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Novel Approaches for Targeting Thymidylate Synthase To Overcome the Resistance and Toxicity of Anticancer Drugs

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1. Thymidylate Synthase

The antifolate drug, methotrexate, was introduced to the clinic as an anticancer agent in the early 1950s.[1] Subsequently, its mechanism of action was elucidated and it was found to bind in mono- and polyglutamated forms to dihydrofolate reductase (DHFR[a]),[2,3] thymidylate synthase (TS),[4] and aminoimidazolecarboxamide ribonucleotide transformylase (AICARTF).[5] A fluoropyrimidine, 5-fluorouracil (5FU), was conceived in 1957[6] following the observation that uracil was utilized preferentially in malignant over nonmalignant cells[7] and has since been a first line drug in cancer chemotherapy. Subsequently, 5-fluoro-2′-deoxyuridine 5′-monophosphate (5FdUMP), an active metabolite of 5FU, was found to inhibit TS by forming a covalent ternary complex with the enzyme and 5,10-methylenetetrahydrofolate (mTHF).[8] These discoveries marked the dawn of exploiting TS as an anticancer target.

TS (EC 2.1.1.45), which is encoded by the TYMS gene in humans, catalyzes the conversion of 2′-deoxyuridine 5′-monophosphate (dUMP) to 2′-deoxythymidine 5′-monophosphate (dTMP) by using mTHF as a cosubstrate. dTMP is then phosphorylated by thymidylate kinase to 2′-deoxythymidine diphosphate (dTDP) and then to 2′-deoxythymidine triphosphate (dTTP) by nucleoside diphosphate kinase for use in the synthesis of new DNA. Thus, in human cells, TS plays a key role in the biosynthetic pathway that provides the sole de novo source of thymidylate, an essential precursor required for DNA replication and repair.[9] In addition to its catalytic function, TS acts as a regulator of translation for some mRNAs. One of these is its own mRNA,[10] and others include p53,[11] which is a tumor suppressor, and c-myc,[12] which is oncogenic. Binding of the TS protein to its own mRNA leads to the formation of an autoregulatory feedback loop for repression of the translation of TS mRNA (Figure 1). A 36 nucleotide sequence (75−110, site I),[13] encompassing the start codon, and a 70 nucleotide sequence (480−550, site II)[14] within the coding region have been identified as the most essential regions in the TS mRNA for binding to the TS protein. On the p53 mRNA, the nucleotide sequence from 531 to 1020 in the protein coding region,[15] and for the c-myc mRNA, the C terminal coding region covering nucleotide positions 1625−1790,[12] have been identified to be important for binding to the TS protein. On the basis of the observation that overexpression of TS sets the cells into a neoplastic phenotype, oncogenic behavior is a novel role that has been attributed to TS recently.[16]

1.1. Resistance and Toxicity: Need for New Approaches. TS has two substrates, dUMP and mTHF, both of which bind in the catalytic site to enable the synthesis of dTMP. Known inhibitors of TS, e.g., fluoropyrimidines or the antifolates such as raltitrexed[1] (Figure 2), act as analogues of one of these substrates and compete for the catalytic pocket to inhibit the enzyme. However, soon after exposure, the cells develop resistance to these chemotherapeutic agents. Multiple mechanisms have been proposed to explain this effect.

One proposed mechanism is disruption of the autoregulatory repression of translation (Figure 1). Only apo-TS can bind the mRNA,[10] whereas TS bound to ligands, such as TS inhibitory drugs, cannot interact with the mRNA.[17] Ligand binding thereby alleviates the translational repression by TS protein, inducing overexpression of TS protein. Increased levels of cellular TS thereby negate the therapeutic effect of TS inhibitory drugs, leading to resistance. In contradiction, though, is the observation that, after treatment of human colon tumor cell lines with 5-fluorodeoxyuridine (5FdUrd), there is no increase of the ribosome/TS mRNA ratio that could be responsible for higher TS expression but instead the half-life of the TS protein is increased.[18] Gene amplification is another TS-inducing mechanism employed by cells for developing resistance to TS inhibitors.[19]

Moreover, some individuals are inherently more resistant to TS targeted therapy than others. In some cases, this can be attributed to the polymorphism of the TS gene. TYMS is a polymorphic gene having two (2R) or three (3R) repeats of a 28 base sequence in the 3′ untranslated region (UTR).

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[a]Abbreviations: αFR, α folate receptor; CE, carboxylesterase; CDK, cyclin dependent kinase; CD, cytidine deaminase; Cyp 450, cytochrome P450; DHFR, dihydrofolate reductase; DPD, dihydropyrimidine dehydrogenase; ECTA, enzyme catalyzed therapeutic activation; EGFR, epidermal growth factor receptor; FPGS, folylpolyglutamate synthetase; GI, gastrointestinal; GARFT, glycinamide ribonucleotide formyl transferase; HDACi, histone deacetylase inhibitors; ODN, oligodeoxynucleotide; OPRT, orotate phosphoribosyltransferase; PKC, protein kinase C; RFC, reduced folate carrier; siRNA, small inhibitory RNAs; TK, thymidine kinase; TP, thymidine phosphorylase; TS, thymidylate synthase; UK, uridine kinase; UP, uridine phosphorylase.

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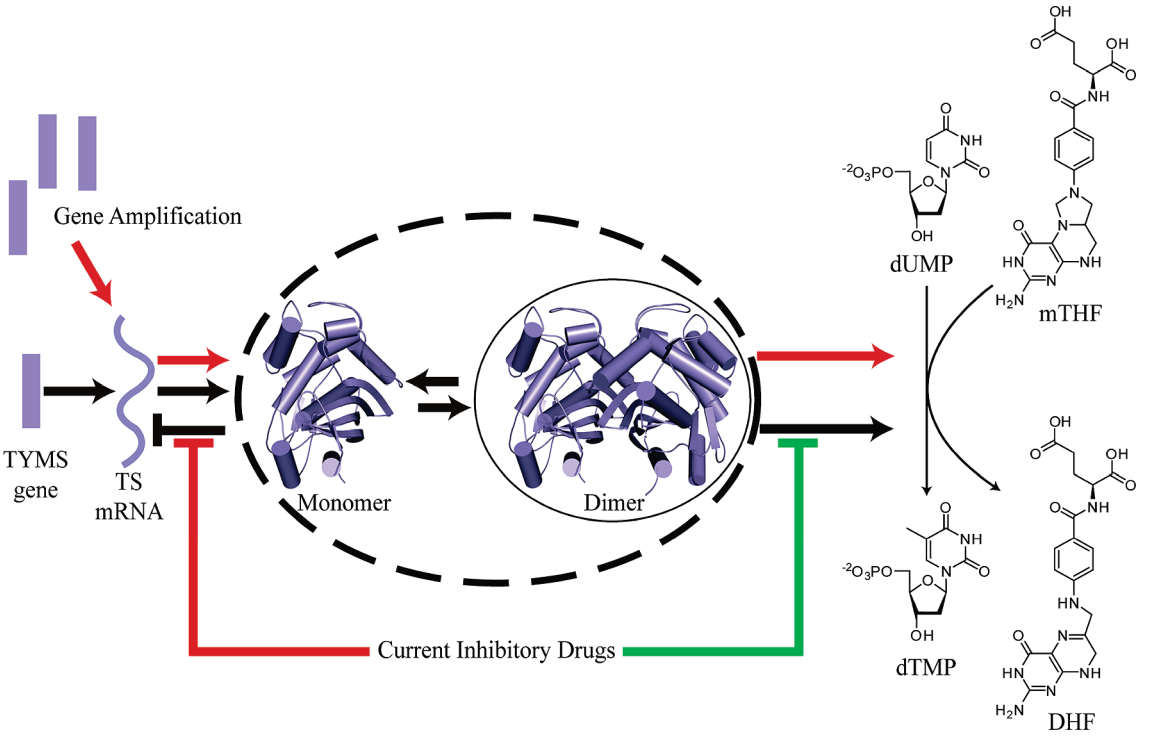


Figure 1. Mechanisms of inhibition of hTS by drugs (green) and of drug resistance (red). From left to right: The TYMS gene is transcribed to TS mRNA which is translated to hTS protein which catalyzes the conversion of dUMP and mTHF to dTMP and dihydrofolate (DHF). The TS protein is shown in its monomeric and dimeric forms, as it has been suggested to exist in a monomer−homodimer equilibrium (black arrows) in nature (Voeller et al. *Biochem. Biophys. Res. Commun.* **2002**, *297*, 24−31). The dimeric form is obligatory for its catalytic activity; which of the two forms binds to the TS mRNA is, however, debatable. Drug resistance by gene amplification and by interruption of the protein−mRNA binding can occur independently of each other.

Humans can be homozygous for either one of these forms, i.e., 2R/2R or 3R/3R or heterozygous 2R/3R. The individuals with 3R tandem repeats express higher levels of TS and could be intrinsically more resistant to TS inhibitory drugs than those with the 2R type.[20−22]

Like other chemotherapeutic drugs, the TS inhibitory anticancer agents also have toxic effects on healthy tissue. Myelotoxicity, thrombocytopenia, neutropenia, mucositis, and diarrhea caused by damage to bone marrow cells, blood cells, or the intestinal lining[23−25] are a few examples of the various toxicities observed upon administration of TS inhibitors. Most of these toxic effects can be avoided by specific delivery of the drugs to the tumors, thereby sparing the remaining tissues. Advances in this direction have led to the design of drugs such as capecitabine[26] (Scheme 1) and pemetrexed[1] (Scheme 2) which show superior toxicity profiles. However, there is still a vast scope for improvement before the severe side effects are completely abrogated.

A PubMed search carried out on March 31, 2010, using "thymidylate synthase" and "cancer" as keywords, retrieved 373 papers published in the past 3 years, illustrating the fact that despite the challenges presented, the status of TS as an anticancer target remains undiminished and it continues to be a focus for cancer therapy research. This Perspective aims to give a brief overview of various strategies that are being employed to overcome the hurdles of resistance and toxicity while targeting TS.

2. New Chemical Strategies for Targeting the Enzyme Thymidylate Synthase

The first structure of TS to be solved was that from *Lactobacillus casei* in 1987.[27] Since then, many structures for human (hTS) and other TS proteins in free and liganded forms have been determined, giving insights into the structural and dynamic features of the protein. The understanding of the molecular mechanisms involved in the catalytic action of TS and its role in pyrimidine synthesis, and folate and related pathways has also improved immensely over the years since it was recognized as an anticancer target. This improved knowledge is now being applied to identify new compounds that could inhibit the enzyme while bypassing the toxicity and resistance problems. This section will deal with some of the strategies being used, some of which have already shown success in the clinic, while others are still at an early experimental level.

2.a. 5FU Prodrugs. The concept of a prodrug is widely employed as a means to improve the pharmacology and pharmacokinetics of an active drug molecule. 5FU, which is one of the first choice anticancer drugs, particularly against colorectal cancer, is itself a prodrug (Scheme 1) (see review by Longley et al.[28]). 5FU is bioactivated to active metabolites, e.g., 5FdUMP (a TS inhibitor), 5-fluorouridine triphosphate (5FUTP, which gets incorporated into the RNA). However, since the enzymes for activating 5FU are not tumor selective, toxicity is a major issue with this drug, e.g., activation in the intestines leads to diarrhea. Moreover, it is rapidly degraded by dihydropyrimidine dehydrogenase (DPD), which is abundantly found in liver, thereby limiting its oral bioavailability. Despite this, 5FU is still being used in a clinical setting in part because of its low therapeutic cost. A prodrug for 5FU, capecitabine (N^4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine), was designed by Miwa et al.[26] and was approved by the FDA in 1998, with a recommended dose of 2500 (mg/m^2)/day (www.accessdata.fda.gov/Scripts/cder/DrugsatFDA/).

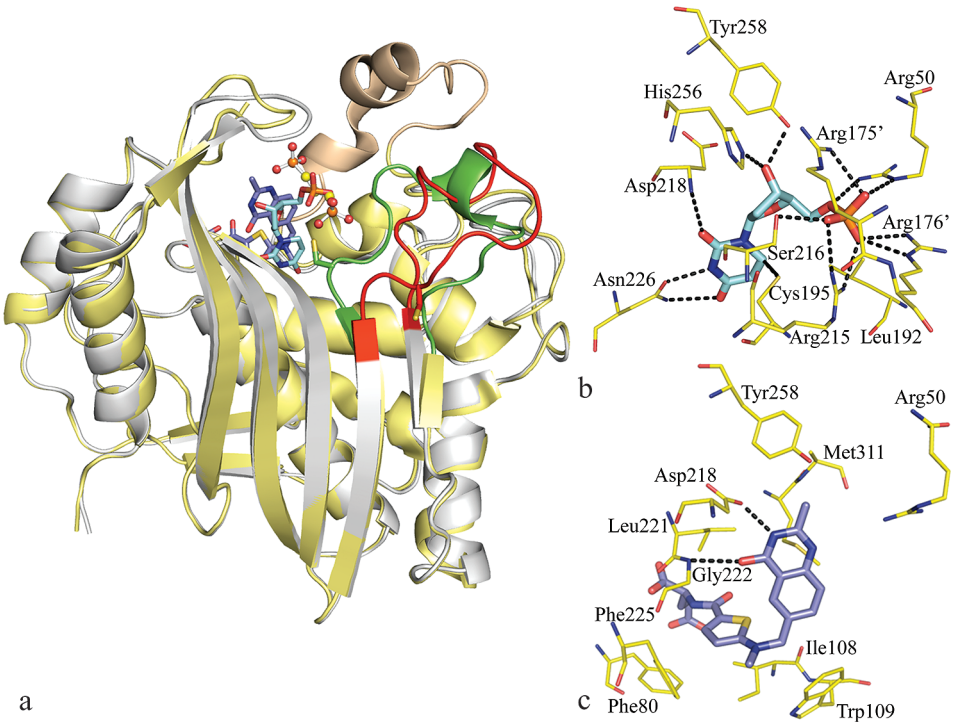


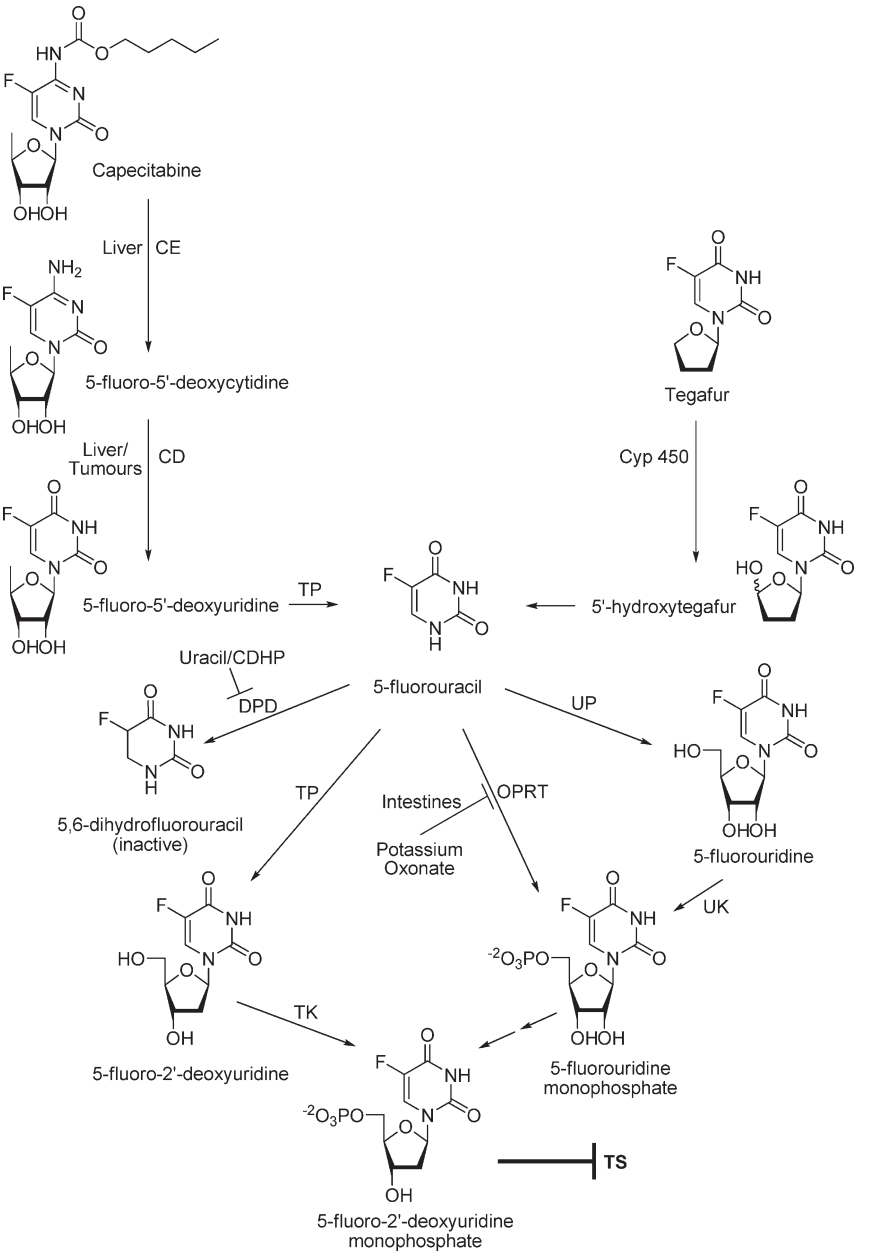
Figure 2. (a) Cartoon representation of the superimposed monomeric subunits (from the crystallized dimers) of human thymidylate synthase in active (PDB code 1HVY) (yellow) and inactive (PDB code 2ONB) (gray) conformations. The active conformation of the active site loop is shown in green and the inactive conformation in red. The catalytic cysteine, C195, is highlighted with stick representation on the loops. The small domain visible in the active crystal structure is shown in brown. dUMP (cyan sticks) and a folate analogue raltitrexed (dark-blue sticks) are present in the active site, whereas PDPA (ball-and-stick representation) is located in an allosteric position. (b) Interactions of dUMP with the protein. Dashed lines represent direct hydrogen bonds between amino acid residues and the ligand. Solid line between Cys195 and dUMP represents a covalent bond. (c) Interactions of folate analogue raltitrexed with the protein.

Capecitabine is selectively converted to 5FU in tumor cells by a cascade of three enzymes, hepatic carboxylesterase (CE), cytidine deaminase (CD) mainly localized in liver and tumor tissues, and thymidine phosphorylase (TP) which is more highly active in tumorous than in normal tissues (Scheme 1), thereby making its action tumor-specific. Improved oral bioavailability and reduced incidence of diarrhea are other major advantages of capecitabine over 5FU,[29] but incidences of a dose-limiting side effect hand-and-foot syndrome have been reported.[30] Tegafur (5-fluoro-1-(tetrahydro-2-furanyl)-2,4(1*H*,3*H*)-pyrimidinedione)[31] is another prodrug of 5FU (Scheme 1), bioactivated in the liver by cytochromes P-450 (Cyp 450).[32] It is administered in combination with modulators such as uracil[33] and 5-chloro-2,4-dihydroxypyridine,[34,35] which compete for DPD, consequently improving the bioavailability of the released 5FU. Combination with potassium oxonate, which inhibits orotate phosphoribosyltransferase (OPRT) in the gastrointestinal (GI) tract, helps in reducing the GI toxicities.[34,35]

Another experimental approach to designing prodrugs for specific delivery employs the antibody directed prodrug therapy (ADEPT) or gene directed prodrug therapy (GDEPT) methodology. The first step is to specifically deliver enzymes to the surface of tumor tissues. Next the prodrugs that are metabolized by the enzymes are administered, thereby leading to activation of the drug molecule only at the tumor mass.[36] Application of cephem conjugation and release of the active molecule by β-lactamase is a well established strategy in the literature for tumor specific delivery of chemotherapeutic agents such as mitomycin and platinum derived drugs. Recently, an experimental 5FU-cephem conjugate that remains stable until activated by β-lactamase has been reported by Phelan et al.[37] Another type of compound with potential as a prodrug is the FdUMP[N] oligodeoxynucleotide, which can be simply cleaved by enzymes with 3′-*O*-exonucleolytic activity such as p53 to release FdUMP.[38]

2.b. Enzyme Catalyzed Therapeutic Activation. Conventional drugs against TS act by inhibiting the enzyme and are ineffective at high concentrations of the protein, as observed in resistant cells. The enzyme catalyzed therapeutic activation (ECTA) approach utilizes the "target" enzyme to activate the ligand, thus turning high concentrations of the enzyme, as observed in resistant cells, to advantage. (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine 5′-monophosphate (BVdUMP), a metabolite of the antiviral agent (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU), is known to be a competitive substrate of TS, which converts it to cytotoxic products intracellularly. Lackey et al.[39] have designed a pronucleotide analogue of BVdU, (*E*)-5-(2-bromovinyl)-2′-deoxy-5′-uridylphenyl L-methoxylalaninylphosphoramidate **1** (NB1011, Chart 1), which is converted to BVdUMP by intracellular enzymes and subsequently to cytotoxic products by TS. It has been demonstrated that **1** is at least 10 times more cytotoxic to cells overexpressing TS, including 5FU resistant cells, and its activity is abolished in the presence of TS inhibitors such as raltitrexed.[40,41] Synergistic antitumor effects have been demonstrated with other nucleoside transport inhibitory chemotherapeutic agents, such as dipyridamole and *p*-nitrobenzylthiosine. The mechanism of synergy is, however, not well understood.[42] **1** has recently completed phase 1 clinical trials on patients with metastatic colorectal cancer with fluoropyrimidine failure[43] (e.g., clinicaltrials.gov/

Scheme 1. Mechanism of Activation of Capecitabine and Tegafur to TS Inhibitors[a]



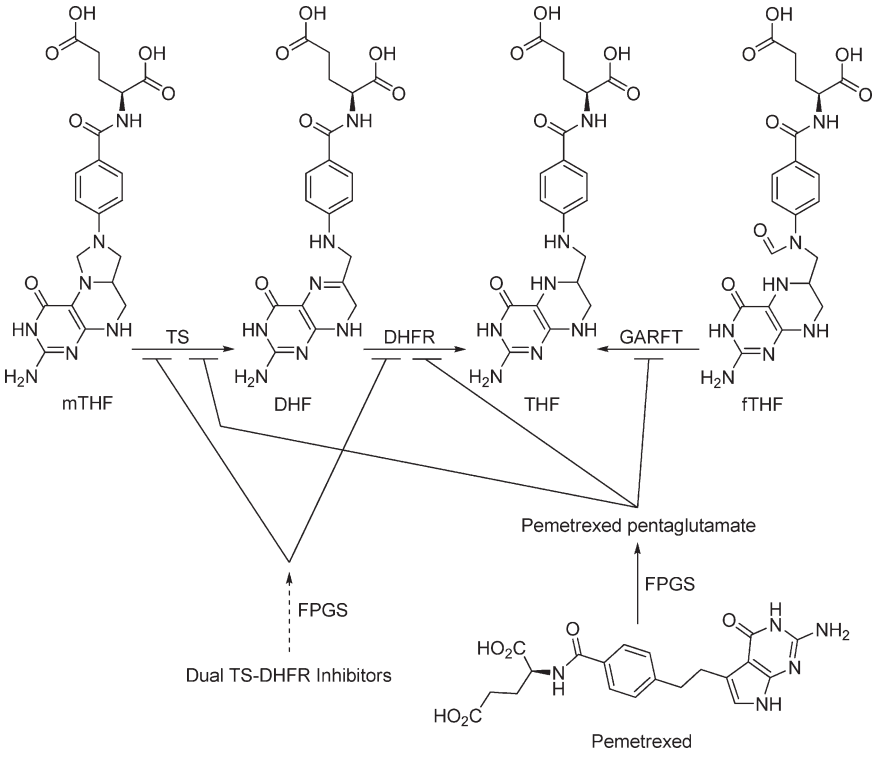
[a] CDHP: 5-chloro-2,4-dihydroxypyridine. The enzymes involved are CE, CD, TP, Cyp 450, UP, UK, OPRT, and TK.

ct2/show/NCT00031616). The success of **1** has stimulated efforts to design new and more potent derivatives of BVdU.[44]

2.c. Multitarget Inhibitors. Pemetrexed, (*N*-[4-[2-(2-amino-4,7-dihydro-4-oxo-1*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]-benzoyl]-L-glutamic Acid, Scheme 2) provides one example of successfully targeting more than one enzyme for anticancer activity. Many detailed reviews can be found on its activity (e.g., see Adjei[45]). In short, the drug enters the cells via a reduced folate carrier and is polyglutamated by folylpolyglutamate synthetase (FPGS) to the pentaglutamate form which is 60 times more potent than the parent compound and has enhanced intracellular retention. It then additively inhibits at least three enzymes in the purine and pyrimidine synthesis pathway, namely, TS (K_i = 1.3 nM), DHFR (K_i = 7.2 nM), and glycinamide ribonucleotide formyl transferase (GARFT) (K_i = 380 nM),[46] all of which have folate derivatives as their natural substrates.

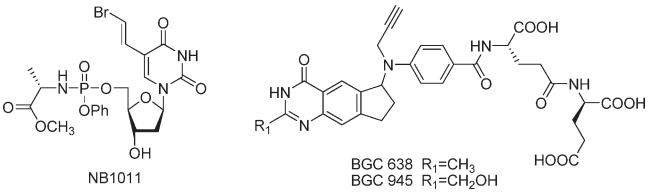
The enzymes TS and DHFR are coupled on the folate pathway such that the product of TS is the substrate for DHFR. Folate analogue inhibitors of TS and DHFR have independently found utility in the clinic as anticancer agents. The substitution on the pyrimidine ring seems to determine whether the molecule will be a TS or DHFR inhibitor; TS inhibitors generally contain a 2-amino-4-oxo or 2-methyl-4-oxo substitution on the pyrimidine ring, whereas the inhibitors of DHFR generally carry a 2,4-diamino substitution.[47] A common inhibitor for both TS and DHFR could show additive effects while circumventing the pharmaceutical disadvantages of the simultaneous application of two drugs. In view of this idea, Gangjee et al. have been working on the structure-based design and synthesis of potent dual inhibitors of TS and DHFR (Scheme 2, Chart 2), such as pyrrolo-[2,3-*d*]pyrimidines **2**, **3**,[48] thieno[2,3-*d*]pyrimidines **4**,[47] and pyrrolo[3,2-*d*]pyrimidines **5**,[49] some of which do not require

Scheme 2. Mechanism of Activation and Targeting Pathway of Multitarget Folate Analogue Inhibitors[a]



[a] The known inhibitor pemetrexed is pentaglutamated by FPGS for potent inhibitory activity, whereas some of the experimental dual TS-DHFR inhibitors do not need glutamation. THF: tetrahydrofolate. fTHF: formyltetrahydrofolate.

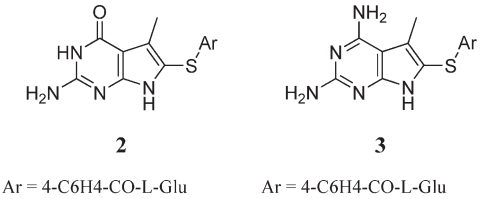
Chart 1



polyglutamylation for activity, e.g., the 2-[(benzoylamino)-4′-yl]-L-glutamic acid derivative of **5** (**5i**).[49] This latter aspect is important for avoiding one route to developing resistance, that via reduced FPGS activity.[50] Some of these molecules, e.g., *N*-{4-[(2-amino-6-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-5-yl)sulfanyl]benzoyl}-L-glutamic acid (**4bi**)[47] have shown potent dual inhibitory activity, not only against human enzymes (IC_{50} of 40 and 20 nM, respectively, for hTS and DHFR) but also against *Escherichia coli* and *Toxoplasma gondii* enzymes, with IC_{50} values nearly in the same range, and they could thus be possible leads for antiparasitic and anticancer agents. A further step could be to design molecules that would additionally inhibit other folate enzymes but not be dependent upon polyglutamation by FPGS for activity.

2.d. Stabilizing the Inactive Conformer and Allosteric Inhibitors. When Schiffer and co-workers[51] first crystallized the unliganded form of hTS, they found that the active site loop (residues 181−197) containing the catalytic cysteine (Cys195) was twisted about 180° compared to the corresponding loop conformation in the liganded hTS. Since in unliganded hTS, Cys195 is outside the active site, the enzyme must be inactive (Figure 2). The authors suggested that the inactive conformation might serve to protect the catalytic

Chart 2. Examples of Dual TS-DHFR Inhibitors



a) X = NH and Ar = 4-C_6H_4-CO-L-Glu

b) X = S and

i) Ar = 4-C_6H_4-CO-L-Glu

ii) Ar = Ph

iii) Ar = 4-Cl-Ph

iv) Ar = 4-NO_2-Ph

v) Ar = 4-F-Ph

i) Ar = 4-CO-L-Glu-Ph

ii) Ar = 4-NO_2-Ph

iii) Ar = 3,4-dl-Cl-Ph

cysteine from cellular modification. Three phosphate/sulfate ions were observed to be bound near the active site, suggesting that inactive hTS could bind to TS mRNA, thereby repressing TS protein synthesis. In addition, the disordered small domain (residues 107−128) of the inactive hTS likely increases its proneness to cellular degradation, further reducing the cellular TS levels. They demonstrated through

fluorescence studies that there is an equilibrium between the active and inactive states; phosphate ions were shown to shift the equilibrium toward the inactive state and binding of dUMP toward the active state.[52] The R163K mutant which stabilizes the active conformer is at least 33% more active than WT hTS, suggesting that at least $^1/_3$ of hTS populates the inactive state.[53] In another crystal of the inactive conformer obtained under low salt conditions,[54] a clear density was observed in a hydrophobic pocket unique to the inactive conformation (formed by residues Phe137, Gln138, Phe142, Gly143, Trp182, and Leu187). It was interpreted as a valine or leucine residue, either from the flexible N-terminus or from the neighboring molecule in the crystal. As this allosteric pocket is located about 16−18 Å from the phosphate/sulfate ions binding site, ligands binding to it could possibly stabilize the inactive conformation.

On the basis of structural considerations, Lovelace et al.[55] designed an anionic inhibitor of hTS, 1,3-propanediphosphonic acid (PDPA), which binds partially to the previously detected phosphate/sulfate sites (Figure 2). Since this position is distinct from the dUMP or folate binding sites, PDPA is an allosteric inhibitor. It shows a complex inhibition profile for the WT hTS, uncompetitive inhibition at low concentrations (K_i = 0.26 μM), and mixed (noncompetitive) inhibition at higher concentrations (K_i = 2.7 μM). On the other hand, the R163K mutant and mouse TS are only weakly but competitively inhibited by PDPA (most likely by binding to the site of the dUMP phosphate moiety). Since these proteins probably do not populate the inactive state, the complex inhibition profile of PDPA for the WT hTS is proposed to be related to the presence of the inactive conformer. Further, PDPA also demonstrated positive cooperativity with an antifolate inhibitor, (*S*)-2-[4-[*N*-[(3,4-dihydro-2,7-dimethyl-4-oxo-6-quinazolinyl)methyl]-*N*-prop-2-ynylamino]-2-fluorobenzamido]-4-(1*H*-1,2,3,4-tetrazol-5-yl)butyric acid (ZD9331).[53] Unfortunately, this small ligand is very polar in nature and is not an ideal lead structure for drug development.

The structural and kinetic features involved in the active−inactive transition and stabilization of either state are still not well understood. Experimental studies, such as design of the fully active R163K variant and the recently reported fully inactive M190K variant,[56] can aid in designing inhibitors to stabilize the inactive state. Thus, ligands designed to prevent the conformational change from the catalytically inactive to the active form might provide a novel approach to inhibiting the enzyme and may also help to avoid the development of cellular resistance associated with higher TS protein levels. The allosteric sites mentioned above may be too small to be targeted by druglike molecules. However, it might be possible to usefully exploit them by additionally targeting neighboring pockets. There may also be other, as yet undiscovered binding sites in hTS that can be targeted to affect the amount of active enzyme.

2.e. Exploiting the Overexpressed Transporters. The reduced folate carrier (RFC) is ubiquitously expressed in cells for the transport of folate into the cells and is also used by a large number of antifolate drugs. These drugs also show high affinity for the α folate receptor (αFR), which is a low capacity folate transporter, highly overexpressed in some cancerous tissues. The β isoform of the folate receptor is instead overexpressed in activated macrophages and some tumors. The high overexpression of the specific isoform of the FR can be utilized for specific delivery of antifolate drugs into the cancerous cells, thereby sparing the remaining tissue. For detailed accounts of folate receptor based targeting, see reviews from Low et al.[57] and Salazar and Ratnam.[58] This approach has been realized for TS inhibition with cyclopenta[*g*]quinazoline derivatives such as 2(*R*)-(4(*S*)-carboxy-4-{4-[*N*-(2-methyl-4-oxo-4,6,7,8-tetrahydro-3*H*-cyclopenta[*g*]quinazolin-6-yl)-*N*-(2-propynyl)amino]benzamido}-butyramido)pentanedioic acid (BGC 638) and 2(*R*)-[4(*S*)-carboxy-4-[4-[*N*-(2-hydroxymethyl-4-oxo-4,6,7,8-tetrahydro-3*H*-cyclopenta[*g*]quinazolin-6-yl)-*N*-(2-propynyl)amino]-benzamido]butyramido]pentanedioic acid **6** (BGC 945, Chart 1) which have unexpectedly high affinity for αFR and low affinity for RFC.[59] The K_i value of **6** for isolated TS is 1.2 nM, and the IC_{50} for αFR overexpressing human tumor cells is ∼1−300 nM; in contrast, the IC_{50} for α FR negative mouse L1210 or human A431 cells is 7 μM. Following its success in mice models, **6** has been taken up for clinical development.[60] Since the underlying mechanism for the selective uptake by αFR is not understood, this approach can as yet not be rationally exploited for drug development.

2.f. Peptidic Inhibitors of the TS Protein. On the basis of an analysis of a *L. casei* TS crystal structure and counting the number of contacts between residues of both subunits, three peptides, N22, M17 and C20, were selected corresponding to the *L. casei* TS sequences 17−38, 174−190, and 201−220, respectively.[61] After incubation of *L. casei* TS with the C20 peptide, inhibition of enzyme activity and aggregation were observed. This effect was not detected for the N22 and M17 peptides, or after incubating the ternary complex of TS, FdUMP, and mTHF, with C20. The ability to use peptides to inhibit TS may be a good starting point for developing peptide-like or peptidomimetic inhibitors.

3. Targeting the Step before: mRNA

Various techniques using antisense oligodeoxynucleotides (ODNs), oligoribonucleotides, small inhibitory RNAs (siRNA), and microRNAs are known to be useful for modulating gene expression at the post-transcriptional level. In 1998, the first antisense ODN drug, fomivirsen was approved for treatment of cytomegalovirus retinitis;[62] many others are now in clinical trials for an array of diseases[63] including cancer (e.g., clinicaltrials.gov/ct2/show/NCT00689065). Since interruption of the autoregulatory mechanism for inhibiting the translation of mRNA is proposed to play a role in development of resistance against TS targeting drugs, therapies targeted at silencing the TS mRNA could be of importance in tackling this resistance problem. Even though the delivery and specificity of RNA inhibitory molecules to the tumor cells remain an issue, targeting the TS mRNA is a possible strategy for development as standalone or, more likely, as adjuvant therapy together with conventional TS inhibitory chemotherapeutic agents.

3.a. Antisense Oligodeoxynucleotides (ODNs). ODNs are designed to anneal against the target mRNA, forming an RNA−DNA duplex leading to abolition of protein synthesis either by direct inhibition of translation or by stimulation of the degradation of mRNA. From the TS protein−mRNA binding studies, it would be expected that since regions of the mRNA important for protein binding, sites I and II, should be important for regulating the translation of TS mRNA in cells, these would be effective antisense targets as well. Surprisingly, this does not seem to be the case.

Several regions throughout the length of the TS mRNA have been investigated for targeting by the antisense technology,

both in vitro and in vivo. Application of ODNs to target TS has been extensively reviewed by Berg et al.[64] Interestingly, the ODN found to be most effective in cell proliferation assays is the one targeting nucleotides 1184−1203 in the 3′ UTR of hTS mRNA.[64,65] Contrary to expectations, the ODNs targeting the translational start site encompassed in site I led to up-regulation of the TS gene transcription and were ineffective in decreasing the TS protein levels.[66] Whether the ODNs bound specifically to the intended target or made any additional interactions affecting this discrepancy remains to be investigated.

It is commonly accepted that use of an ODN leads to insufficient decrease in cellular protein levels to present any significant translation repression effect on cell proliferation. Thus, ODNs are generally considered as an option for adjuvant therapy. However, the effectiveness of using a standalone combination of ODNs targeting different mRNAs was first demonstrated by Normanno et al.[67] for the epidermal growth factor family. Subsequently, the effectiveness of combinations of ODNs targeting the same mRNA has been demonstrated by Berg et al., by using the example of TS mRNA. Combining the ODN targeting nucleotides 1184−1203 with ODNs targeting either 1081−1100 or 1436−1455 showed an additive antiproliferative action, whereas the combination of the latter two failed to show any additivity.[65] Whether ODNs can eventually be developed into an independent treatment for cancer is still questionable. However, they are surely candidates to be considered for combination chemotherapy.

3.b. Small Interfering RNA (siRNA). Small double stranded RNA sequences called siRNA are widely used for selective post-transcriptional silencing of gene expression in functional genomics experiments.[63,68] The siRNAs are about 21 nucleotides long and are reported to be 100- to 1000-fold more efficient than the corresponding ODNs. The specificity of gene silencing by siRNA depends on the length and the concentration of the RNA. Longer siRNAs tend to induce interferon response, and the probability of off-target effects is high at higher concentrations.

Not much literature on identification of effective siRNA targets for TS is available, but as for the ODNs, the general trend seems to be that targeting the sequences toward the 3′ end, i.e., beyond the putative protein binding sites, is more effective. Transfection of cells with siRNA targeting nucleotides 978−996 (R1), 991−1109 (R2), or 1058−1077 (R3) has been reported to cause 90−95% decrease in TS levels,[69−71] whereas targeting the sequences 95−122 and 208−226 had negligible effects on TS expression.[71] Although the observed reduction in TS levels is insufficient to have any significant effect on cell proliferation of HeLa and RKO cell lines, it is enough to resensitize the resistant cells to TS inhibitors.[69] In another study carried out on ACC3 cells from salivary adenoid carcinoma, targeting nucleotides 978−996 effectively inhibited the cell growth and also induced apoptosis in a xenograft model in nude mice.[70]

Direct transfection of cells with siRNA leads to a temporary reduction in gene expression, which resumes after the degradation of the inserted siRNA. A retroviral infection that encodes for the siRNA can lead to stable introduction of the gene and a "permanent" or longer lived effect, but the choice of the plasmid could be a crucial determinant of the effectiveness of the cloned siRNA.[71]

Site 1 is not only implicated in the protein−mRNA binding for translational repression, it also contains the start codon, which is responsible for initiation of translation, so the ineffectiveness of the ODN and siRNA sequences targeting site 1 comes as a surprise. This observation brings into question the significance of site 1 in translational repression and, additionally, the role it plays in translation initiation and mRNA stability.

3.c. Peptides. Though sites I and II sequences of the TS mRNA have been found to be important for the protein−mRNA binding, the mRNA binding regions of the TS protein are still unknown. Identification of these sites would be useful for developing new inhibitors of TS translation, for example, by mimicking the structural features of the RNA binding moieties of the protein. Chu et al. showed that enzymatically active exogenous human recombinant TS protein, but not that synthesized directly in vitro in a rabbit reticulocyte lysate system, is sufficient for binding to the full-length TS mRNA.[10] The presence of oxidizing agents blocked mRNA binding, whereas reducing agents increased the binding.[72] Mutating each cysteine residue in TS to alanine led to partial and drastic reduction in mRNA binding, respectively, for the C199A and C180A mutants, suggesting the impact of residue C180 on translation control.[73]

By screening a series of overlapping 17-mer peptides spanning the complete sequence of hTS, Voeller and co-workers identified five peptides capable of binding the full-length as well as the site I sequence from hTS mRNA.[74] All of the five peptides correspond to sequences located in the interface region between the two monomers of the homodimeric protein, and four of them contain at least one arginine that is conserved in several TS species. One of the peptides corresponds to the sequence of the C20 peptide (see section 2.f above) whose binding resulted in TS protein aggregation. From the location of the peptides at the interface region, Voeller et al. concluded that a monomeric form of TS must exist to make those regions accessible to mRNA. They confirmed the existence of the monomeric form by equilibrium dialysis in which only the monomeric form was able to pass the membrane because of its smaller size. Afterward, it still showed enzymatic activity, presumably in the reformed dimeric form.

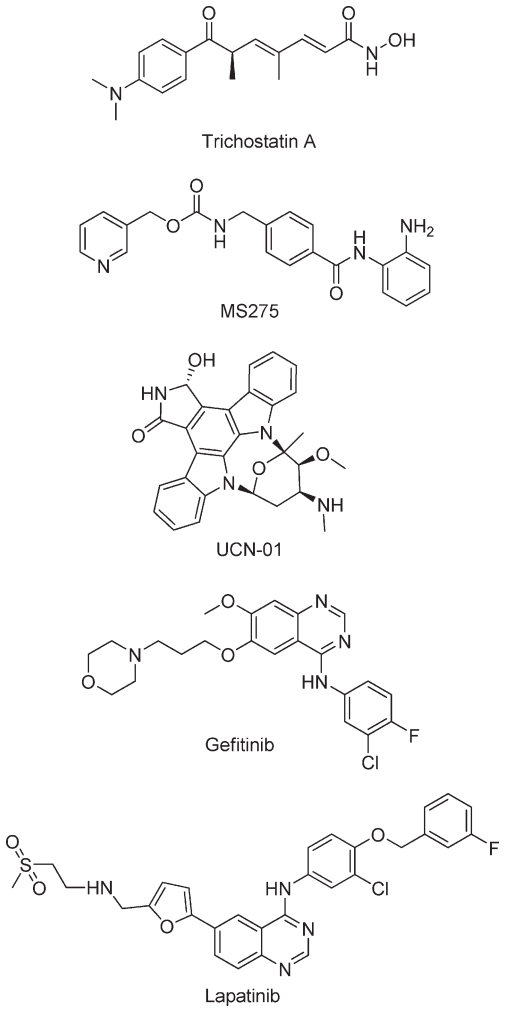
In a more recent paper, peptides binding to the site I sequence from TS mRNA were selected from a large peptide library using mRNA display.[75] After 12 rounds of amplification, an analysis of the amino acid composition of 18 totally random sequence positions within the yielded peptides suggested that mainly basic side chains took part in mRNA binding. Specific sequences were not found, and it was suggested that nonspecific interactions were involved in mRNA−peptide binding.

So far, no clearly defined sequence and structural characterization of the TS mRNA−protein/peptide binding complex has been achieved; therefore, further investigations are important for the rational design of peptides or peptidomimetics to specifically inhibit TS translation.

4. Indirect Thymidylate Synthase Expression Regulators

The techniques discussed so far deal with direct targeting of the TS protein or mRNA. However, targeting other proteins that act as TS expression regulators could also affect the in vivo levels of the TS protein or mRNA and consequently synergize with the TS inhibitors. We shall here discuss histone deacetylase inhibitors and E2F-1 inhibitors as examples from this category.

Chart 3



4.a. Histone Deacetylase Inhibitors (HDACi). HDACi have recently emerged as potent and selective anticancer agents and are undergoing clinical trials. These agents inhibit histone deacetylases, leading to altered acetylation of histone and non-histone proteins. Through microarrays, HDACi have been shown to modulate transcription of about 5% of the genome.[76,77] Potent transcriptional repression of the TYMS gene encoding TS has been demonstrated making TYMS one of the most prominently down-regulated genes following HDACi treatment.[78] Mechanisms of TS mRNA down-regulation by the HDACi, trichostatin A (Chart 3), have been elucidated by Lee et al.[79] They observed that trichostatin A induced TS mRNA down-regulation was abrogated by cycloheximide, which inhibits the synthesis of new proteins, suggesting that trichostatin A induced the expression of a transcriptional repressor of TS mRNA. Additionally, proteasomal degradation of the TS protein, mediated by acetylation of chaperonic Hsp90, was also induced by trichostatin A. HDACi have been shown to synergize the antiproliferative effects of TS inhibitors in in vitro and in vivo mice models and to aid in resensitizing the resistant cells.[80,81]

The synergism of HDACi to TS inhibitors, however, is not universal, as there is at least one report in which the HDACi, valproic acid[82] and *N*-(2-aminophenyl)-4-[*N*-(pyridin-3-ylmethoxycarbonyl) aminomethyl]benzamide (MS275,[83] Chart 3) antagonize the antiproliferative effects of the folate analogue methotrexate, at least in part by up-regulating TS expression.[84] It should, however, be noted that this study was carried out on choroid plexus cells, which, in order to maintain the folate levels in cerebrospinal fluid, have a highly differentiated folate metabolism and transport system, unlike in other cells where the folate pathway is involved in proliferation and dedifferentiation. Further studies to detail the mechanism in play and to study the cell line specificity of the observed effect need to be carried out.

4.b. E2F-1 Modulators. By regulating the transcription of genes that encode for proteins required for DNA synthesis, the E2F family, consisting of six transcription factors, plays a key role in progression of cells from late G1 into S phase of the cell cycle.[85] However, only the E2F1 factor has been noted for its role in apoptosis. It has also been suggested to be one of the regulators of TS levels in the cell. Though this regulation has not been demonstrated directly, TS expression has been found to be inversely correlated with E2F1 expression in various cell lines.[85,86] Thus, the importance of E2F1 for TS gene expression has been generally accepted. To our knowledge, no specific inhibitors of E2F1 are known; however, many known and potential antitumor compounds have been shown to induce E2F1 mediated down-regulation of TS; protein kinase C/cyclin dependent kinase (PKC/CDK) inhibitors and epidermal growth factor receptor (EGFR) inhibitors are two such classes of molecules.

4.b.1. Protein Kinase C/Cyclin Dependent Kinase Inhibitors. 7-Hydroxystaurosporine **7** (UCN-01, Chart 3)[87] is a selective PKC/CDK inhibitor undergoing clinical trials as a standalone drug or in various combination chemotherapies (clinicaltrials.gov). Significant synergism with 5FU has been reported, and attempts at deciphering the mechanism of its action have been made. Though the activity of TS remains unaffected, a decrease in normal and 5FU-induced TS mRNA and consequent protein levels has been observed on administration of **7**.[87] This decrease is preceded by a decrease in E2F1 protein level, while the E2F1 mRNA level remains unaltered.[88] This phenomenon can be explained by a cascade of events triggered by the CDK inhibition caused by **7**. Members of the CDK family are responsible for the phosphorylation of the retinoblastoma protein (pRb); pRb inhibits E2F1 by forming a pRB−E2F1 complex. Phosphorylation of pRb dissociates the E2F1−pRb complex, releasing functional E2F1.[89] CDK inhibitors such as **7**, prevent the release of E2F1 by preventing phosphorylation of pRb. The decrease in the free and functional transcription factor E2F1 results in a decrease in TS gene expression and thus the down-regulation of TS mRNA and protein levels.[88]

4.b.2. Epidermal Growth Factor Receptor Inhibitors. The EGFR inhibitors, gefitinib and lapatinib (Chart 3), have also been reported to cause down-regulation of TS. EGFR belongs to a family of receptor tyrosine kinases and is being established as an anticancer target.[90,91] Since EGFR signal transduction is involved in the activity of E2F1, inhibitors of EGFR could mediate interference of TS expression. Okabe et al.[91] have shown that gefitinib synergizes with 5FU by specifically down-regulating TS and does not affect the expression of other 5FU modulators like DPD or OPRT.

5. Conclusions and Perspectives

Even 37 years after the discovery of TS as an anticancer target, resistance and toxicity continue to pose a challenge to the development of a foolproof TS inhibitory drug. The

various approaches being employed to address these problems from the three levels, the protein, the mRNA, and the gene, have been summarized in this Perspective. While approaches aimed at tumor-specific concentration or activation of TS protein active-site inhibitors have considerably reduced toxicity to normal tissues, these do not overcome the development of resistance. Effective inhibition of the TS mRNA or the TYMS gene could play a crucial role in tackling the problem of resistance. Allosteric inhibitors of the TS protein might also be useful in preventing the development of resistance, but this hypothesis remains to be validated. The ECTA approach, on the other hand, requires TS overexpression, as observed in resistant cells, for activating a cytotoxic agent. Improved understanding of cancer biology has highlighted the involvement of TS in a complicated network of different pathways. Identification of TS expression regulators produced by such TS-interconnected pathways opens new possibilities for TS function repression. The strategies for targeting TS for treating cancer should not focus exclusively on protein inhibition but also consider the concept of protein down-regulation to have a more effective and forceful impact in the clinical setting. Consequently, TS continues to present ample opportunities for drug discovery scientists.

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