Minireview

Thymidylate Synthase Inhibitors

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Abstract

Folate analogues that inhibit thymidylate synthase (TS) selectively were developed based on TS and folate molecular structures and properties. The structure-activity relationship, preclinical and clinical development, and issues of potential importance in the future success of these TS inhibitors are reviewed herein. Properties of these new compounds depend mainly on the use of the reduced folate carrier (RFC) proteins for cellular entry and on intracellular polyglutamation, which augments TS inhibitory function and cellular retention. CB3717 [Zeneca (formerly ICI Pharmaceuticals), Macclesfield, United Kingdom], the first selective TS inhibitor developed, demonstrated antineoplastic activity in Phase I trials, but its development was abandoned due to nephrotoxicity. ZD1694 (Tomudex; Zeneca), a water-soluble, nonnephrotoxic quinazoline, demonstrated activity in colorectal, breast, and pancreatic cancer. Phase III trials of Tomudex in advanced colorectal cancer were completed recently. LY231514 (Eli Lilly Research Labs, Indianapolis, IN) uses the RFC and polyglutamation to exert its selective TS inhibitory action. Phase I trials of this compound have been completed. ZD9331 (Zeneca), currently in preclinical studies, was designed to obviate the use of the RFC for cellular entry. 1843U89 (Glxoso-Wellcome, Research Triangle Park, NC), currently in Phase I trial, does not require the RFC; polyglutamation of this potent TS inhibitor leads to its higher cellular retention without augmenting its TS inhibitory activity. Currently in clinical trials, AG337 (p.o. and i.v. forms) and AG331 (both by Agouron, La Jolla, CA) are lipophilic potent TS inhibitors with action independent of the RFC and polyglutamation. Multiple mechanisms through which cells may overcome TS inhibition have been described. Combining TS inhibitors with other agents that affect TS, interfere with TS gene and mRNA regulation, or inhibit salvage mechanisms of thymidylate depletion may potentially enable greater clinical utility of this class of compounds.

Introduction

TS inhibitors are a new class of compounds that target TS, an enzyme in the folate metabolic pathways necessary for thymidylate synthesis, specifically. By inhibiting TS, these agents decrease de novo thymidylate synthesis, which is necessary for DNA synthesis and repair. Contrary to prior empiric approaches to anticancer drug discovery, the development of new TS inhibitors was based on the targeted and specific design of new molecules and relied on extensive knowledge of the structure, conformation, and properties of TS and the mode of action of folate analogues. These compounds are being tested currently in preclinical and clinical settings, and their role in clinical practice as single agents or in combination regimens remains to be defined. This review will provide a background of the mechanism of TS inhibition, the development and current status of folate analogues that inhibit TS selectively, the mechanisms of resistance to TS inhibition, and the current approaches to augment the efficacy of these new compounds.

TS

A critical step in the de novo pathway of DNA synthesis is the production of the pyrimidine nucleotide TMP from dUMP. This reaction is catalyzed by the enzyme TS using the folate cosubstrate CH2;THF (Fig. 1) and is the only source of de novo cellular thymidylate.

TS (Fig. 1) is a two-substrate enzyme that accepts both dUMP and CH2;THF to form a ternary complex through which reductive methylation of dUMP to TMP occurs. In this process, tetrahydrofolate is oxidized to dihydrofolate. This reaction provides the de novo cellular thymidylate nucleotides necessary for DNA synthesis and repair. TS inhibition results in a thymine-less state, which is toxic to actively dividing cells. This cytotoxicity is effected likely by DNA fragmentation that results from dTTP depletion, which increases misincorporation of dUTP (1). Cells containing the salvage enzyme TK, which uses circulating thymidine, can circumvent this cytotoxicity. However, in normal tissues and in some tumor cells, the circulating thymidine concentrations may not be sufficient to achieve a significant effect (2).

The enzyme involved in regeneration of tetrahydrofolate from dihydrofolate is DHFR (Fig. 1). Folate analogues (antifolates), such as MTX, inhibit DHFR, deplete tetrahydrofolate, and cause a marked decrease in pyrimidine and purine synthesis (3-5). Mechanisms of resistance to MTX include reduced drug transport across cell membranes (6-8), reduced drug polyglu-
Thymidylate Synthase Inhibitors

MTX (Glun)

FH4 (Glun)

MTX

FPGS

MTX

Thymidylate Synthase

FH2 (Glun)

FH4 (Glun)

N5-10 methylene FH4 (Glun)

FH2 (Glun)

dUMP

dTMP

de novo Pyrimidine Synthesis

DNA Synthesis

N5 formyl FH4 (Glun)

FH4 (Glun)

Purines

N10 formyl FH4 (Glun)

Fig. 1 Folate metabolic pathways. Inner square, role of TS in DNA synthesis. Dashed arrows, inhibitory effect. FH4, tetrahydrofolate; FH2, dihydrofolate; (Glun), polyglutamate; MS, methionine synthase; CYCLO, cyclo,5,10,methenyl-FH4-cyclohydroxylase; GAR, glycaminide ribotide transformylase; AICAR, aminoimidazole carboxamide ribotide transformylase.

tamation (polyglutamation confers intracellular drug retention), gene mutation resulting in altered forms of DHFR to which MTX cannot bind effectively, and an increase in cellular DHFR via DHFR gene amplification (9). The development of antifolates that inhibit TS (rather than DHFR) selectively may circumvent MTX tumor resistance, especially that resulting from increased levels of DHFR.

TS inhibition can be achieved with either fluoropyrimidines or folate analogue compounds. The first compounds to have clinically significant TS-inhibiting activity were the fluoropyrimidines 5-FU andFdUrd; these are metabolized to 5-fluoro-2-deoxyuridylate (the most efficacious analogue of deoxyuridylate) and form a ternary complex together with TS and CH2THF, resulting in potent TS inhibition. These drugs also may become incorporated into RNA or DNA via fluoro-UTP (10, 11) or 5-fluoro-dUTP (12), respectively. These events may obscure quantitation of TS inhibition. Despite this, potentiation of the cytotoxic effects of 5-FU andFdUrd was predicted and achieved successfully by the addition of folic acid to increase the intracellular level of CH2THF, resulting in the stabilization of the ternary complex and thus a higher degree and duration of TS inhibition (13, 14).

Selective TS inhibition is of interest in drug development for a variety of reasons. Immunological quantitation using monoclonal antibodies against TS (15, 16) revealed an inverse association between the treatment response to 5-FU and the cellular expression of TS; thus, TS overexpression of tumor cells can be a marker and prognostic indicator for 5-FU resistance. An association between the degree of TS inhibition and the clinical response to chemotherapy in colorectal and breast cancers has been noted (17, 18). Moreover, expression of TS was found to predict for disease-free survival and survival in patients with rectal cancer independently (19). Folate analogues are less susceptible to metabolic degradation than fluoropyrimidines and are not incorporated into RNA or DNA (3). Finally, tetrahydrofolate is a relatively large molecule providing a variety of sites amenable to manipulation in the design of novel TS-specific inhibitors.
To reach and inhibit target enzymes such as DHFR or TS, antifolates enter the cell via the RFC protein transport system on the cell membrane. Efficient or poor cellular antifolate uptake is determined by the molecular structure and polarity of the antifolate, the presence of reduced folates or antifolates (which may compete for the use of RFC), the intracellular folate depletion, and RFC deficiency (20). Once antifolates are inside the cell, glutamate residues are added by the enzyme FPGS in a γ-carboxyl linkage (Fig. 2). Polyglutamates with as many as seven or eight glutamyl residues have been identified (21). These add more negative charges to the antifolate molecule and increase solubility (22), resulting in the agent’s marked reduction in efflux from cells and its prolonged retention in tissues having high FPGS activity, such as the liver. Formation of ternary complexes with dUMP and folate protects the enzyme from carboxypeptidase inactivation. Moreover, polyglutamation increases the affinity of folate analogues for folate-dependent enzymes (but not for DHFR) markedly and results in a potentiation of TS inhibition that may exceed by 100-fold that of the monoglutamated form (23, 24).

Cellular uptake of antifolates via the RFC and the ability of antifolates to undergo polyglutamation have major roles in TS inhibition. A compound that has weak TS inhibitory activity in enzyme assays may undergo rapid cellular uptake by the RFC and extensive polyglutamation by FPGS in cell cultures or in vivo, resulting in potent TS inhibition. Alternatively, a compound that is a potent TS inhibitor in enzyme assays may show only weak activity in vivo if it does not enter the cell readily or is an extremely poor substrate for FPGS. Folate analogues may be regarded as prodrugs with polyglutamation as an activation step (24).

**TS Structure.** Iterative protein crystallographic analysis enabled the study of *Escherichia coli* TS and, subsequently, human TS in both the native form (25, 26) and the complex with dUMP and folate cofactor or a folate analogue (26-31). The primary sequence of the *E. coli* enzyme has high homology...
(46% identity) to the human TS sequence (25), and in the human enzyme, the active site residues are conserved, with 75% identity (29).

TS is a symmetric, obligate dimer of chemically identical units of about 35 kilodaltons each (25). Each of the two subunits folds into a three-layer domain anchored by a large, six-stranded, mixed β sheet. A β sandwich is created by the backside of one sheet in juxtaposition against the corresponding face of the equivalent sheet in the second protomer. This back-to-back apposition of the two β sheets forms the interface between the two subunits. Although the left-handedness of the β sheet twist in each monomer is normal, the twist of one sheet relative to the other is unexpectedly right-handed, in contrast to that of other proteins of similar structure (25, 32). Each β sheet is distorted by a series of stacked β bulges, dividing each sheet into two smaller classical β sheets orthogonal to one another. The stacked β bulges are stabilized by hydrogen bonding and account for the unusual right-handed twist of the opposing β sheets. An extended structure is created that anchors the phosphate of bound dUMP and controls the precise orientation of the catalytically essential sulfhydryl group on a conserved cysteine residue in the active site (32).

The unique TS portion of the ternary complex crystal form is a dimer. However, in unliganded TS crystal structures of humans, E. coli, Lactobacillus casei, and T4 bacteriophage, this unit is a monomer. Thus, structural differences between monomers can be observed only in the ternary complex crystal form. The monomers in this form are similar to one another but differ from the unliganded structures in mobility, reflecting mechanistic nonequivalence of active sites in TS (32).

The substrate-binding site is a funnel-shape cleft extending into the interior of each subunit. Components of each monomer contribute to each of two active sites (25), which are separated widely on either side of the molecule. This fact accounts for the inability to dissociate the native enzyme into catalytically active monomers. Moreover, it suggests a way for the active sites to communicate in the process of ligand binding (25, 33). There is no conclusive evidence for cooperativity between monomers. However, ligand binding is associated with large conformational changes and involves most of the domains of the protein. Thus, the liganded state at both active sites will play a role in ligand affinity (34). Binding studies using equilibrium dialysis and spectroscopy suggest that the two active sites may not react equivalently, although both active sites can be filled through covalent attachment of an inhibitor and folate. Binding studies of FdUMP suggest that both active sites contribute to this process in an asymmetric fashion, wherein one site is preferred over the other and, in some cases, only one active site is used at a time (33). In the presence of folates or folate analogues, binding of FdUMP to both active sites is enhanced greatly (35, 36). However, the novel folate analogue and TS inhibitor 1843U89 (Glaxo-Wellcome, Research Triangle Park, NC) promotes the formation of ternary complexes with TS and dUMP as well as with selective purine nucleotides (36). There has been no prior evidence that purine nucleotides bind to TS in the presence or absence of folates or other folate analogues. Surprisingly, 1843U89 not only enhanced the binding of FdUMP or dUMP to TS but also enabled that of dGMP, GMP, and IMP, which did not bind TS in its absence. The significance of these findings remains to be determined.

The tetraglutamated forms of ZD1694 [Tumexud; Zeneca (formerly ICI Pharmaceuticals) Macclesfield, United Kingdom] or CB3717 (Zeneca) bind to both active site regions, whereas CH3THF and 1843U89 prefer one of the two sites (35). The number of binding sites for dUMP, dGMP, and GMP was only one per TS dimer in the presence of 1843U89, with the second site being extremely weak even in the presence of polyglutamatimes (36). These results suggest that each of the two TS subunits binds folate substrates or analogues differently, and that fact has a major role in the inhibition kinetics of each folate analogue. However, such studies are difficult to perform; interpretation of the data has in the past produced conflicting results; and this issue has been a subject of considerable debate (27, 36).

**TS Catalytic Activity.** Methylene tetrahydrofolate is the source of the methyl group to be transferred to dUMP. It is a one-carbon donor as well as the subsequent reductant of the transferred methylene group. The details of this process have been deduced by studying the ternary complex of TS with FdUMP and methylene tetrahydrofolate, a stable structural analogue of a true catalytic intermediate. Comparison of the three-dimensional structure with that of the apoenzyme provided insight into the conformational changes that accompany ternary complex formation and their significance in the reaction process (26). In a different approach, the ternary complex of TS with dUMP and CB3717 (a selective TS inhibitor discussed below) has been used (31).

The above studies suggest that TS may activate dUMP by the formation of a covalent bond between the sulphydryl group of the conserved cysteine residue at the TS active site and C-6 of dUMP (see Fig. 3). This binding is induced by the folate (37) and converts C-5 of dUMP to a powerful nucleophile (38). Monoglutamylated folates bind to TS in an ordered fashion in which dUMP binds first. However, the intracellular pool of folates consists mainly of polyglutamylated molecules with affinities for TS that may exceed 100-fold (39). This high binding affinity of the polyglutamates results in a random binding order of ligands to TS. Moreover, folate analogues that, as a result of their structures, have a high affinity for TS even in the monoglutamated form can bind to the enzyme before dUMP (31). The binding of the polyglutamyl moiety to TS involves only a few localized interactions, such as hydrogen bonds and van der Waals forces, and seems to depend on more diffuse electrostatic forces, which permit flexibility and freedom of motion (30). Only the first glutamate residue near the p-aminobenzoic acid ring of the folate is fixed rigidly by its interaction with TS. The additional glutamate molecules are progressively more disordered. The polyglutamyl chain thus originates within the confines of the active site cavity and stretches out along the surface of TS exposed to solvent (30). This chain acts mainly to constrain the motion of the cofactor but has no significant effect on the conformation of the ternary complex compared with that of the monoglutamate. This action leads to augmented binding affinity of the cofactor for TS, whereas the $V_{max}$/[$K_{m}$] value is left unaffected (40).

Ternary complex formation is associated with a large conformational change involving four residues at the carboxyl terminus of the protein that close down on the distal side of the
Fig. 3 Proposed chemical mechanism for TS. Reproduced with permission from The FASEB Journal (34).

quinazoline ring of the inhibitor, capping the active site and sequestering the bound ligands from bulk solvent. These changes are due mainly to the binding of the folate and not to dUMP (26, 31); they involve segmental accommodation of several helices, β strands, and loops that move as units against the β sheet interface between monomers. After CH₂THF binds, its imidazolidine ring opens, and a covalent bond is formed between its N-5 methylene group and C-5 of dUMP (27). The steady state intermediate breaks down with a hydride transfer from C-6 of the cofactor to the transferred methylene group on dUMP, thus forming dTMP and releasing dihydrofolate (Fig. 3). This reaction process has been studied also in the presence of FdUMP or folate analogues (26, 28). In the presence of the fluorinated dUMP analogue FdUMP, a stable covalent complex forms between FdUMP, TS, and folate, and a hydride shift cannot occur; this results in enzyme inactivation. The fluoride of FdUMP cannot be extracted by TS to force collapse of the ternary complex that leads to the reaction products. With the folate analogue CB3717, the formation of a covalent bond between TS and dUMP is still induced. However, CB3717 cannot form a covalent bond with dUMP, as is the case with folate and dUMP, and a methylene group transfer cannot occur. Details of these processes are discussed extensively elsewhere (26, 27, 34).

Development of TS Inhibitors

**CB3717**

Extensive analysis of TS inhibition at the molecular level prompted the planning, synthesis, and development of an antifolate compound, CB3717 (N¹°-propargyl-5-8-dideazafolic acid; Zeneca; Fig. 2).

**Preclinical Data. In Vitro Studies.** CB3717 is a 2-amino-4-hydroxy quinazoline carrying a propargyl group (-CH₂-C≡CH) on N¹° that increases the affinity of this antifolate for the catalytic site of TS greatly (41). *In vitro* studies with isolated murine (L1210) and human (W1-L2) TS demonstrated that CB3717 was the tightest binding, monoglutamate, folate-based TS inhibitor at the time of its discovery, with a Kᵢ of 2.7 nM (41, 42). CB3717 inhibits TS, competing with CH₂THF for the same site on the enzyme, and has affinity for DHFR in enzyme assays. However, cell culture studies and clinical trials indicated that the latter finding was not significant, because the inhibition of TS was far more potent than that of DHFR (41, 43). CB3717 does not use the RFC for cellular entry but undergoes extensive polyglutamation by FPGS. Maximal potentiation of TS inhibition (114-fold) was achieved by the triglutamate form of CB3717 (43). The cytotoxicity was completely reversible with the addition of thymidine to the culture medium but was
only partially reversible with folinic acid, a finding corroborating prior data showing potent TS inhibition to be the sole cytotoxic action (41, 44, 45). CB3717 caused a reduction in intracellular thymine nucleotide pools and an elevation of dUMP levels. Moreover, CB3717 did not induce significant overproduction of DHFR in tumor cells, and cells resistant to MTX as a result of raised DHFR levels were not cross-resistant to CB3717. Accordingly, CB3717 was a potent growth inhibitor in both MTX-sensitive and -resistant cell lines (41).

**Animal Studies.** L1210 leukemia-bearing mice administered CB3717 achieved a long-term survival rate of 90% without significant toxic effects, whereas optimal doses of MTX elicited a long-term survival rate of only 20% (41). A daily × 5 dose schedule of CB3717 produced dose-related increases in mean survival time. CB3717 caused elevated deoxyuridine and depleted thymidine levels in plasma (45). Biliary excretion was the major route of drug elimination. In mice, the DLt was renal, with drug accumulation and retention in the kidneys (46). Precipitation within the renal tubule resulting from the poor solubility of CB3717 in normal pH urine may account for its nephrotoxicity (42, 47).

**Clinical Studies. Phase I Studies.** Because of the variable incidence of side effects, the MTD of CB3717 was difficult to determine with precision (48). The DLt of a 1-h infusion was nephrotoxicity. In >50% of patients, significant reductions in glomerular filtration rates occurred at doses >400 mg/m², although milder reductions were observed at lower dose levels (48). Malaise, associated with elevated liver enzymes, resulted in weakness, lethargy, arthralgia, and occasional fever. Only mild hematological side effects were observed. A subsequent Phase I trial found renal toxicity to be severe and unpredictable (49); another trial demonstrated that urine alkalinization decreased the renal toxic effects but noted that profound malaise was present at dose levels as low as 150 mg/m² (50). In these trials, the drug demonstrated antitumor activity in ovarian, breast, and lung cancer and mesothelioma.

**Phase II Studies.** A Phase II study showed the activity of CB3717 in advanced breast cancer, but severe renal failure occurred in 17% of patients (51); urine alkalinization was not used during this study. Other trials described the activity of CB3717 in cisplatin-refractory ovarian cancer (resolution of malignant ascites in 2 and a decrease of CA-125 levels in 1 of 8 patients; Ref. 50) and hepatocellular cancer (6 patients with decreases in tumor sizes and >50% fall in serum α-fetoprotein levels, classified as partial remissions, and 1 patient with stable disease among 14 chemotherapy-naive patients; Ref. 52).

However, because severe renal toxic effects were unpredictable, and profound malaise was noted even at lower doses, further development of CB3717 was abandoned. Nevertheless, this drug served as a prototype for studies of TS inhibition and was the first compound to be developed rationally to inhibit TS selectively.

**ZD1694 (Tomudex)**

Available preclinical and clinical data indicated that a less toxic analogue of CB3717 would be useful in cancer treatment. Because the renal and hepatic toxic effects of CB3717 appeared due to its poor aqueous solubility (46, 53, 54), further research focused on the synthesis of water-soluble analogues. This led to the development of quinazoline compounds, in which the benzene ring of the p-aminobenzoate residue (see Fig. 2) has been replaced with various ring structures, including pyridine, thiophene, or thiazole (24, 54). After extensive biological *in vitro* and *in vivo* evaluation of such analogues, the N10-methylthiophene analogue ZD1694 (Tomudex; Zeneca; Fig. 2) was selected for further development (54). This new quinazoline folate analogue inhibits TS selectively. Similar to CB3717, ZD1694 results in decreased TMP production, which leads to inhibition of DNA synthesis, resulting in cell death. Because Phase III trials of this agent have been completed, its properties and development are discussed in detail below.

**Biochemistry and in Vitro Studies.** ZD1694, or N-(5-[(3,4-dihydro-2 methyl-4-oxoquinazolin-6-ylmethyl)-N-methyl-ylk;lamino]-2-thenyl)-2-glutamic acid, is a yellow-brown to dark green granular powder that is water soluble at pH 7. ZD1694 behaves as a mixed noncompetitive TS inhibitor with respect to tetrahydrofolate (42). As expected of a pure TS inhibitor and as noted previously with CB3717, the addition of thymidine prevents the *in vitro* cytotoxic action of ZD1694 (55). In contrast to CB3717, ZD1694 uses the RFC for cellular entry effectively, and thus, higher intracellular levels are available rapidly for polyglutamation by FPGS (42). ZD1694 is an excellent substrate for FPGS, with an affinity 30 times higher than that of CB3717 (24, 42). In cell cultures, 95% of the drug is present intracellularly in the polyglutamated form within 4 h of ZD1694 exposure, resulting in more potent and prolonged TS inhibition (24, 56). Because of its effective use of the RFC and FPGS, this analogue is 500-fold more active in inhibiting cell growth than CB3717, despite being 20 times less potent as a TS inhibitor in enzyme assays (Kᵣ, 60 nm; Ref. 42). ZD1694 also inhibits DHFR via polyglutamation but is ~100-fold more specific for TS than for DHFR (56). Because the activity of ZD1694 is inhibited completely by thymidine (42), TS inhibition is responsible for limiting the cell growth rate. Folic acid antagonizes this growth inhibition in tumor cell lines in a competitive fashion. *In vitro* data suggest that folic acid competes with ZD1694 for both cellular uptake via the RFC and intracellular polyglutamation.

**Activity.** ZD1694 alone was 1800 times more cytotoxic to MGH-U1 bladder cancer cells after a 24-h exposure than was 5-FU plus LV (57). Progress through the cell cycle was delayed in a dose-dependent manner in HCT-8 ileocecal human carcinoma cells exposed to ZD1694 (58), and further studies showed its significant activity in cisplatin-resistant (59) and ileocecal cancer cells (60). The cell cycle specificity of ZD1694-induced DNA damage was analyzed in HCT-8 cells. Commitment to DNA replication was a prerequisite for damage, observed only in the early to middle S-phase (61).

**Animal Pharmacology.** Experiments in mice bearing a variant lymphoma-L5178Y tumor line showed that a single dose of 10 mg/kg ZD1694 cured the animals, whereas CB3717 exhibited poor activity at the MTD of 200 mg/kg (62). The rationale for a single-dose schedule is the rapid cellular transport of ZD1694 and its metabolism to polyglutamates. ZD1694 did not demonstrate renal and hepatic toxic effects at doses of 500 mg/kg, as observed with CB3717 at 100 mg/kg (63). Human tumor xenograft studies showed ZD1694 to be superior to...
CB3717, 5-FU, and MTX with respect to antitumor activity and therapeutic margin (64).

**Animal Toxicology.** Murine toxic reactions consisted of leukopenia, thrombocytopenia, and damage to the small intestinal mucosa. Unlike the renal and hepatic toxic effects of CB3717, the hematological and gastrointestinal toxic effects of ZD1694 could be prevented by synchronous thymidine administration, suggesting that these effects result from the antiproliferative activity of ZD1694 (42) and not from other physical or chemical properties. Moreover, folinic acid administration conferred some improvement in neutrophil and platelet counts as well as in gastrointestinal toxic reactions in ZD1694-treated BALB/c mice (65, 66), suggesting possible “rescue” activity of thymidine or folinic acid in patients with severe toxic reactions to ZD1694.

In dogs treated with the daily × 5 schedules of ZD1694 (0.5 and 1 mg/m²/day), myelosuppression occurred 4 days after the last dose and had resolved by day 8. At the same dose schedules, reversible histopathological changes in the epidermis, testis, and thymus were observed. These changes included the maturation arrest of spermatogenesis, absence of or reduced spermatozoa, and exfoliated seminiferous epithelial cells in the testis and epidermidis. Thymic lymphocytosis and atrophy were noted (67). In genetic toxicity assays, ZD1694 is not mutagenic but causes DNA strand breaks through its antimetabolite activity. ZD1694 (25 mg/m²) caused a reduction in canine fetal body weight and an increase in postimplantation fetal loss. This dose level was also associated with an increased incidence of major fetal soft-tissue and skeletal abnormalities; therefore, ZD1694 is contraindicated in pregnant women (67).

**Animal Pharmacokinetics.** After the i.v. administration of ZD1694 to rats and dogs, the major and probably only remaining circulatory compound was unchanged drug. Biliary excretion predominates in the rat, whereas renal and biliary routes of elimination are equal in the dog. Rapid drug clearance was noted in both the rat and dog (τ1/2, 0.6 h). The drug disposition was triphasic, with short initial phases and a relatively slow γ phase in both species (τ1/2γ, 22–56 and 14–29 h in the rat and dog, respectively). The long terminal phase is probably due to the slow return of ZD1694 to the plasma from a deep tissue compartment or to the release of polyglutamate-containing RBC from the marrow into the circulation. The latter has been observed at 2–4 weeks in humans. Significant ZD1694 accumulation was not observed with repeated daily administration (63, 68).

Polyglutamates were detected in liver, kidney, and intestinal tissue from mice injected with 5 mg/kg [5-3H]ZD1694 and accounted for at least 75% of the total drug concentration. Polyglutamated forms were retained in tissues even when most of the drug had been cleared from the plasma (69), supporting the use of the infrequent-dose schedule implemented subsequently in clinical trials.

**Clinical Studies. Phase I Studies.** The first Phase I trial of ZD1694 began in Europe in February 1991 and included 61 patients with solid tumors (colorectal, ovarian, head and neck, upper gastrointestinal, and others), most of whom had received prior chemotherapy (70, 71). After single doses of ZD1694, polyglutamation resulted in prolonged TS inhibition in mouse tumors (69); therefore, patients were treated with a single 15-min ZD1694 infusion every 3 weeks at doses ranging from 0.1 to 3.5 mg/m². Pharmacokinetic studies revealed interpatient variability but little intrapatient variability with repeated dosing. The plasma concentration-versus-time profile for ZD1694 showed a triphasic decline following infusion and a prolonged τ1/2γ (50–100 h), as observed in the rat and dog (71).

In this trial, the DLTs for ZD1694 were gastrointestinal, hematological, and malaise or asthenia at an MTD of 3.5 mg/m². No correlation was observed between the pharmacokinetic parameters and the increases in liver enzymes. However, the long τ1/2γ of the drug may be responsible for prolonged hematopoietic stem cell inhibition or gastrointestinal toxic effects. There was no statistically significant difference in the pharmacokinetic parameters (plasma concentration-versus-time profiles, peak plasma concentration, and terminal phase τ1/2γ) or toxic effects between patients with normal versus mild to moderate hepatic dysfunction (bilirubin, 1.25–3 times the upper limit of normal; aspartate aminotransferase or alanine aminotransferase, 3–10 times the upper limit of normal). No dose reduction is thus recommended for patients with hepatic dysfunction. Contrary to this, patients with mild to moderate renal impairment (defined as creatinine clearance <65 ml/min) had statistically significantly higher pharmacokinetic parameters than did patients with normal renal function. The drug manufacturer recommends that patients with renal impairment receive reduced doses of this drug. Among the 28 patients in this trial who received ZD1694 doses of 3.0 and 3.5 mg/m², 43% developed asthenia or malaise, 32% had diarrhea, 32% had grade 3 or 4 reversible elevations of liver transaminase levels, and 21% had grade 3 or 4 leukopenia. Four patients died of complications related to drug exposure at dose levels of 3.0 and 3.5 mg/m²; 3 of these 4 had grade 4 diarrhea. Death was attributed to disease progression in 1 patient, to cardiac decompensation in another who had a prior myocardial infarction, to dehydration in the third patient, and to pulmonary hemorrhage associated with grade 4 thrombocytopenia in the fourth patient (71–74). Three objective PRs were reported, one each in ovarian cancer, breast cancer, and adenocarcinoma of unknown origin. All patients had received prior chemotherapy (71–74). Because patients had poor tolerance of 3.5 mg/m² in this Phase I trial, the recommended Phase II dose was 3.0 mg/m².

A U.S. Phase I trial of ZD1694 conducted at the National Cancer Institute included 50 patients; 76% had colorectal cancer treated previously with 5-FU-based regimens, and the others had lung cancer, sarcoma, or hepatoma. ZD1694 was administered as a 15-min infusion every 3 weeks at dose levels between 0.6 and 4.5 mg/m² (75, 76). In this trial, the DLTs for the drug, first seen at 2.8 mg/m², were grade 3 fatigue and malaise that lasted for several days and increased in severity or duration with repeated dosing. The only other DLT was neutropenia. In contrast to the European Phase I trial results, in this U.S. trial, the MTD was 4.5 mg/m², gastrointestinal and hematological toxic effects occurred less frequently, and no treatment-related deaths were reported. No complete responses or PRs were observed in this trial, but two minor responses were reported (one in a patient with colon cancer and one in another with sarcoma). Based on results of this U.S. trial, the recommended ZD1694 dose for Phase II studies was 4.0 mg/m² (75, 76).
Phase II Studies. Various completed international clinical trials (67, 77–83) together accrued 439 patients with eight different tumor types, including 177 patients with colorectal cancer, 46 with breast cancer, 33 with hepatocellular cancer, 31 with ovarian cancer, 41 with non-small cell lung cancer, 74 with gastric or pancreatic cancer, and 21 with small cell lung cancer. In all Phase II studies, patients received ZD1694 at 3 mg/m². Based on the recommendation of the National Cancer Institute Phase I trial and on the observed lack of toxicity with 3.0 mg/m², 19 patients with non-small cell lung cancer received 4.0 mg/m². The toxicity reported with the higher dose was comparable to the 3.0-mg/m² dose (67).

The most frequently observed adverse events or laboratory abnormalities in Phase II studies were asthenia, diarrhea, nausea and vomiting, leukopenia, and elevations in liver transaminase levels. Twelve percent of patients experienced severe asthenia or malaise. Overall, severe toxic effects (WHO grade 3 or 4) were observed as anemia in 6%, neutropenia in 14%, elevated transaminase levels in 18%, and diarrhea in 8%. Grade 3 or 4 nausea and vomiting were seen in 12% but were controlled easily with antiemetics. Nonspecific cutaneous effects were noted in 14% as maculopapular rashes. Five patients died of severe drug-related hematological suppression and sepsis combined with severe gastrointestinal toxic reactions.

In Phase II trials, objective response rates were noted in 6.5% of patients with platinum-resistant ovarian cancer, 9% with chemotherapy-naïve, non-small cell lung cancer, 25% with advanced breast cancer who had received no prior chemotherapy, and 26% with advanced colorectal cancer who had received no prior chemotherapy (67, 77–80).

The Phase II trial of ZD1694 (3.0 mg/m² every 3 weeks) in 22 patients with previously untreated advanced pancreatic cancer reported PRs in 14%, all of whom continued to respond for 4–5 months (81). The ZD1694 Phase II trial in advanced colorectal cancer involved 177 patients, >80% of whom had hepatic metastases (80, 84) and only 5% of whom had received adjuvant chemotherapy. All received 3 mg/m² ZD1694, which was administered without dose reduction or delay in 82% of the patients (67). Almost 50% of these 177 patients received five courses of treatment, and 20% received more than six cycles. Objective responses were noted in 26%, with an average response duration of 6–7 months. In an additional 26%, however, signs of antitumor activity (minor tumor response or decrease in tumor marker levels) were noted.

Grade 3 or 4 toxic effects included asthenia in 12% and nausea or vomiting in 10%. Grade 4 neutropenia occurred in 3%. In 4% of patients, lower extremity cellulitis that began frequently in one leg and subsequently involved both legs was observed. This problem responded to antibiotic treatment, although microorganisms were not cultured from the areas of inflammation in any of the cases. Most patients who developed cellulitis had history of diabetes, deep venous thrombosis, or concurrent neutropenia. Two patients died of severe diarrhea, mucositis, and hematological toxic effects; one of these had received prior radiation therapy for non-Hodgkin’s lymphoma. An additional patient died; however, he did not receive aggressive treatment for diarrhea and dehydration (67). These trials stressed the need for early hospitalization and vigorous support of patients with severe gastrointestinal toxic effects, especially in association with myelosuppression. Moreover, folinic acid may ameliorate severe, established, ZD1694-induced toxic reactions (66).

Based on its antitumor activity and safety profile in colorectal cancer, ZD1694 seemed to be a promising candidate for comparative Phase III trials. Preliminary data in colon cancer cell lines suggested that ZD1694 may not follow the resistance patterns of 5-FU and thus could prove useful against 5-FU-resistant clones (64). The objective response rate of 26% obtained with 3 mg/m² ZD1694 compares well with response rates recently reported in a meta-analysis of data from advanced colorectal cancer patients treated with 5-FU plus LV (85). The incidence of severe toxicity at this dose of ZD1694 also compares favorably with North Central Cancer Treatment Group data for 5-FU plus folinic acid (86). Contrary to 5-FU, which is extensively metabolized, ZD1694 is eliminated primarily by the kidneys as an intact drug, and its clearance can be predicted by simple studies of renal function. Moreover, ZD1694 is administered easily as a single i.v. dose every 3 weeks, whereas 5-FU plus LV is often given as a weekly or 5-day i.v. regimen.

Phase III Studies. A European Phase III trial comparing 3.0 mg/m² ZD1694 with 5-FU plus LV in 437 patients with advanced or recurrent colorectal cancer was completed recently; preliminary data (with a median follow-up of 5.3 months) show response rates of 20 and 13% for Tomudex and 5-FU, respectively. In addition, 9.4% of patients who received Tomudex and 3% of those in the 5-FU plus LV group had a 40–50% reduction in measurable lesions. Mucositis and WHO grade 3 and 4 neutropenia were lower in the Tomudex group (P < 0.001). The North American Phase III trial opened to accrual in March 1994 and compared 3 mg/m² ZD1694 with 5-FU plus LV in advanced colorectal cancer. Initially, this trial also included a 4-mg/m² ZD1694 arm, but unexpected severe toxic effects and concern about the safety profile led to early closing of that dose arm. The trial continued as a two-arm study and closed to accrual on June 30, 1995. Results from this study are pending. Tomudex was approved in the United Kingdom for treatment of advanced colorectal cancer in August 1995.

Newer TS Inhibitory Agents

In an effort to deal with the various mechanisms of tumor resistance and to increase the activity of TS inhibition, new compounds have been synthesized (see Fig. 2 and Table 1).

ZD9331

Antifolates that are potent TS inhibitors but do not require extensive polyglutamation to exert adequate cytotoxicity are presently under development; such agents can counteract resistance and to increase the activity of TS inhibition, new compounds have been synthesized (see Fig. 2 and Table 1).
Table 1  TS inhibitors currently under development

<table>
<thead>
<tr>
<th>Compound</th>
<th>ZD1694 (Tomudex)</th>
<th>ZD9331</th>
<th>1843U89</th>
<th>AG331</th>
<th>AG337</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Zeneca</td>
<td>Zeneca, Glaxo-Wellcome</td>
<td>Agouron</td>
<td>Agouron</td>
<td></td>
</tr>
<tr>
<td>Use of RFC</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Polyglutamation</td>
<td>Yes</td>
<td>No</td>
<td>Yes (see comment)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Current status</td>
<td>Phase III trial vs. 5-FU and LV in colorectal cancer completed</td>
<td>Preclinical trials</td>
<td>Phase I trials</td>
<td>Phase I trials</td>
<td>Phase II trials</td>
</tr>
<tr>
<td>Comments</td>
<td>One-step polyglutamation to diglutamate only; increases intracellular retention, not potency of TS inhibition</td>
<td>Good p.o. bioavailability</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a γ-tetrazole moiety that inhibits γ polyglutamation. ZD9331 has a $K_i$ of 0.4 nM against isolated TS. The cell growth IC$_{50}$ of ZD9331 is 25 nM in L1210 cells and 7 nM in W1L2 cells. These values increase to $>$4000 nM in the presence of thymidine, confirming the TS locus as the site of action of ZD9331. As expected, this activity of the antifolate is retained against the L1210:RD1694 cell line, which cannot polyglutamate antifolates (87).

Pharmacokinetic studies after i.v. bolus injection of ZD9331 in mice showed a $t_{1/2}$ of 4-6 h (in contrast to 100 h for ZD1694; Ref. 88). Because ZD9331 is not retained in cells by polyglutamation, a prolonged infusion (3 mg/kg over 24 h) is required to exert a curative effect on L5178Y TK-/- tumors. The new compound has a therapeutic ratio of ~8 compared with ~1.5 for ZD1694. Compared with antitumor doses of ZD1694, which produced toxic effects to the bone marrow and small intestine, antitumor doses of ZD9331 produced only hematological toxicity. Murine experiments showed the activity of ZD9331 against a panel of human tumor xenografts, including ovarian, colorectal, gastric, and small cell lung cancers (89). Currently, this compound is in a preclinical stage of development.

1843U89

1843U89 is a novel benzoquinazoline folate analogue (Fig. 2) in which the pteridine nucleus of the folate molecule is replaced by a lipophilic benzoquinazoline moiety. This compound lacks any structural feature corresponding to the p-aminobenzoyl glutamate moiety of folates (90). 1843U89 is an extremely potent, noncompetitive TS inhibitor in enzyme assays ($K_i$, 90 pm), with kinetics suggesting binding to TS at a site independent of the substrate-binding site. Growth inhibition can be reversed by thymidine alone but not by folinic acid, indicating that TS is its exclusive site of action (91). 1843U89 enters the cells via the RFC and, although an excellent substrate for FPGS, is metabolized only one step to a diglutamate form. The monoglutamated form is as potent a TS inhibitor in vitro as the polyglutamated derivatives of ZD1694 (91). In contrast to other antifolates, increasing the number of glutamate residues of 1843U89 enhances the TS binding and inhibitory actions of this compound only slightly. However, metabolism to the diglutamate form increases 1843U89 intracellular accumulation and retention markedly (92).

Experiments with the human TK-deficient GC3TK cell line, used to circumvent problems associated with murine RFC transport and the high circulating thymidine levels that normally counteract the action of the drug in mice, indicate that 1843U89 has marked in vivo antitumor activity (91). Further studies showed its activity in human colon LW5 adenocarcinoma multicellular tumor spheroids (93) and in xenografts of human ovarian SKOV-3 and colonic DLD-1 cancer in CD-1 athymic mice (90, 94). The first Phase I study with this agent is currently underway.

Computer-assisted Drug Discovery

Historically, anticancer drug discovery relied on screening natural or synthetic compounds using receptor- or cell-based assays and on developing antimetabolites based on key biochemical pathways and chemical formulas of involved molecules (95). These compounds are then modified to improve their activity and toxicity profiles. X-ray crystallographic analysis of molecular structures achieves resolution that enables construction of the three-dimensional structure of a protein, ligand, or receptor-ligand complex. Studies of the binding of inhibitors to their targets and of receptor-ligand interactions provide information that then may be used in the de novo drug design. The structure of a receptor-ligand complex can be used in computer-assisted graphics and molecular modeling for the design process of the ideal ligand (29). These methods are changing the process of drug discovery and development.

AG337 and AG331. Using the above approach for drug discovery, TS was a target for the computer-assisted, structure-based design of inhibitors using high-resolution, X-ray crystal-
lography and molecular mechanics to analyze the E. coli TS structure (29, 97–99) and to investigate its interaction with inhibitory ligands, including 5-FU and a classic glutamate-containing folic acid analogue. With computer programming, molecules were then selected that would occupy the maximum space at the TS-active site and use electrostatic forces or hydrogen bonding optimally to stabilize the enzyme-inhibitor complex. The newly synthesized compounds are lipophilic, lack a negatively charged glutamate side chain, and are extremely potent TS inhibitors (IC50s in the nanomolar range). These properties imply that these drugs can enter cells readily without the RFC and thus have activity in tumor clones deficient in this carrier protein. Drug potency is not dependent on polyglutamation. These drugs may exert effective TS inhibition on FPGS-deficient cells; however, the prolonged intracellular retention of the new compounds, conferred normally by polyglutamation and causing prolonged TS inhibition, is lost.

The new compounds, AG337 and AG331 (Agouron, La Jolla, CA; Fig. 2), are potent TS inhibitors with purified human TS Ks of 16 μM (100) and 2 nM (99), respectively. Both compounds have minor structural similarity to the natural folate cofactor, and both inhibit the growth of a variety of tumor cell lines; the IC50s for L1210 in vitro ranged from 0.18 to 3.5 μM (101). The addition of thymidine reversed both growth inhibition and cell cycle arrest in the early S-phase, findings consistent with TS as the primary site of action (100–102). Moreover, both AG337 and AG331 were active in vitro against multidrug-resistant cell lines (102).

**AG337**. Animal Pharmacokinetics of AG337. AG337 had excellent p.o. bioavailability (96). p.o. absorption of AG337 was rapid, with peak plasma levels as measured by high-performance liquid chromatography achieved in <1 h. Comparison of the areas under the curve after administration of i.v. or p.o. AG337 at 75 mg/m² demonstrated the drug to have p.o. bioavailabilities of 100% in mice and >80% in dogs.

**Animal Pharmacology and Toxicology**. p.o. AG337 demonstrated 100% curative activity against the i.p.- or i.m.-implanted L5178Y/TK murine lymphoma and produced long growth delays in the GC3 M/TK human colon carcinoma i.p. xenograft. The murine antitumor activity of AG337 was elicited at dose levels that were well tolerated, even on a repeated-dose schedule; high dose levels also were tolerated in dogs (100). Five-day infusion schedules of AG337 in mice were well tolerated and induced substantial growth retardation in 90% of tumors, with a median delay of 14 days (103). These data corroborate in vitro AG337 studies demonstrating that a 24-h exposure to this agent was not sufficient to induce a significant rate of cell death but that a 72- to 120-h exposure produced concentration-dependent cytotoxicity (102).

**Clinical Studies of p.o. AG337**. Preliminary data from an ongoing Phase I trial demonstrate that p.o. AG337 has a bioavailability of >80%, with peak levels achieved within 1 h and the drug still detectable at 6 h. Mild nausea but no myelosuppression was observed at 360 mg/m² (104).

**Clinical Studies of i.v. AG337**. In a Phase I study of AG337 administered as a 24-h infusion, plasma deoxyuridine levels increased to 60–390% of pretreatment levels (an event that suggests significant TS inhibition) but normalized rapidly after the infusion ended. No tumor antiproliferative effects were observed in that trial (102, 103, 105). These observations and murine data on prolonged infusion schedules suggest that prolonged infusions are necessary to optimize antiproliferative effects.

Phase I studies of AG337 in a 5-day, continuous-infusion schedule were completed recently in the United States and United Kingdom (103, 105, 106). In these trials, the IC50 of the drug was <2 h. Drug clearance decreased from 190 ml/min/m² for the lowest dose to 85 ml/min/m² for the highest dose, indicating the saturable elimination and nonlinear pharmacokinetics of this agent (107). Myelosuppression and mucositis were the DLTs; radiographic and biochemical evidence of antitumor activity also was noted. Based on these trials, the recommended Phase II dose was 1000 mg/m²/day (108). Phase II trials in malignant tumors of the head and neck, lung, pancreas, prostate, liver, and colon were initiated at multiple clinical centers in the United States in October 1994 and are currently ongoing.

**AG331**. Treatment of L1210 cells with 4–10 μM AG331 in vitro elicits a >85% reduction in TS activity. AG331 was active against leukemia and colon cancer cell lines, and its antitumor activity was observed in vivo against the i.p.-implanted murine L5178Y lymphoma (102). In animal studies, plasma AG331 concentrations of >20 μM were tolerated without irreversible toxic effects. The toxic effects seen most frequently in preliminary animal investigations are mild to moderate grades of local vein irritation, transient weight loss, and histamine release resulting in flushing or swelling (102).

AG331 is currently being evaluated in Phase I trials. A study of AG331 administered as a 10-min i.v. infusion to 18 patients with refractory tumors has been completed (102). The IC50 of the drug was found to be ~20 h; with each dose increase, a steady increase in the area under the curve and a dose-dependent decrease in drug clearance were noted, indicating the saturable elimination kinetics of AG331 (88). Mild to moderate nausea and vomiting and mild flushing were observed at 130 and 225 mg/m², respectively, and may be attributed to histamine release (102). The MTD has not yet been reached, and thus far, objective tumor responses have not been observed in the ongoing Phase I trial of AG331 (109).

Recent preclinical data on AG331 in H35 rat and HEPG2 human hepatoma cells revealed that at higher doses of this antifolate, a loss of the TS inhibitory effect occurred, yet cytotoxic effects were preserved. This suggests an alternative mode of action for AG331 and the need to investigate the possibility that in these hepatoma cells, this drug undergoes biotransformation into other, as yet unidentified, toxic metabolites (110).

**Resistance to TS Inhibition**

Multiple mechanisms of resistance to the new selective TS inhibitors have been described, with many of the studies using ZD1694 as their prototype. The active transport of ZD1694 via the RFC and subsequent polyglutamation by FPGS, resulting in increased TS affinity and intracellular polyglutamate retention, are responsible for the potent TS inhibition and antitumor activity of the drug in vitro and in vivo. Decreased uptake via the RFC, failure to polyglutamate ZD1694, and elevation of intracellular TS levels by TS gene amplification have been described in studies of ZD1694-resistant cell lines (111, 112) and are
similar to observed mechanisms of resistance to 5-FU (113) or MTX (114–116). Some issues have been addressed in developing new folate analogues that are more potent TS inhibitors than ZD1694. LY231514 (Eli Lilly Research Labs, Indianapolis, IN), with a \( K_i \) of 3.4 \( \mu \)M, also uses the RFC and polyglutamation to inhibit selectively TS (117, 118). A Phase I trial of this drug has been completed. Neutropenia was the DLT, and nonhematological toxicities included mild fatigue, anorexia, and nausea. No antitumor responses were observed (119). Other drug development strategies led to compounds such as ZD9331 (which does not require polyglutamation; Refs. 87–89), 1843U89 (which requires only one-step polyglutamation; Refs. 36, 91), and AG331 (99, 109) and AG337 (which require neither transport by the RFC nor polyglutamation to exert their cytotoxic action; Refs. 108, 120).

**TS Induction.** Induction of TS leading to increased TS activity levels as a result of TS protein overexpression has been noted as a resistance mechanism to 5-FU (18). Gene amplification is the primary mechanism by which DHFR levels increase after cell exposure to MTX, resulting in MTX resistance, and this has been described also with TS in cell lines resistant to ZD1694 (112). In addition, overexpression of TS protein as a result of deregulation of TS mRNA translation during treatment with a TS inhibitor is another mechanism of TS induction.

Translation of human TS mRNA is controlled by its own end product (TS) in a negative autoregulatory manner (121, 122). TS protein inhibits TS mRNA by binding of the protein to specific TS mRNA sequences critical for the initiation of translation. TS protein can regulate its own mRNA translation. This TS-specific process does not affect translation of unrelated mRNA transcripts. The intracellular level of TS protein is thus critical in the regulation of TS mRNA translation. This model could explain *in vitro*, *in vivo*, and clinical observations describing TS level increases within cancer cells following acute exposures to 5-FU (18, 123–125). FdUMP, dUMP, and selective TS inhibitors (ZD1694) may bind to TS protein, producing a conformational change that inhibits its binding to TS mRNA. Translation of TS mRNA then proceeds uncontrolled, resulting in TS accumulation and drug resistance (122, 126). Coadministration of a translational (but not a transcriptional) inhibitor overcome TS protein accumulation. In addition, TS protein accumulation induced by ZD1694 was not reversed by thymidine coadministration despite a complete reversal of cytotoxicity. This explains observations of increases in TS levels of up to 40-fold in normal breast epithelial cells and up to 10-fold in human breast cancer cells after ZD1694 exposure, whereas TS mRNA levels remain unchanged (126). In these synchronized cells, TS protein induced normally during the S-phase and decreasing subsequently during \( G_1 \) was found to be expressed constitutively throughout the entire cell cycle after ZD1694 treatment. No overexpression of TS mRNA could be demonstrated. Once accumulated by ZD1694, TS was not induced in the S-phase, nor did it decrease in \( G_2 \), and it remained expressed throughout the cell cycle. Thus, TS accumulation was potentiated by TS expression throughout the entire cycle after ZD1694 treatment.

These effects were attributed to a loss of inhibition of TS mRNA translation, whereas TS gene transcription remained unchanged. Subsequent studies of human mammary epithelial cells treated with 1843U89 or AG331 and LY231514 demonstrated that it is possible to achieve higher rates of TS protein synthesis and resistance by the same mechanism described for ZD1694 (127). These *in vitro* data obtained using synchronized cells suggest that TS induction may develop in such cell populations during treatment with most, if not all, TS inhibitors. Accumulation of TS protein may result in clinical drug resistance in patients treated with 5-FU or folate analogues, and these data may explain in part the low response rates to these drugs in the clinical setting.

**TS Interaction with Cancer-related Genes.** Normal or abnormal expression of certain genes, including p53 and bcl-2, can affect the ultimate response of tumors to cytotoxic drugs (128–132). TS may affect these genes specifically. *In vitro*, translation of p53 mRNA was repressed specifically with the addition of exogenous human TS in human colon cancer H630 cells, and TS was demonstrated to complex with p53 and c-myc mRNAs (133, 134). The role of this mRNA-protein interaction, although not yet clear, suggests the possibility of a regulatory role of TS in p53 and c-myc cellular expression. In addition, c-myc protein decreased and p53 and Rb proteins increased after exposure of HCT-8 cells to ZD1694 (135). Although preliminary, these data are intriguing, because such interactions may have a critical role in retarding cell progression through the S-phase and in initiating apoptosis after drug exposure. If the cytotoxic activity of TS inhibitors is mediated ultimately by the induction of apoptosis, then mutations in the genes encoding these proteins or interference with their activity could decrease the expected cytotoxicity. Studies of bcl-2 expression in human lymphoma cells demonstrated that this is possible *in vitro*. Following a 36-h exposure to cytostatic drugs, includingFdUrd, cells transfected with a vector alone underwent apoptosis readily. In contrast, cells transfected with a bcl-2 vector and expressing this protein showed markedly decreased levels of apoptosis (8 versus 67%) independent of TS levels and TS enzymatic activity that were unaffected by bcl-2 expression (136). Preliminary reports from studies of p53 function in patients with advanced colorectal cancer suggest that patients with point mutations in p53 may not respond to chemotherapy with 5-FU and LV (137). Taken together, these data emphasize the increasing importance of a molecular understanding of intrinsic insensitivity to TS inhibitors as well as of TS interaction with cell cycle regulatory genes.

**Pretreatment TS Levels.** Pretreatment TS protein levels and TS gene expression predicted for responses of metastatic colorectal and primary gastric cancer to treatment with a combination of 5-FU and LV and a combination of cisplatin, 5-FU, and LV, respectively (15). Tumors with high levels of TS protein (measured by Western blot using the TS106 monoclonal antibody and densitometry scanning) and TS gene expression (quantitated by reverse transcription PCR and expressed as a TS:β-actin mRNA ratio) showed poor responses to therapy, whereas tumors with low TS protein and gene expression had more responsive disease. These data are preliminary, are based on small numbers, and will require validation in larger clinical trials. However, they do suggest that, in the future, clinicians may be able to predict responses to 5-FU-containing regimens based on pretreatment TS protein levels or TS gene expression. Together with the data from TS induction after exposure to TS.
inhibitors before and after therapy, quantitation of TS levels may help in further defining of the role of TS regulation in drug resistance. These correlations will need to be investigated for applicability with the newer TS inhibitors.

The role of each of the numerous and diverse mechanisms of TS regulation and resistance to TS inhibition remains to be defined. It is not yet clear which factors predispose cells to develop a certain resistance mechanism or which mechanism(s) may at least in part account for the relatively low response rates seen with ZD1694. However, the data presented above suggest that TS inhibition alone may not be sufficient to result in clinically significant cytotoxicity. A limited number of strategies (currently in preclinical development) attempting to improve the efficacy of TS inhibitors are presented briefly below. Moreover, elucidation of intrinsic tumor properties pertinent to altered signal transduction and metastatic potential (including tumor-induced angiogenesis) may lead to new therapeutic strategies.

Improving Drug Efficacy. To counteract TS induction, the development of an antisense RNA that targets the TS mRNA sequences responsible for TS translation has been suggested (126). In another approach using gene therapy, W1L2:R179 cells resistant to TS inhibitors through a 30-fold increase in TS activity over that of their parental W1L2 cell line were transfected with the TS ribozyme using an EBV-based vector. This hammerhead ribozyme cleaves the CUC sequences in a triple, tandemly repeated sequence located in the 5' untranslated region of TS mRNA. Thus, the transfected cells became sensitive to TS inhibitors (138). IFN-γ was shown to enhance the sensitivity of colon cancer cells to 5-FU by interfering with acute TS induction associated with 5-FU exposure and thus may prove to have a role in dealing with this problem (123). Whether these interventions will prove clinically useful in efforts to overcome the increased TS mRNA translation noted with TS inhibition requires further investigation. Alternatively, a 40-fold increase in TS levels in normal cells as mentioned above could render them theoretically more resistant to the TS inhibitor, thus resulting in a host-protective effect, offering the possibility for cell-selective treatment strategies.

Combining the inhibition of de novo and salvage pathways of thymidylate synthesis can provide a synergistic effect. The activity of salvage enzymes can be markedly higher than that of the rate-limiting enzymes of de novo biosynthesis (139). AZT inhibits TK, an enzyme that is up-regulated and involved in salvage pathways of TS inhibition, effectively. Although AZT alone did not inhibit TS, studies combining ZD1694 with AZT in MGH-U1 human bladder cancer cells showed the combination to be more cytotoxic (140). Up-regulation of nucleoside transporter may lead to increased uptake of preformed deoxythymidine or its analogue IdUrd in vitro (141). IdUrd alone did not inhibit TS activity in MGH-U1 and HCT-8 cells. DNA incorporation of this nucleoside analogue increased with administration of ZD1694 due to up-regulation of nucleoside transporter. IdUrd did not increase the TS inhibitory activity of ZD1694, but its DNA incorporation resulted in an increase in DNA single-strand breaks that paralleled cytotoxicity. Moreover, the therapeutic index was greater for the combination than for either agent alone (141). Sequential combinations of 5-FU and ZD1694 also have been tested for inhibition of salvage pathways. Pretreatment of HCT-8 colon carcinoma cells with ZD1694 was reported to enhance the cytotoxicity of 5-FU by potentiating the efficacy of TS inhibition (142). Following cell treatment with ZD1694, a strong TS inhibition leads to an altered balance of dUMP and dTTP pools and allows for an increased incorporation of 5-FU into RNA (2.5–3.0-fold that of cells not exposed to ZD1694). This event has an immediate effect on short-lived mRNAs (such as that of TK), leading to cell death, and this approach potentiated the effect of ZD1694 on sensitive cell lines markedly (143). Plans are in development to test this sequence-dependent synergistic combination in the clinic.

Other strategies have focused simply on potentiating TS inhibition or using agents synergistic with TS inhibitors. Because folate analogues bind to TS at a site different from that of FdUMP, combining 5-FU with selective TS inhibitors could potentiate the action of these agents. TS inhibition may be enhanced by using both a dUMP and a folate analogue to stabilize the TS-cofactor-substrate ternary complex. When AG331 or polyglutamated ZD1694 was combined with 5-FU, enhanced FdUMP binding to TS was observed. The ternary complex consisting of TS, FdUMP, and AG337 or ZD1694 was stable for at least 2 h. This led to potentiation of the growth-inhibitory effect of 5-FU, AG337, or ZD1694 on colon cancer cell lines (144).

In agreement with the above studies, a Phase I trial of Tomudex in combination with 5-FU, folic acid, and IFN-α for recurrent adult solid tumors is currently underway (145).

Conclusion

Inhibition of TS results in a decrease of the de novo thymidylate synthesis that is necessary for DNA synthesis and repair. The development of ZD1694 is an example of a lengthy, targeted approach to the design of new cancer chemotherapeutic agents; ZD1694, a potent, selective TS inhibitor, has demonstrated antitumor effects in both preclinical and Phase II clinical trials and is undergoing Phase III evaluation currently in the United States. More detailed data from the European Phase III trial in colorectal cancer will be available for critical review soon. The outcome of these studies may contribute to more convenient and less toxic alternatives to the currently available chemotherapy for colorectal cancer. Newer antifolate compounds with diverse structures and properties designed and developed by computer-assisted techniques are also undergoing preclinical and clinical testing. However, given the limitations posed by the variety of mechanisms through which cells bypass TS inhibition, it is questionable whether each of these drugs will be more successful than single-agent 5-FU or MTX. Combining TS inhibitors with other cytotoxic agents, agents that interfere with TS gene and mRNA regulation, or agents that inhibit salvage mechanisms following thymidylate depletion may provide greater clinical utility of these new compounds.

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4 J. R. Bertino, personal communication.
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References


242 Thymidylate Synthase Inhibitors


Thymidylate synthase inhibitors.

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