted in sublines with defective polyglutamation irrespective of whether resistance was raised using continuous or pulsatile drug treatment.[39] In addition, MOLT-3 cells developed CB3717 resistance through TS gene

Table 2. Characterization of the sublines made resistant to folate-based TS inhibitors

Subline (reference)	Drug used for the selection of resistance	Degree of resistance (fold resistance)					Primary mechanism(s) of resistance
		CB3717	ZD1694	MTX	TMQ	5-FdUrd	
L1210:C15[51]	CB3717	> 200		5.4		1.2	increase in TS activity
L1210:R^{D1694}[50]	ZD1694	2.6	> 11000	2.2	0.89	1.5	defective polyglutamation
MOLT-3/CB3717$_{40}$[34]	CB3717	30	12	2.2	0.060		impaired RFC; possibly defective polyglutamation
MOLT-3/ZD1694 · C[39]	ZD1694	1.5	1600	0.53	0.033		defective polyglutamation
MOLT-3/ZD9331*	ZD9331	4.8	63	120	0.88		increase in TS activity; impaired RFC
K562/ZD1694 · C[52]	ZD1694	> 11	4200	170	1.6		impaired RFC
W1L2:R179[33]	ZM249148[a]	100	12	0.40	0.33		increase in TS activity
W1L2:R^{D1694}[50]	ZD1694	> 38	> 22000	0.26	1.3	1.4	increase in TS activity
W1L2:C1[32]	ICI198583[b]	> 190	> 17000	0.28	0.38	1.3	increase in TS activity; defective polyglutamation
CH1:R^{D1694}[50]	ZD1694	10	14	1.7	1.5	9.3	increase in TS activity
41M:R^{D1694}[50]	ZD1694	1.4	120	36	0.45	2.1	impaired RFC

[a] *p*-[*N*-(7-bromo-3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-(prop-2-ynyl)amino]-*N*-(2-pyridylmethyl)benzamide.
[b] C^2-desamino-C^2-methyl-N^{10}-propargyl-5,8-dideazafolic acid.
*Kobayashi H, *et al.*; in preparation for publication.

amplification in medium supplemented with pteroylglutamic acid (PGA), but through impaired membrane transport for CB3717 (either FBP or RFC system) in medium with LV.[38]

Several studies have elucidated, in part, the molecular mechanisms of resistance to the TS inhibitors. Increases in TS activity in the resistant cell lines to CB3717, ZD1694 or ICI 198583 were accompanied with increases in TS gene copy number and mRNA levels.[32,33,69] The degree of resistance of W1L2:R^{D1694} and CH1:R^{D1694} cells to ZD1694 (Table 2) was significantly higher than the fold increase in gene copy number, mRNA or protein levels. Thus, small changes at the TS gene level may translate into clinically significant alterations in sensitivity to the drug.[69] This suggests that specific suppression of the amplified gene at the DNA or mRNA levels may efficiently reverse the resistance. This is supported by the fact that a hammerhead ribozyme against human TS mRNA succeeded, in part, in the reversal of resistance to TS inhibitors in W1L2:R179 cells with TS overproduction.[33] In polyglutamation-defective cell lines,[39,50] despite the virtual absence of polyglutamate forms of the ZD1694 used for the selection of the resistance, the growth of the resistant cells was only minimally affected, suggesting that physiological folates essential for cell survival and proliferation could be effectively polyglutamated. This is consistent with the fact that FPGS activity was decreased to about 10% of that observed in the parental cells.[50] Similarly, there were moderate decreases in FPGS mRNA expression in the polyglutamation-defective ZD1694-resistant MOLT-3 cells.[39] For both examples, the reduction in FPGS (activity or mRNA, respectively) did not correlate with the virtual absence of polyglutamate forms of ZD1694. The findings above may indicate a genetic mutation of FPGS, which results in the altered affinity of the enzyme only for the drug or class of drugs used for the selection of resistance, but not for physiological folates. Other investigators also demonstrated that cells resistant to antifolates by virtue of decreased FPGS activity do not necessarily have reduced FPGS mRNA levels.[70] Recently it was shown that there are several different splice forms of FPGS mRNA and variable expressions of the different forms in different cells.[71,72] Some of the spliced mRNA do not encode functional FPGS, associating with acquired resistance to antifolates (B Shane, personal communication). The molecular characterization of FPGS in the polyglutamation-defective MOLT-3 cells is currently underway.

It has been noted that TS levels in tumor cells increased following acute exposure to 5-FU.[73,74] Recent investigations have demonstrated that the TS protein is capable of binding to and thereby controlling the translational efficiency of its own mRNA in a negative autoregulatory manner.[75,76] Occupancy of substrate sites of TS by either the physiological substrates or either antifolates or nucleotide inhibitors produces a conformational change that inhibits its binding to TS mRNA and results in the impairment of the translational regulatory ability of the protein with a consequent increase in total cellular

TS. There were 10- to 40-fold increases in TS protein levels in normal and tumor-derived human mammary epithelial cells following treatment with ZD1694, while TS mRNA levels remained unchanged.[77] The acute induction of TS protein may result in clinical insensitivity in patients treated with 5-FU or folate-based TS inhibitors. Approaches to overcome the acute TS induction are presently under investigation, including co-administration of translational inhibitors[77] or the design of small molecules that retain an ability to inhibit TS but do not interfere with the protein–RNA interaction that maintains intracellular TS levels.[78] Interferon-γ was also shown to repress the acute TS induction accompanying enzyme inhibition.[74,78] In another approach using gene therapy, antisense or ribozyme specifically targeted at TS mRNA may be useful as a tool for the suppression of acute TS induction.[33,78]

In vivo studies and clinical investigations of ZD1694 ('Tomudex')

A pharmacokinetic study of ZD1694 in both mice and rats demonstrated a rapid clearance of the drug from plasma with a half-life of approximately 30 min, which was significantly faster than CB3717 clearance ($t_{1/2\beta} = 93$ min).[79] Drug clearance was triphasic with a prolonged final elimination phase, which may result in a persistence of low plasma drug levels, the significance of which is unknown.[35] As predicted from *in vitro* studies, rapid drug accumulation and retention due to extensive polyglutamation was reported in some mouse tissues, resulting in much higher drug concentrations in tissues than in the plasma. The drug concentration in liver, kidney and small intestinal mucosa was approximately 50- to 100-fold higher than plasma, when mice were injected i.p. with 5 mg/kg [^{3}H]ZD1694 and tissues removed 24 h later.[53]

A single dose of 10 mg/kg ZD1694 (i.p. administration) cured DBA2 mice bearing a thymidine kinase (TK)-deficient mutant L5178Y mouse lymphoma.[23,80] When given in a 10 mg/kg/daily × 5 days i.p. schedule, which produced cures in mice bearing the L1210:ICR ascitic mouse tumor,[24] some toxicities against the gastrointestinal tract and bone marrow were manifested.[80] Co-administration of either thymidine or LV prevented the antitumor activity and toxicity of ZD1694.[24] The reversal effect produced by LV probably relates to the antagonistic effect which it may have on drug uptake and polyglutamate formation, as outlined earlier. ZD1694 did not induce nephro- and hepatotoxicity after 500 mg/kg i.v. bolus or repeated doses, and contrasted with that observed with CB3717 at 100 mg/kg i.v. bolus.[22] Human tumor xenograft studies demonstrated a superior antitumor efficacy of ZD1694 over CB3717, 5-FU and MTX, although the same daily × 14 protocol was used for all three drugs.[81].

Following animal studies, ZD1694 ('Tomudex') entered phase I trials in Europe in 1991. Determination of a safe starting dose was not easy to predict from rodent studies because of the high level of plasma thymidine in rodents relative to humans, which essentially circumvents TS inhibition.[31] Dogs, which have thymidine levels similar to humans, were used to predict a safe dose for the phase I study. The dose chosen was 0.1 mg/m^2 to be given via a 15 min infusion once every 3 weeks. Since extensive polyglutamation of ZD1694 was predicted to occur in both tumors and normal tissues leading to drug retention, it was believed that an infrequent bolus (short-infusion) dosing regimen would (i) give antitumor activity and (ii) reduce the likelihood of unacceptable toxicity occurring in clinical studies.[35] A European phase I study with 61 patients with a range of tumors defined a recommended dose of 3.0 mg/m^2 given once every 3 weeks for phase II evaluation.[82,83] Toxicity encountered at doses of 1.6 mg/m^2 or above included gastrointestinal (diarrhea) and hematological (mainly neutropenia) toxicities, malaise, and reversible asymptomatic elevation in liver transaminases, and a maximum tolerated dose of 3.5 mg/m^2 was established. No drug-induced nephrotoxicity was observed. Pharmacokinetic studies showed a triphasic elimination with a prolonged third phase of up to 105 h.[83] On the one hand, the results of a US phase I trial conducted by the National Cancer Institute recommended 3.0–4.0 mg/m^2 as starting dose for phase II trials.[84]

Although 'Tomudex' (3.0 mg/m^2), in phase II studies, had demonstrated antitumor activity in a range of tumor types[25] including advanced breast,[85] pancreatic,[86] non-small cell lung[87] and refractory ovarian cancer after platinum-based therapy,[88] the most notable activity was seen in advanced colorectal cancer, with an objective response rate of 26%.[25,89] Toxicity profiles reported across all these studies were similar to those reported in the European phase I study. The significant activity of 'Tomudex' as a single agent against colorectal cancer had led to a randomized multicenter, international phase III study comparing 'Tomudex' given 3.0 mg/m^2 as a single i.v. bolus infusion once every 3 weeks with an accepted regimen of 5-FU (425 mg/m^2) and low dose LV (20 mg/m^2) (given five consecutive

daily i.v. bolus injection, repeated every 4–5 weeks; the Mayo regimen).[26] The analysis of 434 evaluable patients with previously untreated advanced colorectal cancer resulted in complete or partial responses in 20% of patients who received 'Tomudex' and in 17% of patients with 5-FU plus LV.[27] Median survival was about 10 months for both treatments. However, patients who received 'Tomudex' spent a substantially shorter time in hospital for dosing and had significantly lower rates of grade 3 and 4 toxicities such as leucopenia and mucositis, although in the 'Tomudex'-treated patients there was a higher incidence of reversible elevation of transaminases of limited clinical significance. The final results of the phase III study of 'Tomudex' concluded that 'Tomudex' has similar efficacy and an acceptable safety profile, and has a more convenient administration schedule when compared with a standard regimen of 5-FU plus low dose LV against advanced colorectal cancer.[26,27] The US phase III trial of 'Tomudex' (3.0 mg/m^2) was completed in 1995 and the results are pending.

'Tomudex' has been recently registered in the UK and several other countries for the first-line treatment of advanced colorectal cancer.

Investigational new folate-based TS inhibitors

ZD1694 requires metabolic activation by FPGS and it is possible that extensive polyglutamation of the drug in normal tissues may be responsible for the occasional unacceptable degree of toxicity, such as diarrhea and bone marrow suppression. This prompted further design and development of an alternative class of TS inhibitors which are not substrates for FPGS. Non-polyglutamatable drugs may be desirable for two reasons. First, such compounds will not be retained intracellularly (at least through polyglutamation) so that TS inhibition may be more transient, which in turn may give more control over normal tissue toxicity. Secondly, these new drugs may display a different spectrum of antitumor activity and will overcome antifolate-resistance due to impaired polyglutamation. However, they may require less convenient administration protocols than those used for 'Tomudex', e.g. continuous infusion.

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; Figure 2), which uses the RFC but is not a substrate for FPGS, is a highly water-soluble non-polyglutamatable potent TS inhibitor (K_i = 0.4 nM; Table 3).[90–95] As expected, ZD9331 overcame the ZD1694-resistance in L1210:R^{D1694} cells with defective polyglutamation.[91] ZD9331 is active in experimental tumor models and toxicities observed include myelosuppression and gastrointestinal toxicities (ref. 93 and unpublished observation). ZD9331, which possesses a profile of antitumor activity discretely separate from that of ZD1694, is in phase I clinical study. 1843U89, another new selective TS inhibitor, enters cells via RFC and is an excellent substrate for FPGS, but does not require polyglutamation for its TS inhibitory potency.[96] Of interest is the fact that its diglutamate metabolite is a very poor substrate for FPGS so that higher chain-length polyglutamates are not formed. In addition, the diglutamate shows no higher potency for TS inhibition than the parent monoglutamate.[97] Therefore, reduced polyglutamation capacity as a resistance mechanism should be less important for the 1843U89 than for the compounds requiring polyglutamation for activity. The dose-limiting toxicity of 1843U89 is the small intes-

Table 3. Cellular pharmacological properties of the newer class of folate-based TS inhibitors

Drug	K_i value for TS (nM)	Substrate activity for:		Current clinical studies
		RFC	FPGS	
ZD1694	62 (monoglutamate) 1.0 (tetraglutamate)	(+)	(+)	licensed in several countries
ZD9331	0.4	(+)	(−)	phase I
1843U89	0.09 (monoglutamate) 0.13 (diglutamate)	(+)	(+)[a]	phase I (1843U89/folic acid combination)
LY231514	263 (monoglutamate) 4.2 (pentaglutamate)	(+)	(+)	phase I, II
AG337	11	(−)	(−)	phase I, II
AG331	2	(−)	(−)	phase I

[a]One-step polyglutamation to diglutamate.

tine (diarrhea) in dogs and mice. The finding that co-administration of folic acid resulted in the effective reduction of the drug-induced gut toxicity along with lack of reversal of its antitumor efficacy strongly supports the clinical evaluation of the 1843U89/folic acid combination in humans, and this strategy is now under phase I trial.[97] LY231514, another new class TS inhibitor which is a substrate for RFC and FPGS, and targets multiple enzymes in the folate-dependent pathways, is also currently in phase I and phase II trials.[98–100]

Newer folate-based TS inhibitors with diverse structures and properties have been rationally developed, using an X-ray crystal structure-based design approach founded on the knowledge of the three-dimensional structure of the TS protein.[101–104] AG337 and AG331, lipophilic selective TS inhibitors, were specifically designed by using such computer-assisted technology. Such compounds were designed to circumvent some of the intrinsic or acquired resistance to which the 'classical' antifolates might be subjected.[105] These are not substrates for RFC and FPGS, but retain their cytotoxic activity through TS inhibition.[105,106] It is expected that these non-classical, lipophilic TS inhibitors would enter cells by passive diffusion and would require prolonged administration at higher dose levels than the 'classical', polyglutamatable TS inhibitors such as ZD1694 to elicit maximal antitumor efficacy, because of the absence of significant intracellular retention. Clinical evaluations of these compounds are currently underway in the US and UK.[107–113] Excellent p.o. bioavailability of AG337 led to clinical investigations of orally-administered AG337, which are also presently underway.[114,115]

Conclusion

In this review, we provide the background for the design and development of ZD1694 and other folate-based TS inhibitors being investigated, and describe some of their biochemical and pharmacological properties, and activities in sensitive and resistant cells. Rational design of the folate-based TS inhibitors was based on an extensive understanding of the relationship between chemical structure and biological properties, and of analog interactions with TS, transport proteins and FPGS. The development of these folate-based TS inhibitors exemplifies a targeted approach to the design of new cancer chemotherapeutic agents. The phase III study of 'Tomudex' against advanced colorectal cancer recently demonstrated that as a single agent, it has good activity and compares favorably with the standard regimen of 5-FU plus low dose LV. The uncomplicated administration protocol for 'Tomudex' (given i.v. bolus once every 3 weeks) may contribute to the quality of life in patients. Although ZD1694 requires metabolic activation by FPGS to exert its antitumor activity, its cytotoxic effect is mediated solely through TS inhibition. This distinguishes it from both 5-FU and MTX. ZD1694 may have an incompletely overlapping spectrum of antitumor activity compared with 5-FU and thus may show activity against 5-FU-resistant tumors. However, although the critical role of uptake and polyglutamation of ZD1694 for its biological activity may be associated with therapeutic selectivity, it may also be associated with drug resistance (intrinsic or acquired). Since impairment of RFC function or defective polyglutamation in tumor cells is expected to compromise the utility of the RFC-mediated, polyglutamatable TS inhibitors, an alternative class of TS inhibitors, which are independent of RFC function and polyglutamation for biological activity, have been under full clinical development. The selective TS inhibitors with diverse chemical structure and different biological properties may find important positions in cancer chemotherapeutic regimens.

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