Novel Chemical Strategies for Thymidylate Synthase Inhibition

W.H. Gmeiner*

Department of Cancer Biology, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA

Abstract: Thymidylate synthase (TS) is a well-validated target for cancer chemotherapy. TS was established as the principal target of the widely used anticancer drug 5-fluorouracil (5FU). The 5FU metabolite FdUMP forms a covalent complex with TS that is stabilized by 5-formyl tetrahydrofolate (leucovorin; LV). Numerous chemical strategies have been employed to develop novel TS inhibitors that are superior to 5FU/LV. 5FU is non-ideal as a TS-inhibitory drug because it is only inefficiently converted to FdUMP, while the remainder of the administered dose is converted to toxic metabolites. My laboratory has explored the utility of FdUMP[N] compounds (oligodeoxynucleotides comprised of FdUMP nucleotides) as FdUMP pro-drugs. FdUMP[N] compounds result in potent TS-inhibition, and display many advantages relative to 5FU/LV. A number of other chemical strategies have also been employed to develop pro-drugs, or metabolic precursors of FdUMP, and several of these strategies will be reviewed. In addition to chemical strategies to develop FdUMP pro-drugs, a number of chemical strategies have been devised to develop molecules that resemble the reduced folate co-factor required for TS catalysis. The synthesis of antifolates that have TS-inhibitory activity, such as Raltitrexed, has resulted in compounds that are effective and specific TS-inhibitors and, in some cases, have clinical potential. Chemical strategies that target TS mRNA for destruction are also being explored as potential chemotherapeutics. These diverse chemical approaches to control TS activity in tumor cells for the treatment of cancer will be reviewed.

Keywords: Thymidylate synthase, antifolates, 5FU, cancer chemotherapy, structure-based drug design.

INTRODUCTION

Thymidylate synthase (TS) inhibitors constitute an important class of anticancer drugs [reviewed in 1-4]. Although TS inhibition is one of the most mature strategies for treating human cancer [5-7], several new approaches for TS inhibition have been described in recent years. These new approaches include further advances in the design and synthesis of novel TS-inhibitory antifolate compounds [8-10], the targeting of TS mRNA with antisense DNA or siRNA [11-13], and other novel strategies that are reviewed in the following sections. My own laboratory has pioneered the use of FdUMP[N] compounds as a new class of TS-inhibitors [14-19]. These new approaches, collectively, hold the promise for the development of a more efficacious and less toxic TS-directed chemotherapy.

Thymidylate synthase enzymes (TS) catalyze the reductive methylation of dUMP by N^5,N^{10}-methylene tetrahydrofolate [20]. The product of TS catalysis is 2'-deoxythymidine-5'-O-monophosphate (dTMP). TS is required for the *de novo* synthesis of dTMP (Fig. **1**). dUMP, the substrate for TS catalysis, is derived from either the deamination of dCMP (catalyzed by dCMP deaminase), or from the hydrolysis of dUTP (catalyzed by dUTPase) [21]. The product of TS catalysis, dTMP, is readily phosphorylated to dTTP, one of the four principal building blocks required for DNA synthesis. dTTP is also a negative regulator of dCMP deaminase and ribonucleotide reductase [21]. Since dTTP is required for DNA synthesis, inhibition of TS effectively limits DNA replication and cell division to what can be achieved using dTMP obtained *via* thymidine kinase and the salvage pathway [22]. TS inhibition is an important strategy for anticancer drug design because rapidly proliferating malignant cells require the *de novo* synthesis of deoxynucleotides to a greater extent than do cells that are less highly proliferative [23]. TS inhibition is also an important strategy for treating infectious disease because many pathogens express a TS enzyme that is structurally distinct from their host [24].

Thymidylate synthase enzymes are highly conserved evolutionarily both in terms of structure and mechanism [25]. The enzymes are active as homodimers consisting of two identical subunits each having a molecular weight of 30-35 kDa [26]. The dimer interface consists of an extensive β-sheet [27]. The binding site for substrate and co-factor consists mainly of residues from a single sub-unit with two Arg residues (R175 and R176 of human TS) from the second sub-unit contributing to binding [28]. TS is a "half the sites reactive enzyme" with catalysis proceeding at only one binding site at a time [29]. Binding of substrate and co-factor to TS proceeds in an ordered fashion with the substrate binding first [20] (Fig. **2**). A substantial conformational change occurs in TS following the binding of the co-factor [30]. The dUMP substrate is activated at the C5 position by nucleophilic attack by a cysteine sulfhydryl (C198 in TS from *L. casei*, C195 from human TS) [31] (Fig. **3**). Binding of N^5,N^{10}-methylene tetrahydrofolate to the TS active site then occurs with formation of an N^5 iminium ion [20]. Bond formation proceeds by the nucleophilic attack of the C5 enolate of dUMP upon the N^5 iminium ion of the reduced folate co-factor. An unidentified proteinaceous base then abstracts a proton from O4 of dUMP, promoting the formation of an exocyclic C5 methylene. The enzymatic reaction is completed by the

*Address correspondence to this author at the Department of Cancer Biology, Wake Forest University School of Medicine, Winston-Salem, NC 27157 USA. Tel: (336) 716-6216; Fax: (336) 716-7671; E-mail: bgmeiner@wfubmc.edu

0929-8673/05 $50.00+.00

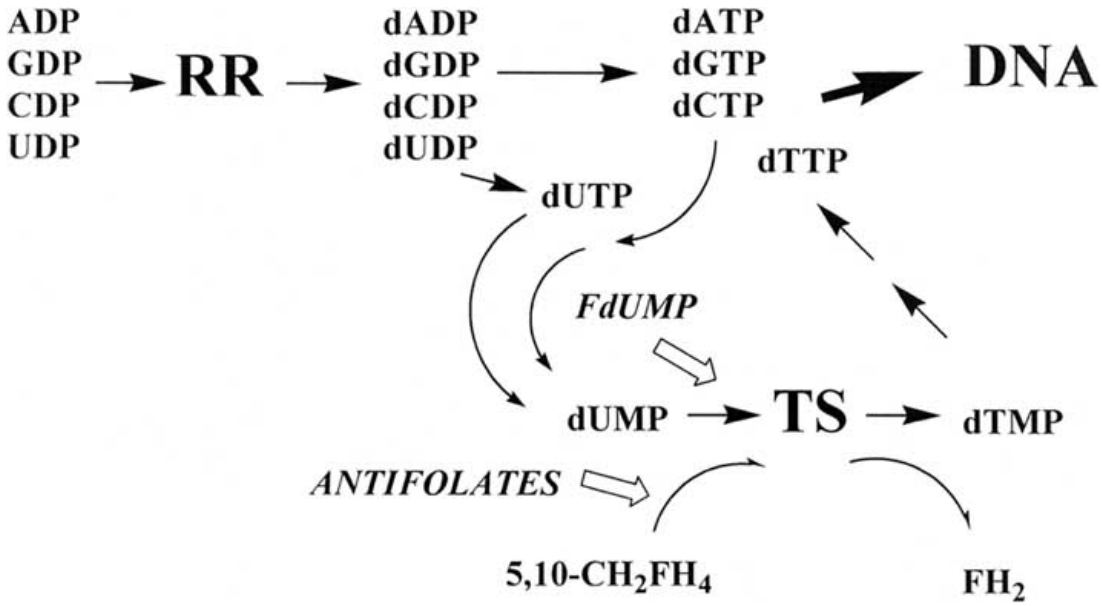

Fig. (1). The role of thymidylate synthase (TS) in dNTP biosynthesis. TS is required for the de novo synthesis of dTMP and is inhibited by substrate analogs such as FdUMP and co-factor analogs (antifolates).

reduction of the methylene *via* hydride transfer and β-elimination of the sulfhydryl anion from C6 [20]. The catalytic cycle is completed by release co-factor followed by the release of product.

CONSEQUENCES OF TS INHIBITION

Inhibition of TS in cells that have committed to cell-cycle progression and cell-division results in "thymineless cell death" [32]. Thymineless cell death refers to the induction of programmed cell death in thymidine-depleted cells attempting to undergo cell division. The mechanism of thymineless cell death has not been totally elucidated in any system. In *E coli*, thymineless cell death is mediated by the *mazEF* suicide module [33]. Programmed cell death in colon carcinoma cells, subsequent to thymidine depletion, results from the activation of Fas [34-36]. Inhibition of TS results in the accumulation of dUMP, the substrate for TS, and subsequently the misincorporation of dUTP into DNA [37]. Misincorporation of dUTP into DNA leads to the initiation of DNA repair by uracil DNA glycosylase (UDG) [38]. However, under conditions of TS inhibition and thymidine depletion, UDG-mediated repair is likely to be futile [39]. Ultimately, DNA double-strand breaks occur at a frequency that leads to the initiation of apoptotic cell death [40].

STRATEGIES FOR TS INHIBITION

The design of TS inhibitors has proven to be an important strategy for the development of drugs to treat both microbial infection [24], and diseases of uncontrolled proliferation, especially cancer [1-4]. Strategies to use TS inhibition for the design of antimicrobial agents rely on structural differences between TS expressed by the targeted microbe compared with TS expressed by the host organism (*e.g.* human TS (hTS)) [24]. Although highly conserved mechanistically and structurally, TS proteins from microbes are sufficiently distinct structurally from hTS to permit the design of species-specific TS inhibitors. Recently, a family of TS proteins that is both structurally and evolutionarily distinct from the highly conserved ThyA family of TS proteins has been identified (ThyX) [41]. The ThyX TS proteins are expressed in certain microbes, but have not yet been identified in sources from higher organisms. This review article will focus on the design of inhibitors that target hTS, a ThyA protein. Mammalian TS enzymes (including hTS) differ from prokaryotic TS proteins by the presence of an N-terminal insertion and the presence of insertions in two surface loops [42].

The present review focuses on the development of TS inhibitors for the treatment of human cancer. Perhaps the most widely studied molecule that functions as a TS inhibitor is 5-fluoro-2'-deoxyuridine-5'-O-monophosphate (FdUMP) [7]. FdUMP is the TS-inhibitory metabolite of the widely used anticancer drug 5-fluorouracil (5FU) [43,44]. FdUMP is an analogue of the natural substrate for TS, dUMP. A number of other dUMP analogs also have TS-inhibitory properties. This class of TS inhibitor is described below under the heading, "Nucleoside Analogs." A new class of TS-inhibitory compounds that are related to the nucleoside analogs are polymers of FdUMP, FdUMP[N] compounds. FdUMP[N] compounds are currently under pre-clinical development in my laboratory [14-19]. Another class of compounds that have TS-inhibitory activity is the antifolates. Antifolates are structural analogues of the reduced folate co-factor that serves as the one-carbon donor and reductant for the reaction catalyzed by thymidylate synthase. Recent developments in antifolate design, synthesis, and biological activity are reviewed in the following section. In addition to the nucleoside analogs and antifolates, each of which inhibit TS activity by binding to the enzyme, several laboratories have investigated the potential utility of antisense and antigene approaches to

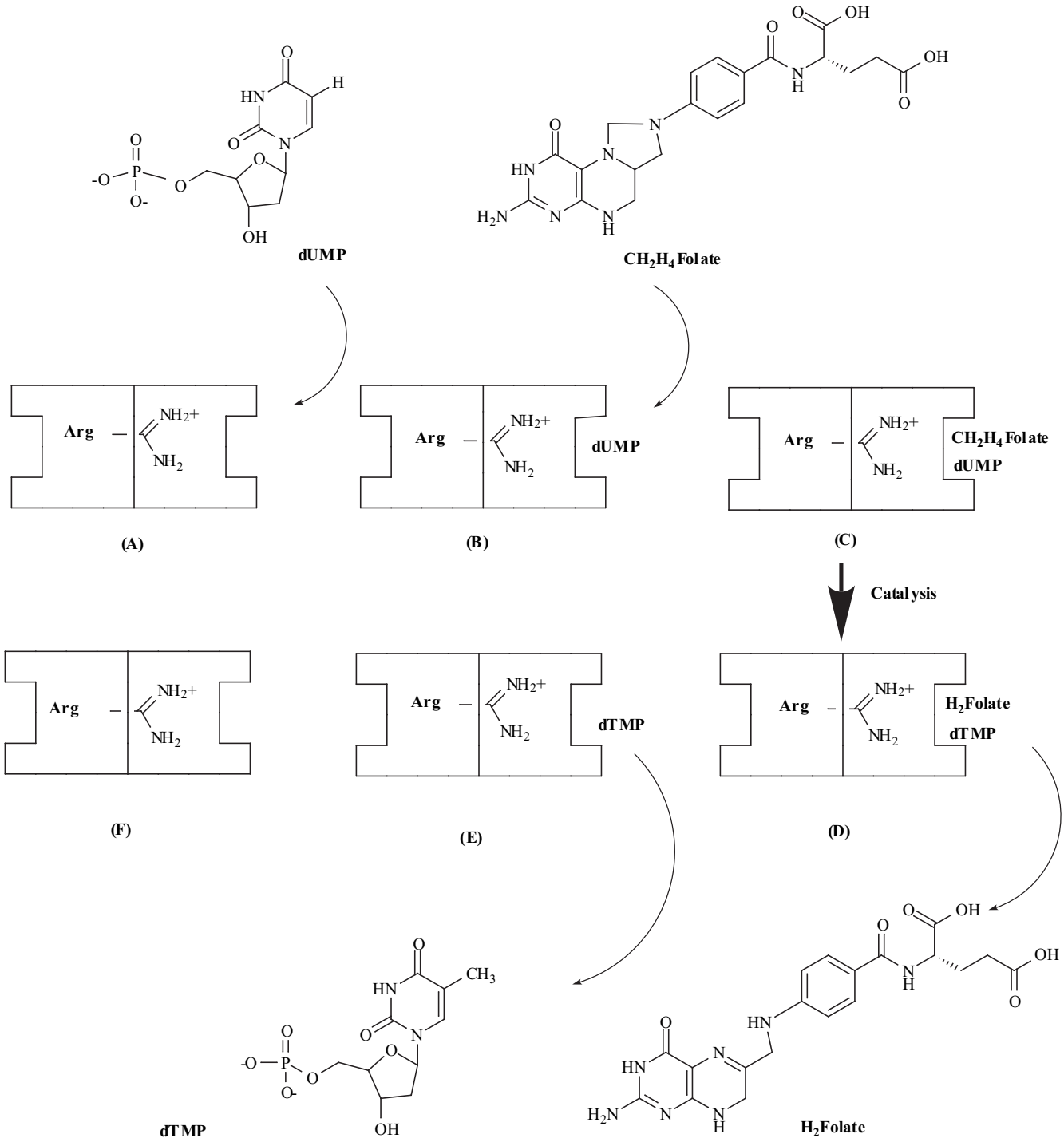

Fig. (2). Depiction of a catalytic cycle for thymidylate synthase (TS). TS is a "half-the-sites reactive" enzyme. Arginine residues are involved in both both interdomain and intradomain stabilization of substrate binding. For simplicity, only one of the two interdomain substrate:Arg interactions is depicted. Binding of substrate precedes binding of the co-factor. Following catalysis, modified co-factor is releases prior to release of the product, dTMP.

inhibit TS activity by reducing the expression of TS protein. These approaches will also be reviewed.

ANTIFOLATES

The biosynthesis of thymidylate by TS proceeds *via* the formation of a ternary complex consisting of the substrate dUMP, the reduced folate co-factor (5,10-methylenetetrahydrofolate), and the enzyme TS. The 5FU metabolite FdUMP forms a covalent complex with TS [43,44]. The TS:FdUMP binary complex is inhibited from further catalytic activity, however this complex is reversible [45,46]. Formation of a ternary complex upon binding 5,10-methylenetetrahydrofolate results in the formation of the highly stable ternary complex. The critical role of the reduced folate moiety in TS catalysis spurred many

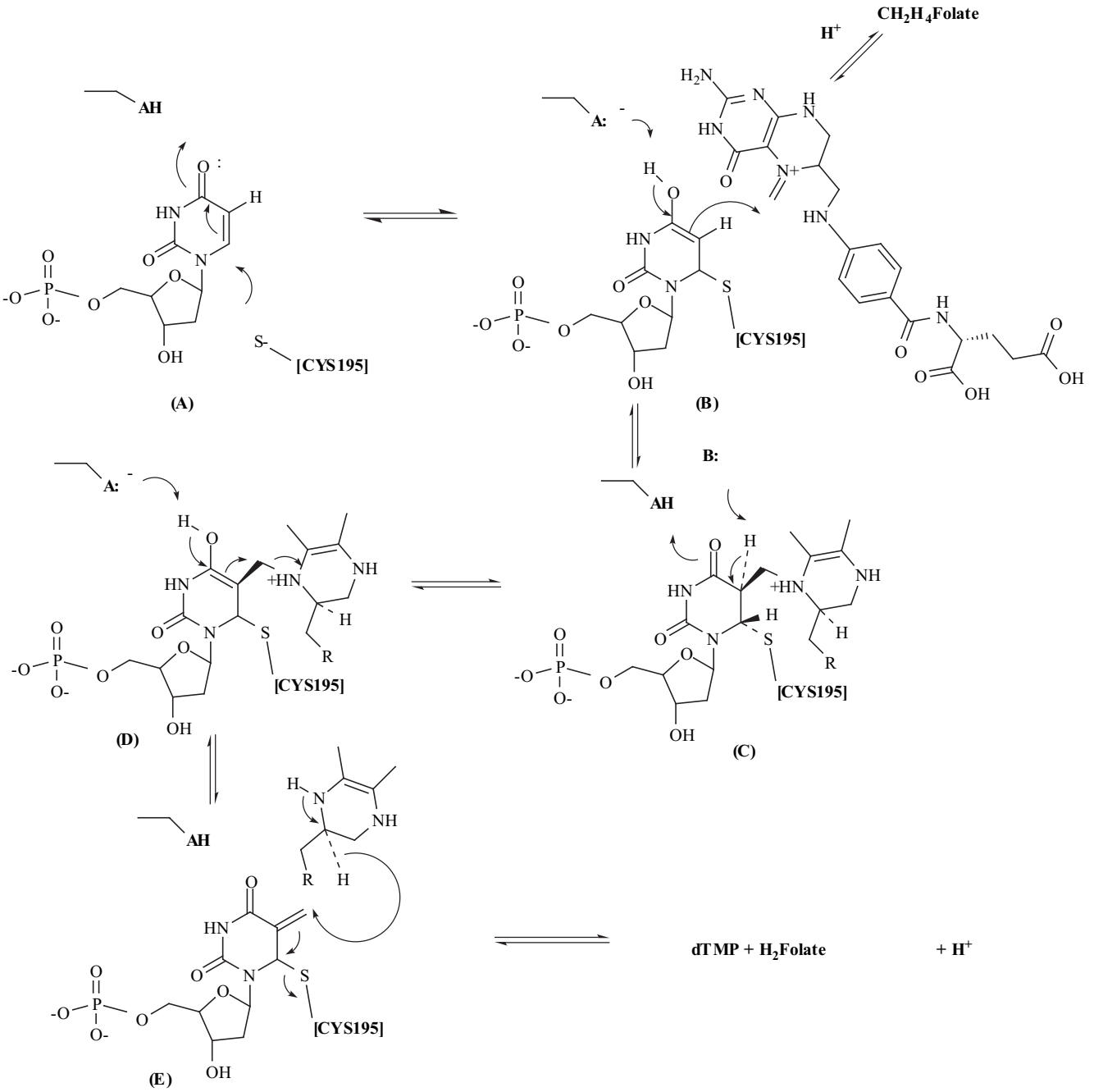

Fig. (3). Mechanism of catalysis for human thymidylate synthase (TS). Cys 195 (human TS) attacks C6 of dUMP to form an enolate, which then abstracts a proton from an unidentified basic residue of the protein to form an enol in step (**A**). The co-factor, N^5,N^{10}-methylene tetrahydrofolate (CH_2H_4folate) is activated by iminium ion formation at N5. The C5 position of dUMP, activated by enol formation, reacts with the methylene of the activated co-factor in step (**B**). H5 of dUMP is abstracted and the enol is re-generated in (**C**). Abstraction of a proton from O4 of the enol in (**D**) results in formation of an exocyclic methylene and release of the catalytic Cys (Cys 195). The methylene is then reduced by the modified co-factor to produce dTMP. The modified co-factor, which has served as methylene donor and reductant is released from the active site of TS followed by release of product, dTMP. The chemical evidence supporting this mechanism is summarized in [20].

investigations into the design and synthesis of folate analogs (antifolates) that would preferentially inhibit TS, rather than dihydrofolate reductase (DHFR) [47]. Such TS-inhibitory antifolates might prove valuable as alternatives to dUMP analogs as TS inhibitory molecules for the treatment of human cancer. At least four advantages could potentially be derived from the inhibition of TS with folate analogs rather than with dUMP analogs: 1) Inhibition of TS by antifolates should be insensitive to the elevated levels of dUMP that arise following the initial inhibition of TS. Elevated dUMP levels decrease the effectiveness of dUMP analogs due to competitive binding; 2) Antifolates are larger than dUMP analogs, thus there is greater potential for diverse chemical modification; 3) TS-inhibitory antifolates might also be more useful than fluoropyrimidines (FPs) for elucidating the mechanism of thymineless cell death since FPs, especially 5FU, have RNA-mediated effects that also contribute to cytotoxicity; 4) Antifolates also have distinct pharmaco-

kinetic properties and toxicity profiles from FP inhibitors of TS, and this is a potential advantage in their use for anticancer therapy. The chemical structures for folic acid, the antifolate CB3717, and four antifolate anticancer drugs that are currently being evaluated for the treatment of cancer are shown in (Fig. **4**). The structures for four other antifolate anticancer drugs are shown in (Fig. **5**).

EARLY TS-INHIBITORY ANTIFOLATES

One of the first antifolates to be developed as a specific TS inhibitor was CB3717 (Fig. **4**); [48]). The quinazoline ring system of CB37317 has a 2-amino-4-hydroxy substitution pattern. The 2-amino-4-hydroxy substitution pattern has been shown to result in selective preference for TS inhibition [49] relative to the preferential DHFR inhibition displayed by 2,4-diamino quinazolines [50], such as methotrexate (MTX). In addition to the 2-amino-4-hydroxy quinazoline substitution, CB3717 also had a propargyl substituent at N10, but otherwise is a 5,8 dideaza folic acid analog (Fig. **4**). While CB3717 displayed DHFR inhibitory activity (K_i = 2.3 x 10^{-8} M), as well as TS inhibition (K_i = 4.9 nM), evidence that the cytotoxicity of CB3717 resulted from TS inhibition included the complete rescue of L1210 cells from CB317 with 10 micromolar dT [48]. Treatment of L1210 cells (16 h at IC_{50}) with CB3717 resulted in 88% depletion of dTTP and 2,300% enhancement of cellular dUMP concentrations.

The potent inhibition of TS by CB3717 resulted in its evaluation as an anticancer drug. Dose-related nephrotoxicity was observed, however, in a substantial percentage of patients treated with CB3717. Reversible hepatic toxicity was also reported [51]. Although the serious toxicities associated with CB3717 administration prohibited its further clinical development, the strong TS inhibitory activity of CB3717 spurred design efforts to synthesize alternative antifolates with reduced toxicity.

Analogs of CB3717 were designed and synthesized at the Institute for Cancer Research (U.K.) in an effort to preserve the strong TS-inhibitory activity of the parent compound, but to eliminate the nephrotoxicity associated with its clinical administration. A possible cause for the nephrotoxicity of CB3717 was intermolecular hydrogen bond formation involving the hydrogen bond donor/acceptor groups of the 2-amino-4-hydroxy quinazoline ring system. Formation of intermolecular hydrogen bonds would reduce aqueous solubility, and could result in drug precipitation in the acidic environment of the kidney. Subsequent experimentation revealed that the 2-desamino analog of CB3717 [52] and the 2-desamino-2-methyl analog of CB3717 (ICI 198583; [53]; (Fig. **5**) displayed greater solubility and reduced toxicity relative to the parent compound, although at the expense of slightly decreased TS inhibitory activity [54-56]. The 2-methyl-4-hydroxy substituted quinazoline ring system has been retained in the design of many subsequent TS-inhibitory antifolates, including ZD 1694 (raltitrexed – (Fig. **4**), that is clinically approved for the treatment of colon cancer in Europe [57].

CELLULAR TRANSPORT AND RETENTION

The 2-desamino and 2-desamino-2-methyl analogs (ICI 198583) of CB3717 differed from the parent drug not just in toxicity and TS inhibition, but also in transport into cells

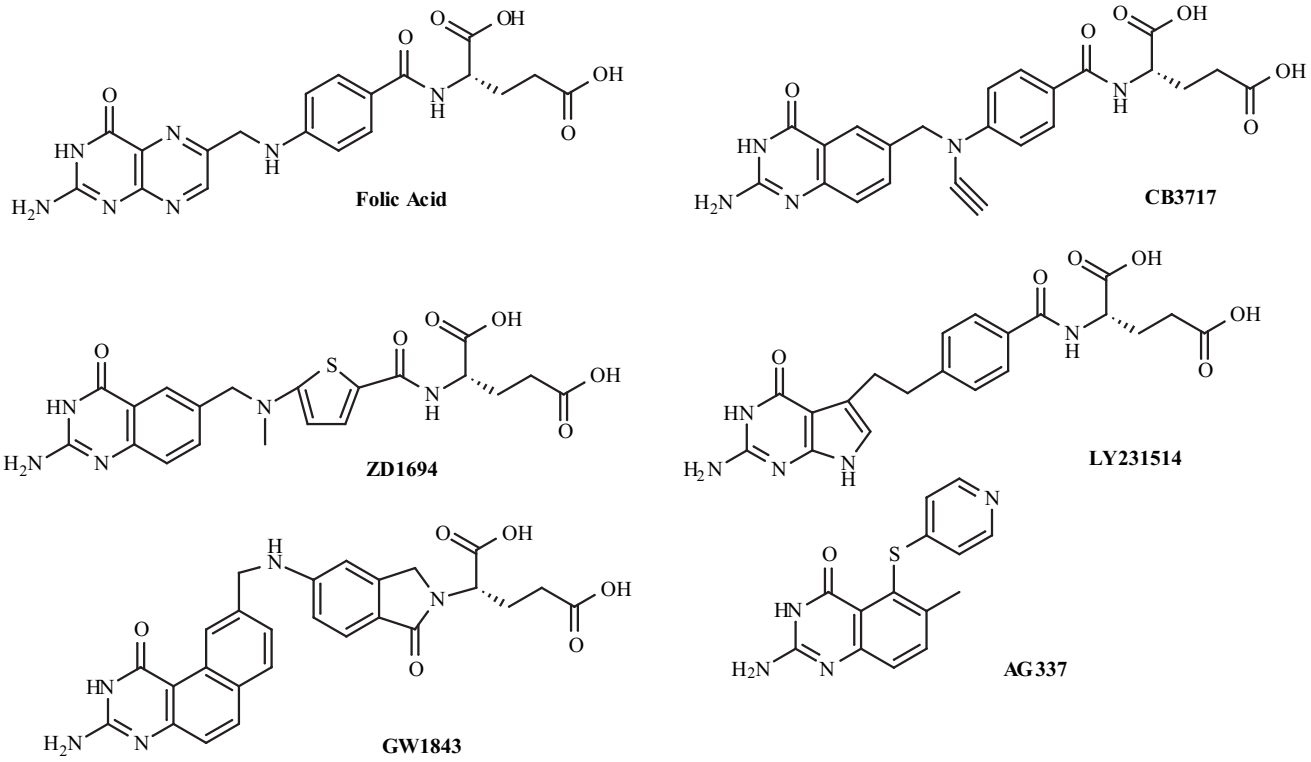

Fig. (4). Structure of folic acid, the antifolate CB3717, and of four antifolates that are currently being evaluated for the clinical treatment of cancer.

and in substrate affinity for polyglutamation by the cellular enzyme FPGS. ICI 198583 was transported more efficiently into L1210 cells *via* the reduced folate carrier (RFC) relative to CB3717 with the 2-desamino derivative of CB3717 displaying intermediate transport properties [55]. It is possible to achieve much higher levels of drug in malignant cells expressing RFC if the drug is transported efficiently by this protein. ICI 198583 was also a better substrate for polyglutamation by FPGS than either CB3717 or the 2-desamino analog of CB3717 [55]. The polyanionic character of drugs that have been polyglutamated by FPGS, which typically adds 2-5 glutamic acids, results in increased cellular retention of these drugs. Polyglutamated antifolates also generally bind more tightly to TS and thus are better TS inhibitors. It was the enhanced transport of ICI 198583 *via* the RFC and its enhanced polyglutamation by FPGS relative to CB3717 that resulted in a 10-fold increase in the therapeutic index for ICI 198583 relative to CB3717 despite the lower TS-inhibitory activity of ICI 198583 *in vitro* [47]. The effects of chemical modification on cellular transport and polyglutamation have been important design considerations for the synthesis of nearly all subsequent TS-inhibitory antifolates [10].

Efficient drug transport *via* the RFC is often considered a desirable trait in many antifolate design strategies since, in principle, this increases intracellular concentrations of drug. Negative features of efficient RFC transport include the possibility of increased toxicity towards non-malignant cells, particularly those that undergo frequent cell division such as those in the GI-tract and bone marrow [58,59], and the possibility of drug-resistance developing in malignant cells due to mutations in the RFC that reduce drug uptake [60], or due to transcriptional silencing of the RFC gene by methylation [61]. Likewise, efficient polyglutamation of TS-inhibitory antifolates is desirable because it increases cellular retention of drug. Polyglutamation of TS-inhibitory antifolates also results in drugs that bind to TS more tightly, and thus are better (~60-fold) TS inhibitors [57]. However, these potential advantages of enhanced polyglutamation are also potential liabilities since enhanced hydrolysis of the polyglutamates due to elevated hydrolase (GGH) in malignant cells can result in drug resistant disease [62]. Mutation or reduced expression of FPGS can reduce drug activities [63]. Cellular resistance to antifolates that are substrates for FPGS can also arise from increased cellular uptake of folates [64]. For these reasons, a number of antifolate design programs have focused on synthesizing drugs that do not require the RFC for uptake and/or, are not substrates for FPGS [63].

The structural basis for the stabilization of antifolate binding to TS by polyglutamation was explored using X-ray crystallography and molecular modeling [65]. A computer model of the γ-L-glutamic acid derivative of ICI 198583 (a dipeptide) in a ternary complex with dUMP and *E. coli* TS showed that the first carboxylate interacted with Lys48 *via* an intervening water molecule while the α-carboxyl oxygen of the terminal Glu interacted with Arg 49 [65]. Initially, dipeptide analogs of ICI 198583 were synthesized in which each amino acid had an L-configuration [66]. These analogs were shown to have increased TS-inhibitory activities relative to the parent molecule, however, these compounds were susceptible to hydrolysis *in vivo* and were substrates for FPGS. Since resistance to TS-inhibitory antifolates that are substrates for FPGS could potentially arise due to the enhanced hydrolysis of polyglutamates or reduced polyglutamation, efforts were made to synthesize dipeptide analogs of ICI 198583, and related antifolates, which were resistant to chemical and/or enzymatic hydrolysis. Molecular modeling studies showed that the second amino acid could have either a D- or an L-configuration and still maintain the stabilizing electrostatic interaction with Arg 49 of TS [65]. A subsequent series of dipeptide derivatives of ICI 198583 were synthesized that included a D-amino acid. These dipeptide derivatives of ICI 198583 showed improved hydrolytic stability yet were still potent inhibitors of TS. The promising results obtained with the dipeptide analogs of ICI 198583 that were not substrates for FPGS, were hydrolytically stable, and were strong inhibitors of TS prompted the design of an analog that would have these properties and would be suited for further clinical development. ZD9331 was identified as a compound that has these desirable properties, and it is currently being

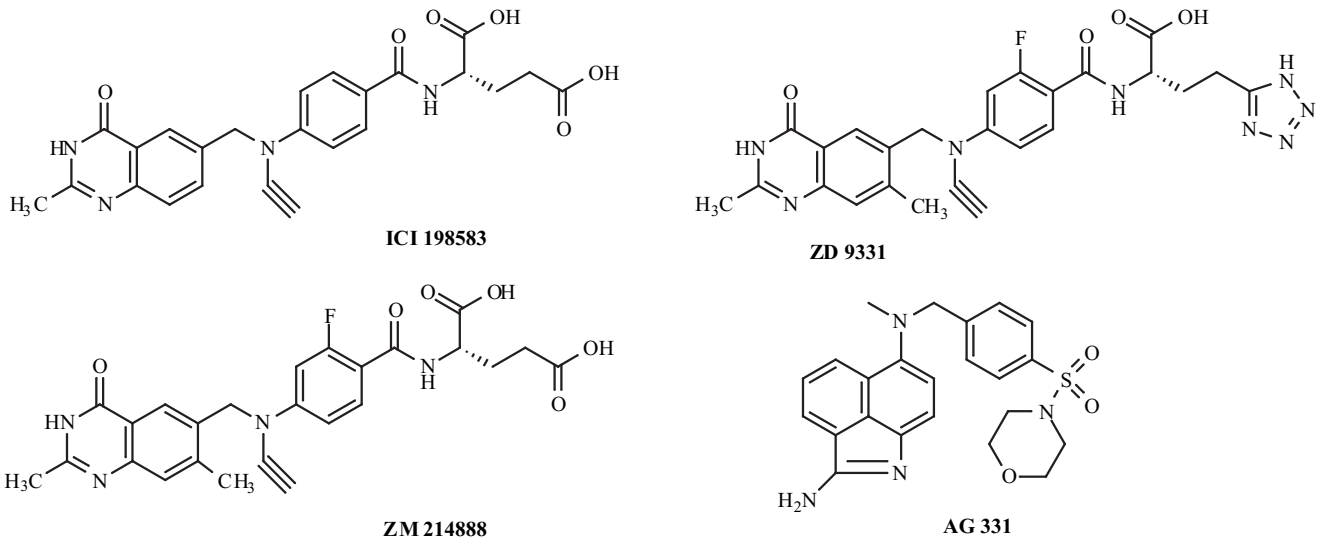

Fig. (5). Structures of four antifolates that have been developed as TS-inhibitory compounds.

evaluated in clinical trials [67,68]. ZD9331 has a carboxylate in an analogous position to the α-carboxylate of ICI 198583, but the γ-carboxylate of L-Glu of ICI 198583 has been replaced with a methylene-spaced tetrazole moiety. ZD9331 is transported into cells by the RFC and also enters cells by passive diffusion. ZD9331 is not, however, a substrate for FPGS [10]. ZD9331 also has 7-methyl and 2'-fluoro substituents that were not present in ICI 198583. The presence of these substituents was shown to enhance the potency of ZM214888 relative to ICI 198583 from which it differed only by these substitutions [69].

STRUCTURE-BASED DRUG DESIGN OF ANTIFOLATES

The X-ray structures of ternary complexes with antifolates bound to *E. coli* TS have provided structural information that has been useful in structure-based drug design (SBDD) of novel TS-inhibitory antifolates [70,71]. The 2-amino-4-hydroxy quinazoline ring system of 10-propargyl-5,8-dideazafolate was bent at approximately a 65º angle relative to the p-aminobenzoate moiety in the X-ray structure of the ternary complex with TS and FdUMP [70]. These X-ray structures also revealed that there was space available in the antifolate binding site of TS for derivatization at C7 of the quinazoline ring system. The 7-methyl analogs of CB3717 and ICI 198583 were synthesized, and displayed stronger TS inhibitory activity than did the parent molecules. Recently, cyclopenta[g]quinazoline-based antifolates in which C7 and C9 of ICI 198583 were incorporated into a cyclopentyl ring have been synthesized and evaluated for TS inhibition [72]. These compounds are potent inhibitors of TS, and this potency may derive from a preference to adopt a bent conformation. The creation of the cyclopentyl ring may also permit stereochemical control at C9 that further restricts the relative spatial relationship of the p-aminobenzoate moiety relative to the quinazoline ring system. Such stereochemical control may permit adoption of a molecular shape that is more ideal for TS binding, and subsequent inhibition of TS enzymatic activity, than are the present generation of antifolates.

Analysis of the structure for the reduced folate binding site of *E. coli* TS with FdUMP covalently bound was also used for the design of the TS inhibitors AG331 and AG337 (nolatrexed – (Fig. **4**) [73]. "Humanized" forms of the *E. coli* enzyme were also developed, and crystal structures of these "humanized" enzymes were analyzed to better account for the structural differences between the human and bacterial enzymes. The aromatic compound naphthalene was found by computational docking methods to efficiently occupy the pteridine binding site of TS. Consideration of hydrogen bonding interactions between the co-factor analog and TS resulted in the selection of a napthostyril tricyclic ring system for further development. This program resulted in the synthesis and development of AG331 and related compounds [74]. In these compounds, the p-amino benzoic acid moiety of folic acid was modified to a p-tolyl sulfonyl derivative to facilitate attachment to the 5-position of the napthostyril ring system, while a morpholino ring was included in AG331 to increase the water solubility of TS-inhibitory antifolate. AG331 and related compounds were designed to enter cells by passive diffusion, and were designed not to be substrates for FPGS. Subsequent structure-based design of TS-inhibitory folate analogs resulted in the development of 5-(arylthio)quinazolines. The shape of these analogs was designed to be complementary to the co-factor binding site of TS in the ternary complex that takes a sharp turn in the region of the enzyme that interacts near the junction of the quinazoline ring and the p-amino benzoic acid moiety [75]. The compound AG337 (thymitaq) was selected from this program to proceed in further pre-clinical development [76] and clinical trials [77].

Other programs have also reported progress in the use of structure-based drug design to create novel TS-inhibitory antifolates [78,79]. The availability of X-ray structures for human TS should be useful for SBDD studies to design novel antifolates for the treatment of human cancer [80-82]. These structure-based approaches hold the promise for understanding the structural basis for TS mutations that confer resistance to antifolates, and for designing novel antifolates capable of overcoming these sources of drug-resistance [83,84]. The availability of more sophisticated computational tools and greater computer power will expedite these efforts [85].

MULTI-TARGETED ANTIFOLATES

As mentioned above, the 2-amino-4-hydroxy substitution pattern of the quinazoline ring system in tetrahydrofolate analogs results in compounds that are selective TS inhibitors, while the 2,4-diamino substitution pattern results in selective inhibitors of DHFR. A research program to explore the enzyme inhibitory activities of 5-substituted pyrrolopyrimidines revealed that in contrast to the selectivity for TS-inhibition displayed by 2-amino-4-hydroxy substituted quinazolines, the equivalent 2-amino-4-oxo substitution pattern for pyrrolopyrimidines resulted in compounds that target both TS and DHFR (as well as GARFT), and are referred to as multi-targeted antifolates (MTA) [86]. The dual specificity for TS and DHFR inhibition results from two distinct binding modes for 2-amino-4-oxo pyrrolopyrimidines. The classical binding mode results in the expected TS inhibition while in the alternative binding mode, the pyrrole NH plays a similar role as the 4-amino group in 2,4-diamino substituted quinazolines [87]. The compound that is in clinical development is Pemetrexed (LY231514 – (Fig. **4**) [88]. Pemetrexed has a carbon at the position occupied by nitrogen in the p-aminobenzoic acid moiety and is a substrate for polyglutamation by FPGS and is taken into cells by the RFC [89,90].

BENZOQUINAZOLINE ANTIFOLATES

Replacement of the pteridine moiety of folates with a benzoquinazoline ring system has been shown to result in compounds that have potent TS-inhibitory activity and antitumor activity [91]. Due to the poor solubility of substituted benzoquinazolines, a sulfonamide linkage was initially used to link the (p-aminobenzoyly)-glutamate moiety to the benzoquinazoline ring system. The success of

substituted quinazoline antifolates that preserved the methyleneamino linkage present between the (p-aminobenzoyl)-glutamate moiety and the quinazoline ring system resulted in synthesis of the analogous substituted benzoquinazoline compounds [92]. Elaboration of the aromatic ring of the p-aminobenzoyl moiety in the resulting series of compounds resulted in compounds that were substrates for FPGS, but that bound TS tightly without polyglutamation [93]. The compound 1843U89 (GW1843 – (Fig. **4**) was considered for further pre-clinical and clinical development. 1843U89 enters cells *via* the RFC [94]. Binding of 1843U89 to TS distorts the folate binding site, and enhances binding of not only dUMP analogs, but dGMP as well [95]. The benzoquinazoline ring system provided a larger surface for stacking with the purine ring system of dGMP [96]. 1943U89 was effective at disrupting multicellular tumor spheroids at concentrations of thymidine present in human serum [97]. 1843U89 has been reformulated by encapsulation in liposomes (GS7904L), and is being evaluated in clinical trials [1].

NUCLEOSIDE ANALOGS

Analogs of the dUMP substrate have proven to be effective as TS inhibitors. Analog design has, for the most part, focused on maintaining essential chemical functionality to permit tight and specific binding of the analog to the nucleotide binding site of TS. Maintaining the C6 position of uridine, or presenting an equivalent Michael acceptor [98] for the reactive cysteine of TS (C195 of human TS), is essential for the design of nucleotide analogs that form covalent complexes with TS [99]. Effective TS-inhibitory nucleotide analogs also preserve the 5'-O-phosphate that makes several electrostatic contacts with the side chains of four Arg residues that stabilize nucleotide analog binding [100]. In addition to contributing to analog binding, interactions between the 5'-O-phosphate and Arg side chains may also indirectly contribute to the activation of the reactive cysteine [101]. The presence of a planar, aromatic ring system, such as the pyrimidine ring system of dUMP, is essential for aromatic stacking with the quinazoline ring system of the reduced folate co-factor [102].

The C5 position of dUMP has been subjected to extensive modification with electrophilic moieties to create TS-inhibitory nucleotide analogs. In particular, halogen substitution for hydrogen at C5 has been shown to result in TS-inhibitory nucleotides. Halogen substitution at C5 activates C6 towards nucleophilic attack by the reactive Cys of TS and stabilizes the resulting Michael adduct that occurs subsequent to Michael addition [103]. The 5-fluoro substitution (FdUMP) has resulted in the most successful TS-inhibitor in terms of clinical utility. The strength of the C-F bond, the similar size of fluorine and hydrogen, and the electrophilic character of fluorine all contribute to the 5-fluoro analog of dUMP as being an excellent TS inhibitor. 5-chloro-2'-deoxyuridine is also a potent inhibitor of TS [104]. The 5-bromo and 5-iodo substituted dUMP analogs undergo TS-catalyzed dehalogenation in the presence of exogenous thiols that reduces their utility as TS-inhibitors [105]. The 5-fluoro and 5-chloro substituted dUMP analogs do not undergo dehalogenation, and the C-F (C-Cl) bond is too strong for elimination of F- (or Cl-), effectively trapping the enolate. The 5-nitro analog of dUMP is also a potent TS inhibitor [106]. The inhibited complexes are stabilized by the binding of reduced folate. Recently it was shown that TS-inhibitory folates also stabilized TS-inhibitory ternary complexes with FdUMP suggesting that antifolates might be used effectively with FdUMP precursors in combination chemotherapy [107].

Electrophiles other than the halogens that have additional functionality (*e.g.* multiple bonds) may also be placed at the C5 position of dUMP to create mechanism-based TS inhibitors. Among the functionality at the C5 position of dUMP that has been shown to result in the formation of a TS inhibitor is the trifluoromethyl group [108-110], an ethynyl group [111-113], and the E-2-bromovinyl group [114]. Upon Michael addition of the reactive Cys at C6 and enolate formation, these electrophiles undergo further reaction that may result in enzyme inactivation. Loss of fluoride ion from the enolate formed with trifluoromethyl dT results in formation of a difluoromethylene intermediate that reacts with Tyr 146 of *E. coli* TS inactivating this enzyme [108]. Likewise, both the 5-ethynyl and 5-(2-bromovinyl) moieties are susceptible to nucleophilic addition or displacement following Michael addition of the reactive cysteine of TS. The 5-(2-bromovinyl) substituted dUMP has been used to target cells that overexpress TS [115]. Several new mechanism-based TS inhibitors that have substituted alkene or alkyne moieties at the 5-position of dUMP have been described in recent years. These include the 5-(3-fluoropropyn-1-yl) [116] and the 5-imidazolylpropynyl [117] dUMP analogs that have been designed on the basis of structural analysis of TS ternary complexes, and that are efficient mechanism-based TS inhibitors.

While substitution of electrophiles at C5 has been the predominant mode of substitution and has resulted in the design and synthesis of the most successful TS-inhibitors, substitution at other positions of the pyrimidine ring has also been explored for the design of TS-inhibitors. 5-fluoro dCMP, the 4-amino analog of dUMP, serves as a direct TS inhibitor, as well as a precursor to FdUMP [118]. The 2- and/or 4-thio analogs of FdUMP show reduced TS-inhibitory activity relative to FdUMP [119]. Thus, spatial and mechanistic consideration have dictated that design alterations to the pyrimidine ring be focused on the C5 position. Nonetheless, certain molecules that have minimal resemblance to dUMP are good inhibitors of TS. Pyridoxal 5'-phosphate, which resembles dUMP in having a planar aromatic ring and a phosphate group, is an effective inhibitor of *Lactobacillus casei* TS [120]. Glyceraldehyde 3-phosphate also inhibits TS [121].

FDUMP PRO-DRUGS

The 5'-O-phosphate of dUMP is critical for dUMP binding to TS, as well as for activation of the reactive cysteine of TS. Since cellular uptake of nucleotides is, in general, not competitive with extracellular degradation to the respective nucleosides, a number of pro-drug strategies have been designed to deliver nucleotide pro-drugs into cells. 5'-phosphordiamidate derivatives of FdUMP were synthesized and found to inhibit the growth of murine leukemia cells

[122]. The rates of activity corresponded to the rate of hydrolysis of the phosphordiamidate to FdUMP. More recently, novel haloethyl and piperidyl phosphoramidate FdUMP pro-drugs have been described that have biological activity in the nanomolar range [123,124]. Several other strategies have been described to create neutral pro-drugs of FdUMP that are activated by cellular enzymes [125,126].

FDUMP[N] OLIGODEOXYNUCLEOTIDES

FdUMP[N] oligodeoxynucleotides (ODNs) constitute an interesting class of TS-inhibitory molecules with potential clinical utility [14-19] (Fig. **6**). The FdUMP nucleotide unit is present in intact form in the FdUMP[N] ODN [14]. Monomeric FdUMP is released from FdUMP[N] by enzymes that have 3'-O-exonucleolytic activities such as p53 [127], or the recently described TREX proteins [128]. Our laboratory has shown that FdUMP[N] ODNs have biological activities that are significantly more than N-fold greater with respect to both 5FU and FdU [129]. Further evidence that FdUMP[N] molecules enter cells as ODNs and release FdUMP intracellularly was obtained using cells lines that were deficient in the metabolism of 5FU or FdU to FdUMP [17]. FdUMP[N] molecules appear to have interesting mechanistic aspects, such as topoisomerase I cleavage complex formation, that are distinct from 5FU [130]. Exposure of cancer cells to FdUMP[N] results in complete and sustained TS-inhibition [129], and TS inhibition contributes to the antitumor activity of FdUMP[N] compounds [16]. Conjugation of FdUMP[N] molecules with folic acid has been used to target 5FU-resistant human colon cancer cells [19].

5FU PRO-DRUGS

While a number of promising strategies have been put forward or are being developed to deliver the TS-inhibitor FdUMP into malignant cells, the predominant form of fluoropyrimidine in clinical use is still 5FU [6]. The use of 5FU has many drawbacks, chiefly the rapid catabolism to fluoro-β-alanine [131] and the misincorporation of FUTP into RNA that causes GI-tract toxicity [132]. Nonetheless, 5FU penetrates cell membranes readily by passive diffusion [133] and serves as an inefficient pro-drug of FdUMP. One of the chief difficulties with the clinical administration of 5FU has been the requirement for slow intravenous infusion of the drug to achieve maximal TS inhibition [134]. For this reason, a number of 5FU pro-drug strategies have been developed to permit oral bioavailability. The most successful 5FU pro-drug that is used clinically is capecitabine (Xeloda) [135]. 5-fluoro-2'-deoxyuridine could, in principle, serve as an efficient precursor of FdUMP. FdU is rapidly metabolized to 5FU *in vivo*, and thus serves clinically as a pro-drug of 5FU [136]. Other 5FU pro-drugs that are being evaluated for clinical utility are 5'-deoxy-5-fluorouridine [137] and ftorafur [138]. Other, orally-available 5FU pro-drugs are in pre-clinical development [139].

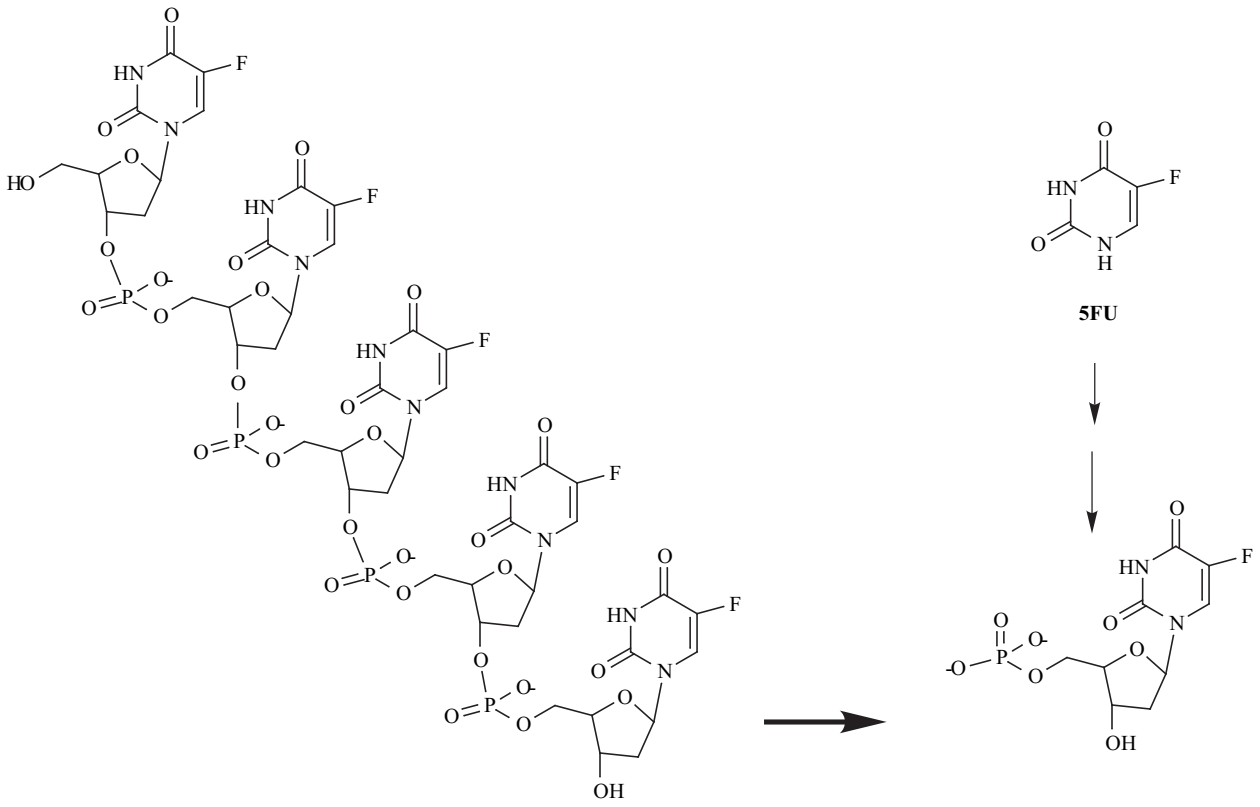

Fig. (6). Structure of FdUMP[5], representative of FdUMP[N] polymeric compounds. FdUMP[5] can be cleaved to release FdUMP while the metabolic conversion of 5FU to FdUMP requires multiple enzymatic steps.

ANTISENSE TARGETING OF TS MRNA

While the targeting of TS protein with either substrate or co-factor analogs has proven very successful for TS inhibition, in principle targeting TS mRNA (antisense strategy) or the TS gene (antigene strategy) could also be effective at reducing TS activity. TS activity is decreased by the transfection of human colon cancer cells with a TS antisense construct [140]. Exposure of cancer cells to exogenous ODNs that are antisense to TS mRNA also decreases TS expression and activity and sensitizes cells to TS-inhibitory drugs [141-143]. A variety of chemistries are possible. Antisense ODNs with the phosphorothioate backbone being the most common. 2'-O-methyl ORNs that were antisense to TS mRNA and targeted the site of TS protein binding specifically inhibited TS expression in colon cancer cells [144]. Antisense ODNs targeting the translation initiation site of TS mRNA also inhibited TS mRNA expression [145]. TS mRNA levels are also regulated by naturally expressed antisense (rTS alpha) [146]. Inhibiting transcription of TS mRNA may also be a viable strategy for reducing TS activity in malignant cells [147].

SUMMARY

TS inhibitors constitute an important class of anticancer drugs. The widespread clinical experience with 5FU and other TS inhibitors make it probable that TS inhibitors will continue to play a significant role in anticancer therapy. In this review, a variety of novel strategies that have been used to devise TS inhibitors have been reviewed. Many of these strategies have employed rational modification of the nucleotide substrate or the reduced folate co-factor. Screening methodologies have also been employed to identify novel TS inhibitors [148,149]. Other strategies have focused on inhibiting transcription of the TS gene or translation of TS mRNA, or disrupting TS protein binding to TS mRNA. Recent cancer mortality statistics reveal that the current generation of TS-inhibitors in clinical use has provided a survival benefit for patients with certain malignancies [150]. Recently, TS has been shown to be an oncogene and that overexpression of TS results in cellular transformation [151]. The development of more potent, more selective, and less toxic TS-inhibitors is likely to be of enormous benefit for cancer chemotherapy.

ACKNOWLEDGEMENT

W.H. Gmeiner acknowledges support of NIH P30 CA12197.

REFERENCES

[1] Chu, E.; Callender, M.A.; Farrell, M.P.; Schmitz, J.C. *Cancer Chemother. Pharmacol.,* **2003**, *52 (suppl 1)*, S80.
[2] Danenberg, P.V.; Malli, H.; Swenson, S. *Semin. Oncol*. **2001**, *26*, 621.
[3] Rustum, Y.M.; Harstrick, A.; Cao, S.; Vanhoefer, U.; Yin, M.B.; Wilke, H; Seeber, S. *J. Clin. Oncol*., **2003**, *15*, 389.
[4] Touroutoglou, N.; Pazdur, R. *Clin. Cancer Res*., **1996**, *2*, 227.
[5] Heidelberger, C.; Chaudhuri, N.K.; Danenberg, P.; Mooren, D.; Griesbach, L.; Duschinsky, R.; Schnitzer, R.J.; Pleven, E.; Schreiner, J. *Nature*, **1957**, *179*, 663.
[6] Grem, J. *Invest. New Drugs*, **2000**, *18*, 299.
[7] Longley, D.B.; Harkin, D.P.; Johnston, P.G. *Nature Cancer*, **2003**, *3*, 330.
[8] Jackman, A.L.; Theti, D.S.; Gibbs, D.D. *Adv. Drug Deliv. Rev*., **2004**, *56*, 1111.
[9] Bavetsias, V; Jackman, A.L. *Curr. Med. Chem*., **1998**, *5*, 265.
[10] Jackman, A.L.; Melin, C.J.; Kimbell, R.; Brunton, L.; Aherne, G.W.; Theti, D.S.; Walton, M. *Biochim. Biophys. Acta*, **2002**, *1587*, 215.
[11] Ju, J.; Kane, S.E.; Lenz, H.J.; Danenberg, K.D.; Chu, E.; Danenberg, P.V. *Clin. Cancer Res*., **1998**, *4*, 2229.
[12] Berg, R.W.; Ferguson, P.J.; Vincent, M.D.; Koropatnick, D.J. *Cancer Gene Ther*., **2003**, *10*, 278.
[13] Lin, S.B.; Ts'o, P.O.; Sun, S.K.; Choo, K.B.; Yang, F.Y.; Lim, Y.P.; Tsai, H.L.; Au, L.C. *Mol. Pharmacol*., **2001**, *60*, 474.
[14] Gmeiner, W.H. *Cancer Watch*, **1995**, *4*, 120.
[15] Gmeiner, W.H.; Sahasrabudhe, P.; Pon, R.T.; Sonntag, J.; Srinivasan, S.; Iversen, P.L. *Nucl. Nuct*., **1995**, **14**, 243.
[16] Liu, C.; Willingham, M.; Liu, J.; Gmeiner, W.H. *Intl. J. Oncol*., **2002**, *21*, 303.
[17] Liu, J.; Skradis, A.; Kolar, C.; Kolath, J.; Anderson, J.; Lawson, T.; Talmadge, J.; Gmeiner, W.H. *Nucl. Nuct*., **1999**, *18*, 1789.
[18] Liu, J.; Kolath, J.; Anderson, J.; Kolar, C.; Lawson, T.; Talmadge, J.; Gmeiner, W.H. *Antisense Nucl. Acid Drug Des*., **1999**, *9*, 481-485.
[19] Liu, J.; Kolar, C.; Lawson, T.A.; Gmeiner, W.H. *J. Org. Chem*., **2001**, *66*, 5655.
[20] Carreras, C.W.; Santi, D.V. *Annu. Rev. Biochem*., **1995**, *64*, 721.
[21] Mathews, C.K.; van Holde, K.E. Biochemistry. Benjamin/Cummings: Redwood City, CA, 1990.
[22] Hatse, S.; De Clercq, E.; Balzarini, J. *Biochem. Pharm*., **1999**, *58*, 539.
[23] van Triest, B.; Pinedo, H.M.; Giaccone, G.; Peters, G.J. *Ann. Oncol*., **2000**, *11*, 385.
[24] Costi, M.P.; Tondi, D.; Rinaldi, M.; Barlocco, D.; Pecorari, P.; Soragni, F.; Venturelli, A.; Stroud, R.M. *Biochim. Biophys. Acta*, **2002**, *1587*, 206.
[25] Deng, T.L.; Li, D.W.; Jenh, C.H.; Johnson, L.F. *J. Biol. Chem*., **1986**, *261*, 16000.
[26] Davisson, V.J.; Sirawaraporn, W.; Santi, D.V.; *J.Biol. Chem*., **1989**, *264*, 9145.
[27] Anderson, A.C.; Perry, K.M.; Freymann, D.M.; Stroud, R.M. *J. Mol. Biol*., **2000**, *297*, 645.
[28] Anderson, A.C.; O'Neil, R.H.; DeLano, W.L.; Stroud, R.M. *Biochemistry*, **1999**, *38*, 13829.
[29] Maley, F.; Pedersen-Lane, J.; Changchian, L. *Biochemistry*, **1995**, *34*, 1469.
[30] Stroud, R.M.; Finer-Moore, J.S. *Biochemistry*, **2003**, *42*, 239.
[31] Liu, Y.; Barrett, J.E.; Schultz, P.G.; Santi, D.V. *Biochemistry*, **1999**, *38*, 848.
[32] Ahmad, S.I.; Kirk, S.H.; Eisenstark, A. *Annu. Rev. Microbiol*., **1998**, *52*, 591.
[33] Sat, B.; Reches, M.; Engelberg-Kulka, H. *J. Bacteriology*, **2003**, *185*, 1803.
[34] Houghton, J.A.; Harwood, F.G.; Tillman, D.M. *Proc. Natl. Acad. Sci. USA*, **1997**, *94*, 8144.
[35] Tillman, D.M.; Petak, I.; Houghton, J.A. *Clin. Cancer Res*., **1999**, *5*, 425.
[36] Petak, I.; Tillman, D.M.; Harwood, F.G.; Mihalik, R.; Houghton, J.A. *Cancer Res*., **2000**, *60*, 2643.
[37] Ladner, R.D. *Curr. Protein Pep. Sci*., **2001**, *2*, 361.
[38] Dinner, A.R.; Blackburn, G.M.; Karplus, M. *Nature*, **2001**, *413*, 752.
[39] Ingraham, H.A.; Dickey, L.; Goulian, M. *Biochemistry*, **1986**, *25*, 3225.
[40] Valerie, K.; Povirk, L.F. *Oncogene*, **2003**, *22*, 5792.
[41] Myllykallio, H.; Lipowski, G.; Leduc, D.; Filee, J.; Forterre, P.; Liebl, U. *Science*, **2002**, *297*, 105.
[42] Sotelo-Mundo, R.R.; Ciesla, J.; Dzik, J.M.; Rode, W.; Maley, F.; Maley, G.F.; Hardy, L.W.; Montfort, W.R. *Biochemistry*, **1999**, *38*, 1087.
[43] Santi, D.V.; McHenry, C.S. *Proc. Natl. Acad. Sci. USA*, **1972**, *69*, 1855.
[44] Santi, D.V.; McHenry, C.S.; Sommer, H. *Biochemistry*, **1974**, *13*, 471.
[45] Danenberg, P.V.; Danenberg, K.D. *Biochemistry*, **1978**, *17*, 4018.

[46] Danenberg, K.D.; Danenberg, P.V. Evidence for a sequential interaction of the subunits of thymidylate synthetase. *J. Biol. Chem*., **1979**, *254*, 4345.

[47] Jackman, A.L.; Boyle, F.T.; Harrap, K.R. *Invest. New Drugs*, **1996**, *14*, 305.

[48] Jackson, R.C.; Jackman, A.L.; Calvert, A.H. *Biochem. Pharmacol*., **1983**, *32*, 3783.

[49] Bird, O.D.; Vaitkus, J.W.; Clarke, J. *Mol. Pharm*., **1970**, *6*, 573.

[50] Hynes, J.B.; Eason, D.E.; Garret, C.M.; Colvin Jr., P.L., Shores, K.E.; Freisheim, J.H. *J. Med. Chem*., **1977**, *20*, 588.

[51] Calvert, A.H.; Alison, D.L.; Harland, S.J.; Robinson, B.A.; Jackman, A.L.; Jones, T.R.; Newell, D.R.; Siddik, Z.H.; Wiltshaw, E.; McElwain, T.J.; Smith, I.E.; Harrap, K.R. *J. Clin. Oncol*., **1986**, *4*, 1245.

[52] Jones, T.R.; Thornton, T.J.; Flinn, A.; Jackman, A.L.; Newell, D.R.; Calvert, A.H. *J. Med. Chem*., **1989**, *32*, 847.

[53] Hughes, L.R.; Jackman, A.L.; Oldfield, J.; Smith, R.C.; Burrows, K.D.; Marsham, P.R.; Bishop, J.A.; Jones, T.R.; O'Connor, B.M.; Calvert, A.H. *J. Med. Chem*., **1990**, *33*, 3060.

[54] Jackman, A.L.; Taylor, G.A.; O'Connor, B.M.; Bishop, J.A.; Moran, R.G.; Calvert, A.H. *Cancer Res*., **1990**, *50*, 5212.

[55] Jackman, A.L.; Newell, D.R.; Gibson, W.; Jodrell, D.I.; Taylor, G.A.; Bishop, J.A.; Hughes, L.R.; Calvert, A.H. *Biochem. Pharm*., **1991**, *42*, 1885.

[56] Jackman, A.L.; Marsham, P.R.; Moran, R.G.; Kimbell, R.; O'Connor, B.M.; Hughes, L.R.; Calvert, A.H. *Adv. Enzyme Regul*., **1991**, *31*, 13.

[57] Jackman, A.L.; Farrugia, D.C.; Gibson, W.; Kimbell, R.; Harrap, K.R.; Stephens, T.C.; Azab, M.; Boyle, F.T. *Eur. J. Cancer*, **1995**, *31*, 1277.

[58] Farrugia, D.C.; Aherne, G.W.; Brunton, L.; Clarke, S.J. Jackman, A.L. *Clin. Cancer Res*., **2000**, *6*, 3646.

[59] Jackman, A.L.; Kimbell, R.; Aherne, G.W.; Brunton, L.; Jansen, G.; Stephens, T.C.; Smith, M.N.; Wardleworth, J.M.; Boyle, F.T. *Clin. Cancer Res*., **1997**, *3*, 911.

[60] Rhee, M.S.; Wang, Y.; Nair, M.G.; Galivan, J. Acquisition of resistance to antifolates caused by enhanced gamma-glutamyl hydrolase activities. *Cancer Res*., **1993**, *53*, 2227.

[61] Worm, J.; Kirkin, A.F.; Dzhandzhugazyan, K.N.; Guldberg, P. *J. Biol. Chem*., **2001**, *276*, 39990.

[62] Cole, P.D.; Kamen, B.A.; Gorlick, R.; Banerjee, D.; Smith, A.K.; Magill, E.; Bertino, J.R. *Cancer Res*., **2001**, *61*, 4599.

[63] Liani, E.; Rothem, L.; Bunni, M.A.; Smith, C.A.; Jansen, G.; Assaraf, Y.G. *Int. J. Cancer*, **2003**, *103*, 587.

[64] Tse, A.; Moran, R.G. *J. Biol. Chem*., **1998**, *273*, 25944.

[65] Bavetsias, V.; Jackman, A.; Kimbell, R.; Gibson, W.; Boyle, F.T.; Bisset, G.M.F. *J. Med. Chem*., **1996**, *39*, 73.

[66] Bisset, G.M.F.; Bavetsias, V.; Thornton, T.J.; Pawelczak, K.; Calvert, A.H.; Hughes, L.R.; Jackman, A. *J. Med. Chem*., **1994**, *37*, 3294.

[67] deJonge, M.J.A.; Punt, C.J.A.; Sparreboom, A.; Planting, A.S.T.; Peters, M.E.W.J.; van de Schraaf, J.; Jackman, A.; de Mulder, H.M.; Verweij, J. *J. Clin. Oncol*., **2002**, *20*, 1923.

[68] Marsham, P.R.; Wardleworth, J.M.; Boyle, F.T.; Hennequin, L.F.; Kimbell, R.; Brown, M.; Jackman, A.L. *J. Med. Chem*., **1999**, *42*, 3809.

[69] Marsham, P.R.; Jackman, A.L.; Barker, A.J.; Boyle, F.T.; Pegg, S.J.; Wardleworth, J.M.; Kimbell, R.; O'Connor, B.M.; Calvert, A.H.; Hughes, L.R. *J. Med. Chem*., **1995**, *38*, 994.

[70] Matthews, D.A.; Appelt, K.; Oatley, S.J.; Xuong, N.H. *J. Mol. Biol*., **1990**, *214*, 923.

[71] Montfort, W.R.; Perry, K.M.; Fauman, E.B.; Finer-Moore, J.S.; Maley, G.F.; Hardy, L.; Maley, F.; Stroud, R.M. *Biochemistry*, **1990**, *29*, 6964.

[72] Bavetsias, V.; Marriott, J.H.; Melin, C.; Kimbell, R.; Matusiak, Z.S.; Boyle, F.T.; Jackman, A.L. *J. Med. Chem*., **2000**, *43*, 1910.

[73] Appelt, K.; Bacquet, R.J.; Bartlett, C.A. ; Booth, C.L.J. ; Freer, S.T. ; Fuhry, M.A.M.; Gehring, M.R.; Herrmann, S.M.; Howland, E.F.; Janson, C.A.; Jones, T.R.; Kan, C-C.; Kathardekar, V.; Lewis, K.K.; Marzoni, G.P.; Matthews, D.A.; Mohr, C.; Moomaw, E.W.; Morse, C.A.; Oatley, S.J.; Ogden, R.C.; Rami Reddy, M.; Reich, S.H.; Schoetllin, W.S.; Smith, W.W.; Varney, M.D.; Villafranca, J.E.; Ward, R.W.; Webber, S.; Webber, S.E.; Welsh, K.M.; White, J. *J. Med. Chem*., **1991**, *34*, 1925.

[74] O'Dwyer, P.J.; Laub, P.B.; DeMaria, D.; Qian, M.; Reilly, D.; Giantonio, B.; Johnston, A.L.; Wu, E.Y.; Bauman, L.; Clendeninn, N.J.; Gallo, J.M. *Clin. Cancer Res*., **1996**, *2*, 1685.

[75] Webber, S.E.; Bleckman, T.M.; Attard, J.; Deal, J.G.; Kathardekar, V.; Welsh, K.M; Webber, S.; Janson, C.A.; Matthews, D.M.; Smith, W.W.; Freer, S.T.; Jordan, S.R.; Bacquet, R.J.; Howland, E.F.; Booth, C.L.J.; Ward, R.W.; Hermann, S.M.; White, J.; Morse, C.A.; Hilliard, J.A.; Bartlett, C.A. *J. Med. Chem*., **1993**, *36*, 733.

[76] Webber, S.; Bartlett, C.A.; Boritzki, T.J.; Hilliard, J.A.; Howland, E.F.; Johnston, A.L; Kosa, M; Margosiak, S.A.; Morse, C.A.; Shetty, B.V. *Cancer Chemother. Pharmacol*., **1996**, *37*, 509.

[77] Stewart, K.; Tessitore, J.; Rudy, J.; Clendennin, N.; Johnston, A. *Cancer*, **1999**, 86, 410.

[78] Tondi, D.; Slomczynska, U.; Costi, M.P.; Watterson, D.M.; Ghelli, S.; Shoichet, B.K. *Chem. Biol*., **1999**, *6*, 319.

[79] Kim, S-G.; Yoon, C-J.; Kim, S-H.; Cho, Y-J.; Kang, D-I. *Bioorg. Med. Chem*., **2000**, *8*, 11.

[80] Schiffer, C.; Clifton, I.; Davisson, V.J.; Santi, D.V.; Stroud, R.M. *Biochemistry*, **1995**, *34*, 16279.

[81] Almog, R.; Waddling, C.A.; Maley, F.; Maley, G.F.; van Roey, P. *Protein Sci*., **2001**, *10*, 988.

[82] Phan, J.; Koli, S.; Minor, W.; Dunlap, R.B.; Berger, S.; Lebioda, L. *Biochemistry*, **2001**, *40*, 1897.

[83] Tong, Y.; Liu-Chen, X.; Ercikan-Abali, E.A.; Zhao, S-C.; Banerjee, D.; Maley, F.; Bertino, J.R. *J. Biol. Chem*., **1998**, *273*, 31209.

[84] Fantz, C.; Shaw, D.; Jennings, W.; Forsthoefel, A.; Kitchens, M.; Phan, J.; Minor, W.; Lebioda, L.; Berger, F.G.; Spencer, H.T. *Mol. Pharm*., **2000**, *57*, 359.

[85] Sapse, D.S.; Tong, Y.; Bertino, J.R.; Sapse, A-M. *Cancer Invest*., **1999**, *17*, 396.

[86] Gangjee, A.; Vidwans, A.; Elzein, E.; McGuire, J.J.; Queener, S.F.; Kisliuk. *J. Med. Chem*., **2001**, *44*, 1993.

[87] Gangjee, A.; Yu, J.; J.J.McGuire, J.J.; Cody, V.; Galitsky, N.; Kisliuk, R.L.; Queener, S.F. *J. Med. Chem*., **2000**, *43*, 3837.

[88] John, W.; Picus, J.; Blanke, C.D.; Clark, J.W.; Schulman, L.N.; Rowinsky, E.K.; Thornton, D.E.; Loehrer, P.L. *Cancer*, **2000**, *88*, 1807.

[89] Sigmond, J.; Backus, H.H.; Wouters, D.; Temmink, O.H.; Jansen, G.; Peters, G.J. *Biochem. Pharmacol*., **2003**, *66*, 431.

[90] Wang, Y.; Zhao, R.; Goldman, I.D. *Biochem. Pharmacol*., **2003**, *65*, 1163.

[91] Pendergast, W.; Dickerson, S.H.; Johnson, J.V.; Dev, I.K.; Ferone, R.; Duch, D.S.; Smith, G.K. *J. Med. Chem*., **1993**, *36*, 3464.

[92] Pendergast, W.; Dickerson, S.H.; Dev, I.K.; Ferone, R.; Duch, D.S.; Smith, G.K. *J. Med. Chem*., **1994**, *37*, 838.

[93] Hanlon, M.H.; Ferone, R. *Cancer Res*., **1996**, *56*, 3301.

[94] Duch, D.S.; Banks, S.; Dev, I.K.; Dickerson, S.H.; Ferone, R.; Heath, L.S.; Humphreys, J.; Knick, V.; Pendergast, W.; Singer, S. *Cancer Res*., **1993**, *53*, 4810.

[95] Weichsel, A.; Montfort, W.R.; Ciesla, J.; Maley, F. *Proc. Natl. Acad. Sci. USA*, **1995**, *92*, 3493.

[96] [96]Chen, C-H.; Davis, R.A.; Maley, F. *Biochemistry*, **1996**, *35*, 8786.

[97] Banks, S.D.; Waters, K.A.; Barrett, L.L.; Dickerson, S.; Pendergast, W.; Smith, G.K. *Cancer Chemother. Pharmacol*., **1994**, *33*, 455.

[98] [98]Kalman, T.I. *Biochem. Biophys. Res. Commun*., **1972**, *49*, 1007.

[99] Kalman, T.I. ; Nie, Z. *Nucl. Nuct. Nucl. Acids*, **2000**, *19*, 357.

[100] Stroud, R.M.; Finer-Moore, J.S. *FASEB J*., **1993**, *7*, 671.

[101] Phan, J.; Mahdavian, E.; Nivens, M.C.; Wladek, M.; Berger, S.; Spencer, H.T.; Dunlap, R.B.; Lebioda, L. *Biochemistry*, **2000**, *39*, 6969.

[102] Montfort, W.R.; Weichsel, A. *Pharmacol. Ther*., **1997**, *76*, 29.

[103] Wataya, Y.; Santi, D.V. *J. Am. Chem. Soc*., **1977**, *99*, 4534.

[104] Brandon, M.L.; Mi, L-J.; Chaung, W.; Teebor, G.; Boorstein, R.J. *Mutation Res*., **2000**, *459*, 161.

[105] Garrett, C.; Wataya, Y.; Santi, D.V. *Biochemistry*, **1979**, *18*, 2798.

[106] Matsuda, A.; Wataya, Y.; Santi, D.V. *Biochem. Biophys. Res. Commun*., **1978**, *84*, 654.

[107] van der Wilt, C.L.; Smid, K.; Peters, G.J. *Biochem. Pharm*., **2002**, *64*, 669.

[108] Eckstein, J.W.; Foster, P.G.; Finer-Moore, J.; Wataya, Y.; Santi, D.V. *Biochemistry*, **1994**, *33*, 15086.

[109] Danenberg, P.V.; Heidelberger, C. *J. Med. Chem*., **1973**, *16*, 712.

[110] Fujiwara, W.; Oki, T.; Heidelberger, C. *Mol. Pharmacol*., **1970**, *6*, 281.

[111] Barr, P.J.; Robins, M.J.; Santi, D.V. *Biochemistry*, **1983**, *22*, 1696.

[112] Barr, P.J.; Nolan, P.A.; Santi, D.V.; Robins, M.J. *J. Med. Chem*., **1981**, *24*, 1385.
[113] Danenberg, P.V.; Bhatt, R.S.; Kundu, N.G.; Danenberg, K.; Heidelberger, C. *J. Med. Chem*., **1981**, *24*, 1537.
[114] Barr, P.J.; Oppenheimer, N.J.; Santi, D.V. *J. Biol. Chem*., **1983**, *258*, 13627.
[115] Li, Q.; Boyer, C.; Lee, J.Y.; Shepard, H.M. *Mol. Pharm*., **2001**, *59*, 446.
[116] Kalman, T.I.; Nie, Z.; Kamat, A. *Bioorg. Med. Chem. Lett*., **2000**, *10*, 391.
[117] Saxl, R.L.; Reston, J.; Nie, Z.; Kalman, T.I.; Maley, F. *Biochemistry*, **2003**, *42*, 4544.
[118] Liu, L.; Santi, D.V. *Biochim. Biophys. Acta*, **1994**, *1209*, 89.
[119] Jarmula, A.; Anulewicz, R.; Les, A.; Cyranski, M.K.; Adamowicz, L.; Bretner, M.; Felczak, K.; Kulikowski, T.; Krygowski, T.M; Rode, W. *Biochim. Biophys. Acta*, **1998**, *1382*, 277.
[120] Santi, D.V.; Ouyang, T.M.; Tan, A.K.; Gregory, D.H.; Scanlan, T.; Carreras, C.W. *Biochemistry*, **1993**, *32*, 11819.
[121] Bures, A.K.; Daron, H.H.; Aull, J.L. *Int. J. Biochem*., **1991**, *23*, 733.
[122] Phelps, M.E.; Woodman, P.W.; Danenberg, P.V. *J. Med. Chem*., **1980**, *23*, 1229.
[123] Tobias, S.C.; Borch, R.F. *J. Med. Chem*., **2001**, *44*, 4475.
[124] Freel Meyers, C.L.; Hong, L.; Joswig, C.; Borch, R.F. *J. Med. Chem*., **2000**, *43*, 4313.
[125] Farquhar, D.; Chen, R.; Khan, S. *J. Med. Chem*., **1995**, *38*, 488.
[126] Tosqullas, G.; Alvarez, K.; Dell'Aquilla, C.; Morvan, F. ; Vasseur, J.J. ; Imbach, J.L.; Rayner, B. *Nucl. Acids Res*., **1998**, *26*, 2069.
[127] Mummenbrauer, T.; Janus, F.; Muller, B.; Weismuller, L.; Deppert, W.; Grosse, F. *Cell*, **1996**, *85*, 1089.
[128] Mazur, D.J.; Perrino, F.W. *J. Biol. Chem*., **2001**, *276*, 17022.
[129] Gmeiner, W.H.; Trump, E.; Wei, C. *Nucl. Nuct. Nucl. Acids*, **2004**, *23*, 401.
[130] Liao, Z-Y.; Sordet, O.; Zhang, H-L.; Kohlhagen, G.; Antony, S.; Gmeiner, W.H.; Pommier, Y. *Submitted*.
[131] Paff, M.T.; Baccanari, D.P.; Davis, S.T.; Cao, S.; Tansik, R.L.; Rustum, Y.M.; Spector, T. *Invest. New Drugs*, **2000**, *18*, 365.
[132] Pritchard, D.M.; Watson, A.J.M.; Potten, C.S.; Jackman, A.L.; Hickman, J.A. *Proc. Natl. Acad. Sci. USA*, **1997**, *94*, 1795.
[133] Tiraby, M.; Cazaux, C.; Baron, M.; Drocourt, D.; Reynes, F.P.; Tiraby, G. *FEMS Microbial Lett*., **1998**, *167*, 41.
[134] Ren, Q.; van Groeningen, J.V.; Hardcastle, A.; Aherne, G.W.; Geoffrey, F.; Allegra, C.J.; Johnston, P.G.; Grem, J.L. *Oncology Res*., **1997**, *9*, 77.
[135] Hoff, P.M. *Semin.Oncol*., **2003**, *30*, 88.
[136] Uchida, M.; Brown, N.; Ho, D.H.W. *Anticancer Res*., **1990**, *10*, 779.
[137] Ikemoto, S.; Sugimura, K.; Yoshida, N.; Kuratsukuri, K.; Wada, S.; Nakatani, T. *Anticancer Res*., **2002**, *22*, 4023.
[138] Malet-Martino, M.; Martino, R. *Oncologist*, **2002**, *7*, 288.
[139] Rohlff, C.; Watson, S.A.; Morris, T.M.; Skelton, L.; Jackman, A.L.; Page, M.J. *Cancer Res*., **1999**, *59*, 1268.
[140] Matsuoka, K.; Tsukuda, K.; Suda, M.; Kobayashi, K.; Ota, T.; Okita, A.; Watanabe, K.; Suzuki, E.; Murikami, M.; Doihara, H.; Shimizu, N. *Int. J. Oncol*., **2004**, *24*, 217.
[141] Berg, R.W.; Ferguson, P.J.; Vincent, M.D.; Koropatnick, D.J. *Cancer Gene Ther*., **2003**, *10*, 278.
[142] Ferguson, P.J.; DeMoor, J.M.; Vincent, M.D.; Koropatnick, J. *Br. J. Pharm*., **2001**, *134*, 1437.
[143] Lin, S.B.; Ts'o, P.O.; Sun, S.K.; Choo, K.B.; Yang, F.Y.; Lim, Y.P.; Tsai, H.L.; Au, L.C. *Mol. Pharmacol*., **2001**, *60*, 474.
[144] Schmitz, J.C.; Yu, D.; Agrawal, S.; Chu, E. *Nucl. Acids Res*., **2001**, *29*, 415.
[145] Ju, J.; Kane, S.E.; Lenz, H.J.; Danenberg, K.D.; Chu, E.; Danenberg, P.V. *Clin. Cancer Res*., **1998**, *4*, 2229.
[146] Chu, J.; Dolnick, B.J. *Biochim. Biophys. Acta*, **2002**, *1587*, 183.
[147] Powell, C.M.H.; Rudge, T.L.; Zhu, Q.; Johnson, L.F.; Hansen, U. *EMBO J*., **2000**, *19*, 4665.
[148] Wu, Q.; Dolnick, B.J. *Mol. Pharm*., **2003**, *63*, 167.
[149] Erlanson, D.A.; Braisted, A.C.; Raphael, D.R.; Randal, M. ; Stroud, R.M. ; Gordon, E.M. ; Wells, J.A. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 9367.
[150] Bal, G. *CA Cancer J. Clin*., **2001**, *51*, 11.
[151] Rahman, L.; Voeller, D.; Rahman, M.; Lipkowitz, S.; Allegra, C.; Barrett, J.C.; Kaye, F.J.; Zajac-Kaye, M. *Cancer Cell*, **2004**, *5*, 341.