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Edward Chu · Marc A. Callender · Michael P. Farrell
John C. Schmitz

Thymidylate synthase inhibitors as anticancer agents: from bench to bedside

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Abstract Thymidylate synthase (TS) is a folate-dependent enzyme that catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate to 2′-deoxythymidine-5′-monophosphate. This pathway provides the sole intracellular de novo source of 2′-deoxythymidine-5′-triphosphate; therefore, TS represents a critical target in cancer chemotherapy. 5-Fluorouracil (5-FU) was synthesized in 1957 and represents the first class of antineoplastic agents to be developed as inhibitors of TS. While 5-FU has been widely used to treat various human malignancies, its overall clinical efficacy is limited. Therefore, significant efforts have focused on the design of novel, more potent inhibitor compounds of TS. These agents fall into two main categories: folate analogs and nucleotide analogs. Five antifolate analogs are currently being evaluated in the clinic: raltitrexed, pemetrexed, nolatrexed, ZD9331, and GS7904L. Our laboratory has identified a novel mechanism of resistance that develops to TS inhibitor compounds, namely drug-mediated acute induction of new TS synthesis; this mechanism is directly controlled at the translational level. The ability of cancer cells to acutely induce the expression of TS may represent a novel mechanism for the development of cellular drug resistance. The future success of TS inhibitor compounds in the clinic may depend on novel strategies to selectively inhibit TS and on novel combination therapies to overcome cellular drug resistance.

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E. Chu (✉) · M. A. Callender · M. P. Farrell · J. C. Schmitz
Cancer Center – 111D, VA Connecticut Healthcare System,
950 Campbell Avenue, West Haven, CT 06516, USA
E-mail: chueyale@yahoo.com
Tel.: +1-203-9373421
Fax: +1-203-9373803

E. Chu · M. A. Callender · M. P. Farrell · J. C. Schmitz
Department of Medicine and Pharmacology,
Yale Cancer Center, Yale University School of Medicine,
New Haven, CT 06516, USA

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Introduction

Thymidylate synthase (TS) catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP) using 5,10-methylenetetrahydrofolate as the one-carbon methyl donor [8, 17]. dTMP is then phosphorylated by two successive steps to 2′-deoxythymidine-5′-triphosphate (dTTP), an essential precursor for DNA synthesis (Fig. 1). This pathway is the sole intracellular de novo source of dTTP; therefore TS represents a critical target in cancer chemotherapy [8].

5-Fluorouracil (5-FU) is a member of the fluoropyrimidine class of antineoplastic agents and was first synthesized by Heidelberger and coworkers in 1957 [38, 39]. This agent represents the first class of TS inhibitors to be used in the clinic and was rationally designed based on the insightful observation that rat hepatoma tumors incorporate uracil into DNA to a significantly greater extent than corresponding normal tissue. Heidelberger and colleagues postulated that a chemically modified uracil molecule might be effective in disrupting tumor DNA biosynthesis. Since its synthesis some 46 years ago, 5-FU remains an active agent with broad-spectrum activity against many solid tumors, including colorectal, pancreas, breast, head and neck, gastric, and ovarian cancers [64].

5-FU exerts its cytotoxic activity through several mechanisms of action including inhibition of TS, incorporation into DNA, and/or incorporation into RNA [2]. The specific dose, route, and schedule of administration may also play a critical role as to its final mode(s) of action [55, 58, 77]. In its parent form, 5-FU is inactive and must be converted intracellularly to various nucleotide forms. For example, 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) is considered

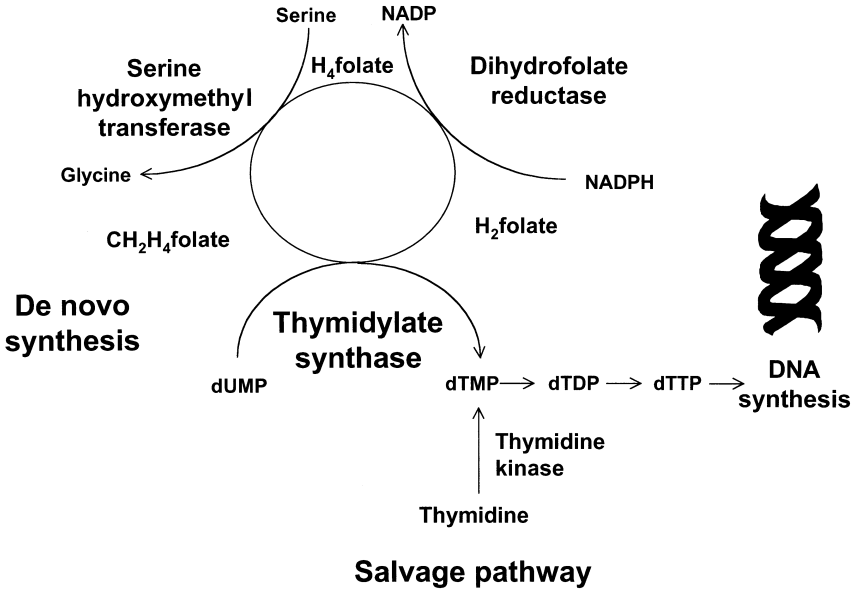

Fig. 1 Thymidylate synthase as a chemotherapeutic target (*dUMP* 2′-deoxyuridine 5′-monophosphate, *dTMP* 2′-deoxythymidine-5′-monophosphate, *dTDP* 2′-deoxythymidine-5′-diphosphate, *dTTP* 2′-deoxythymidine-5′-triphosphate)

to be a critical nucleotide metabolite as it forms a covalent ternary complex with TS in the presence of the reduced folate 5,10-methylenetetrahydrofolate, resulting in inhibition of the enzyme. FdUMP is phosphorylated by a series of enzymatic steps to 5-fluoro-2′-deoxyuridine-5′-triphosphate (FdUTP), which can then be incorporated into DNA leading to inhibition of DNA synthesis and function. The resultant inhibition of TS gives rise to an accumulation of dUMP, which can be subsequently misincorporated into DNA in the form of dUTP, resulting in the formation of single- and double-strand DNA breaks.

There are several lines of evidence to support the concept of TS as an important chemotherapeutic target. Preclinical in vitro and in vivo studies have shown an inverse relationship between the level of TS enzyme activity in tumor cells and 5-FU sensitivity [16, 46]. Similar findings have been extended to the clinical setting, where a strong association between the level of TS expression and response to 5-FU-based chemotherapy has been observed in patients with breast and colorectal cancer. In addition, there is a close association between the level of TS enzyme inhibition in tumor samples from patients and clinical response to 5-FU-based chemotherapy. In patients with early-stage rectal cancer [45], metastatic colorectal cancer [32, 70], non-small-cell lung cancer [40], breast cancer [60], gastric cancer [41, 51], and head and neck cancer [47], pretreatment levels of TS protein appear to be highly predictive for response to 5-FU-based chemotherapy.

Further support for the importance of TS enzyme inhibition comes from the clinical use of the reduced folate leucovorin (LV) in combination with 5-FU. LV is metabolized intracellularly to the reduced folate 5,10-methylenetetrahydrofolate, which forms a ternary complex with the 5-FU metabolite FdUMP and the target TS, and in so doing, helps to maintain the enzyme in a maximally inhibited state. This effect of maintaining the enzyme in an inhibited state is critical as the TS-catalyzed reaction provides the essential nucleotide precursors for DNA biosynthesis. Subsequent work confirmed that 5-FU cytotoxicity was significantly enhanced upon addition of LV [79]. For the past 15–20 years, the combination of 5-FU and LV has been considered the gold standard for treating patients with advanced colorectal cancer. To date, there have been 25 randomized trials comparing the clinical activity of 5-FU and LV with single-agent 5-FU. All these studies demonstrated significantly higher response rates with the combination of 5-FU and LV when compared with single-agent 5-FU [6, 10, 31]. However, with the exception of one study, this combination regimen has not resulted in improved overall survival of patients with advanced colorectal cancer. The combination of 5-FU and LV has also been extended to the adjuvant setting for early-stage colon cancer, where it has resulted in improved disease-free and overall survival when compared with 5-FU alone.

The central role of TS in DNA biosynthesis and tumor biology has been clearly established; however, the clinical experience with 5-FU in a wide range of malignancies has been disappointing. Therefore, significant efforts have focused on designing novel, more potent inhibitor compounds of TS. These agents fall into two main categories: folate analogs and nucleotide analogs. Herein, we discuss the antifolate TS inhibitor compounds (Fig. 2) currently under evaluation in clinical trials, with emphasis on their mechanism of action, toxicity profile, and range of clinical activity.

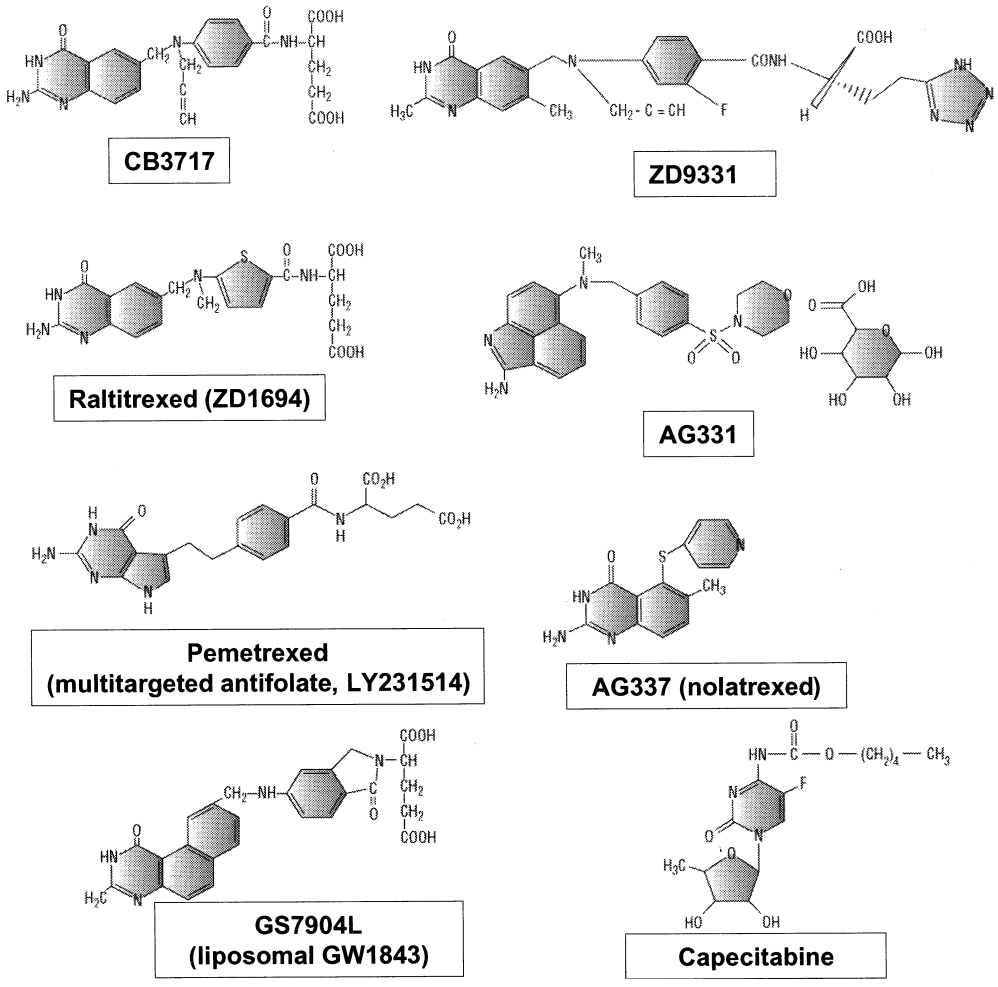

Fig. 2 Antifolate thymidylate synthase inhibitor compounds

Folate analogs

CB3717

CB3717 is a quinazoline-based antifolate analog that was first investigated in the 1980s [11, 42]. It is a potent inhibitor of TS in vitro and phase I studies documented broad-spectrum activity in patients with breast, liver, and cisplatin-refractory ovarian cancers. Unfortunately, severe life-threatening nephrotoxicity developed in several patients, which caused an immediate halt to the further clinical development of this agent. Subsequent analysis revealed that CB3717 was precipitating in the acidic pH of the renal tubules, thereby resulting in renal toxicity [11].

Raltitrexed (ZD1694)

Raltitrexed is a water-soluble analog of CB3717 and a more potent inhibitor of TS. Given its improved water solubility, this agent does not cause renal toxicity. It is transported into cells via the reduced folate carrier (RFC) and undergoes rapid polyglutamation by the enzyme folylpolyglutamate synthase (FPGS). In its monoglutamate form, raltitrexed is a mixed, noncompetitive inhibitor of human TS with a Ki approaching 90–100 n*M*. Polyglutamation to the higher glutamate forms renders it a significantly more potent inhibitor of TS by up to 100-fold. Once metabolized to the higher polyglutamate forms, its retention within cells is significantly prolonged [42].

Two phase I studies were conducted with raltitrexed, one in the UK [26] and one by the National Cancer Institute in the USA [78]. In both studies, raltitrexed was administered as a 15-min infusion every 3 weeks. The dose-limiting toxicities in both trials included anorexia, fatigue, diarrhea, and myelosuppression. A reversible elevation of serum transaminases, as well as increases in alkaline phosphatase and serum bilirubin, were also observed. Based on the European study, the recommended phase II dose was 3.0 mg/m^2, while a dose of 4.0 mg/m^2 was recommended in the USA. Differences in the underlying nutritional status of patients from the two countries, especially as it relates to folate supplementation, may have accounted for the notable difference in the maximally tolerated dose of the drug.

Several phase II trials of raltitrexed were then conducted, and the best activity was observed in previously

untreated patients with advanced colorectal and breast cancers; overall response rates were in the 20–26% range [76, 89]. The major toxicities observed were grade III/IV diarrhea, leukopenia, asthenia, and reversible elevation of serum transaminases. Grade III/IV nausea and vomiting were seen in 12% of patients and a maculopapular rash was noted in 14%.

Based on the promising phase II activity in colorectal cancer, two large phase III randomized trials were conducted comparing single-agent raltitrexed with the Mayo Clinic regimen of 5-FU and LV. In the European study, 439 patients with previously untreated, advanced colorectal cancer were randomized to receive either raltitrexed 3 mg/m^2 every 3 weeks or 5-FU 425 mg/m^2 and LV 20 mg/m^2 for 5 days every 4–5 weeks [28, 30]. Response rates were similar in the two arms: 19% in patients receiving raltitrexed and 18% in patients receiving 5-FU plus LV. Time to progression and median survival were also similar. The incidence and severity of toxicities were significantly reduced in the raltitrexed group, and in particular mucositis (2% vs 16%, $P < 0.001$). Based on these results, raltitrexed was approved as first-line therapy for advanced colorectal cancer in several European countries, Australia, Canada, and Japan.

In the North American phase III trial [63], the same two regimens were compared. While objective responses were similar in both arms (14% for raltitrexed versus 15% for 5-FU plus LV), time to progression and overall survival were significantly longer in patients treated with 5-FU plus LV (survival 12.7 months versus 9.7 months, $P = 0.01$). Because raltitrexed did not offer a survival advantage over standard chemotherapy, it was not approved for use by the US Food and Drug Administration as first-line therapy in metastatic colorectal cancer.

Currently, raltitrexed remains an investigational agent in the USA, and much attention is focused on combining it with approved anticancer agents, including the topoisomerase I inhibitor irinotecan [29, 52]. In a phase I study, the combination of raltitrexed and irinotecan was well tolerated, and 5 of 20 patients with advanced colorectal cancer in this trial had a partial response. Four of these five patients had previously received 5-FU chemotherapy [52]. Encouraging results have been observed in phase I/II studies with raltitrexed combined with oxaliplatin in patients with previously untreated metastatic colorectal carcinoma [72]. This combination showed a 47% response rate with a median overall survival of >14.5 months. Neutropenia, elevation of serum transaminases, peripheral neuropathy, and diarrhea were the main adverse effects observed [72].

Raltitrexed has also been evaluated as a radiosensitizing agent in phase I trials with radiotherapy in previously untreated but surgically resected patients with stage II or III rectal cancer. In these patients, a total of 50.4 Gy at 1.8 Gy per fraction over 5–6 weeks was administered with escalating doses of raltitrexed from 2.0 to 3.0 mg/m^2. The main dose-limiting toxicities included leukopenia and diarrhea at 3.0 mg/m^2, and based on this one study, the recommended dose was 2.6 mg/m^2 once every 3 weeks [7].

Pemetrexed (MTA, LY231514)

Pemetrexed is an antifolate analog in which a pyrrole ring replaces the pyrazine portion folate and a methylene group replaces the benzyl nitrogen in the bridging portion of the molecule. While this compound inhibits TS, it also inhibits other folate-dependent enzymes including dihydrofolate reductase (DHFR), aminoimidazole carboxamide ribonucleotide formyltransferase, and glycinamide ribonucleotide formyltransferase. However, the prevailing evidence suggests that the main site of action is inhibition of TS. Like raltitrexed, it utilizes the RFC for entry into cells and requires polyglutamation for maximal inhibitory effects on the various target enzymes. It has shown activity in vitro against colon, renal, liver, and lung cancers [73].

Based on the phase I clinical studies, the main toxicities associated with pemetrexed include neutropenia, anorexia, thrombocytopenia, fatigue, gastrointestinal toxicity, and a reversible elevation of liver enzymes [68]. The initial dose and schedule selected for phase II studies was 600 mg/m^2 administered intravenously every 21 days [62]. This dose in two Canadian studies was subsequently reduced to 500 mg/m^2 secondary to undue toxicity observed with the higher dose. As a single agent, pemetrexed produced a 23% response rate in previously untreated patients with non-small-cell lung cancer [69] with a slightly higher partial response rate in studies combining pemetrexed with cisplatin (39%) [56]. A phase II study of pemetrexed in advanced colorectal carcinoma showed a response rate of 15% [44]. This study used a dose of 600 mg/m^2, and 22% of patients required a dose reduction secondary to significant hematologic toxicity. A second phase II study in colorectal cancer at a reduced dose (500 mg/m^2) is currently ongoing in Canada.

Phase I studies investigating the combination of pemetrexed plus cisplatin and pemetrexed plus carboplatin were performed, and both combinations showed promising activity [12, 15, 71, 83]. In particular, the combination of pemetrexed and cisplatin showed responses in 5 of 11 patients with malignant pleural mesothelioma, and these results led to a phase III single-blind randomized trial comparing the combination of pemetrexed and cisplatin with single-agent cisplatin. The results of this study were presented at the 2002 American Society of Clinical Oncology meeting. The combination of pemetrexed and cisplatin showed an improved overall survival, time to disease progression, response rate, and improvement in lung function and overall quality of life when compared with single-agent cisplatin [86].

GS7904L (liposomal GW1843)

GW1843 is an antifolate analog in which a glutaric acid moiety replaces the glutamate in the molecule. It is taken up by the reduced folate transporter and is a potent, specific inhibitor of TS not requiring polyglutamation

for its cytotoxicity. Preclinical in vivo antitumor effects were observed in a broad range of human tumor xenografts including colon, breast and ovarian cancers, and osteosarcoma. In these studies, the administration of oral folic acid reduced and/or eliminated toxicity without preventing antitumor activity [75]. A phase I trial performed with and without co-administration of oral folic acid revealed dose-limiting toxicities including pancytopenia, fever, stomatitis, and skin rash. All adverse effects, except for myelosuppression, were reduced in severity by coadministration of folic acid. Specific antitumor activity was observed in patients with gastric, bladder, and colon cancer [9]. GW1843 has been reformulated through encapsulation in liposomes (GS7904L), and preclinical toxicology studies have revealed reduced toxicity to intestinal mucosal and bone marrow. Phase I clinical studies with this liposomal preparation have been initiated in Germany. Additional phase I trials are being developed in the USA.

ZD9331

ZD9331 was developed to be a highly specific TS inhibitor using the X-ray crystal structure of TS, and like raltitrexed, its predecessor compound, it is actively transported into cells via the RFC [43, 57]. ZD9331 does not require polyglutamation by FPGS for activation and therefore retains cytotoxic activity in tumors expressing low levels of FPGS. Preclinical studies have shown ZD9331 to have a broad spectrum of antitumor activity in human cancer cell lines and tumor xenografts. In phase I studies, the dose-limiting toxicities were myelosuppression, nausea, vomiting, skin rash, and diarrhea [36, 65, 84]. In phase II trials, ZD9331 has shown promising activity as second- and third-line therapy in patients with ovarian and colorectal cancer [66, 74]. This agent is currently under investigation in other solid tumors, including non-small-cell lung, gastric, and pancreas cancer both as a single agent and in combination with other cytotoxic agents. Phase I studies of ZD9331 in combination with topotecan, a topoisomerase I inhibitor, have been conducted in refractory malignant solid tumors. Adverse effects have included myelosuppression and grade III asthenia, and one death occurred at the maximum tolerated dose [4]. Other phase II studies are currently underway in combination with gemcitabine, as are various phase I studies combining ZD9331 with cisplatin, carboplatin, or docetaxel.

AG337 (nolatrexed)

AG331 and AG337 are lipophilic, antifolate analogs that were designed specifically on the basis of the crystal structure of human TS. They enter cells via passive diffusion and are not dependent on the RFC or other specific transport systems to cross the cell membrane. Because their cellular half-lives are short, they must be administered via continuous infusion. AG331 was found to result in severe liver toxicity in phase I testing and further evaluation was subsequently terminated [27, 61]. AG337 was well tolerated in the initial phase I studies, and showed promising activity against head and neck, pancreatic, and hepatocellular cancer. A phase II trial of nolatrexed in patients with squamous cell cancer of the head and neck, administered as a continuous infusion over 5 days every 3 weeks with doses initiated at 1000 mg/m^2, showed complete responses in 2 and partial responses in 2 of 22 patients to give an overall response rate of 18% [3]. The most common adverse effects included rash, mucositis, neutropenia, and thrombocytopenia; these toxicities are similar to those observed in the initial phase I studies [13, 67, 81]. One drug-related neutropenic sepsis death occurred during the study. A phase II trial also showed activity in hepatocellular cancer, and as a result a large phase III randomized trial is currently ongoing in North America where patients with unresectable hepatocellular carcinoma are randomized to receive either nolatrexed or doxorubicin (the control arm).

Regulation of TS expression

Several groups have described rapid increases in TS enzyme levels in various in vitro, in vivo, and clinical model systems following short-term exposure to the fluoropyrimidines [80, 82, 85]. It was initially postulated that the drug-mediated induction of TS might represent a mechanism for the acute development of resistance to 5-FU chemotherapy with direct biological and clinical relevance. Although the molecular mechanisms mediating the 5-FU-induced expression of TS were not well characterized in these initial studies, several possibilities were suggested including increased transcription of TS-specific sequences, enhanced stability of TS mRNA, increased efficiency of TS mRNA translation, and enhanced stability of TS protein. Intense research efforts subsequently focused on elucidating the critical biochemical and molecular events that control the 5-FU-mediated acute induction of TS. Keyomarsi et al. [50] demonstrated that treatment of human breast cancer MCF-7 cells with the quinazoline antifolate analog raltitrexed gave rise to an acute 10 to 40-fold increase in levels of TS enzyme activity with no associated change in TS mRNA levels. The presence of the protein synthesis inhibitor cycloheximide effectively blocked the elevation in TS enzyme levels following exposure to raltitrexed, providing suggestive evidence for a translational regulatory event.

Studies from our own laboratory, using a human colon cancer H630 cell line as a model system, revealed that the increase in TS enzyme activity and TS protein expression in response to short-term exposure to 5-FU was not associated with a corresponding change in the level of TS mRNA expression [19, 22]. While the majority of the induced protein was complexed with the

5-FU metabolite FdUMP, a nearly 50% increase in free levels of TS protein was also observed. Thus the induction of TS protein in response to 5-FU exposure allowed free TS to remain at levels 50% above baseline, such that thymidylate and DNA biosynthesis could be maintained in the face of a cytotoxic stress such as 5-FU. Further work revealed that the increase in TS protein expression resulted directly from new synthesis of TS protein and not from alterations in protein stability. This study provided the first direct evidence for the role of translational regulation in an intact biological system and highlighted the biological relevance of this regulatory process.

Welsh et al. [87] investigated the induction of TS in a wide range of human cancer cell lines and non-transformed human fibroblasts following short-term exposure to the antifolate analog ZD9331. While a six- to ten-fold induction of TS protein expression was observed in various human cancer cell lines, a significantly greater level of TS induction was noted in non-transformed human fibroblasts treated with equitoxic doses of drug. Additional in vivo experiments using the L5178Y TK- mouse lymphoma model implanted into DBA2 mice identified significantly higher levels of induction of TS protein in normal intestinal epithelial cells when compared with tumor cells.

Our laboratory has conducted an extensive series of studies to more carefully elucidate the regulation of TS mRNA translation in response to exposure to cytotoxic stress. The current working model for the translational autoregulatory control of TS and the interaction between TS protein and its own TS mRNA is presented in Fig. 3. This regulatory process is well established as an important mechanism controlling the expression of various bacteriophage and prokaryotic systems [1, 5, 14, 37, 39, 88]. However, TS represents the first eukaryotic gene for which expression is controlled in such a manner. The expression of three other eukaryotic genes, namely DHFR [23, 33, 34], p53 [35, 59], and serine hydroxymethyltransferase [54] is controlled by a similar autoregulatory feedback mechanism.

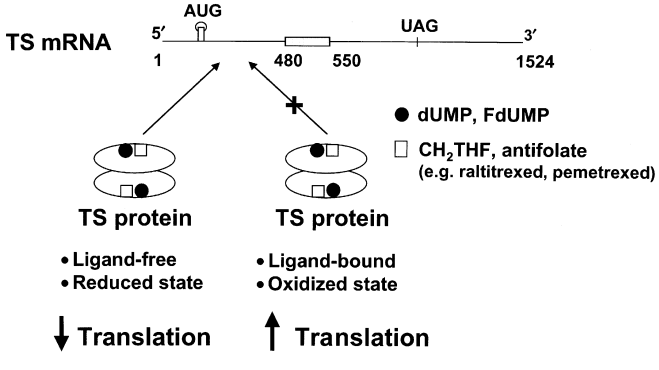

Fig. 3 Model for translational autoregulation of thymidylate synthase (*TS* thymidylate synthase, *dUMP* 2′-deoxyuridine-5′-monophosphate, *FdUMP* 5-fluoro-2′-deoxyuridine-5′-monophosphate)

Two different *cis*-acting sequences have been identified on the human TS mRNA. Each binds to TS with high affinity, on the order of 1–3 n*M* [20, 21]. The first site is a 30-nucleotide sequence corresponding to nucleotides 80–109 and includes the translational start site within the loop aspect of a stable stem-loop structure. The second site is contained within a 70-nucleotide sequence corresponding to nucleotides 480–550 in the protein coding region [53]. This second *cis* element is sufficient to confer the property of translational regulation onto a heterologous luciferase reporter gene and requires the presence of an intact TS protein for its biological effect [53]. While this sequence is able to function independently of the 5′-upstream *cis* element in vivo, our studies suggest that both elements are required for complete translational autoregulatory activity.

Our laboratory has shown that the RNA-binding activity of human recombinant TS is exquisitely redox sensitive and requires the presence of at least one free sulfhydryl group [24]. In addition to the redox state, the state of occupancy of the TS protein represents another critical determinant of RNA binding. Specifically, when TS is ligand-free, maximal RNA-binding activity is maintained, thereby resulting in complete translational repression of TS mRNA. In contrast, when TS is bound by either of its physiologic substrates, dUMP or the reduced folate CH_2THF, or bound by the 5-FU nucleotide metabolite FdUMP, TS is unable to bind to its target mRNA (see Fig. 2). In addition, incubation of TS with various antifolate analogs including raltitrexed and pemetrexed significantly impairs its ability to interact with TS mRNA. The end result of this disruption in RNA-binding activity is abrogation of translational repression, thereby resulting in increased synthesis of new TS protein. Such a condition would exist in cells exposed to direct inhibitor compounds of TS, whether they be nucleotide analogs such as 5-FU or antifolate analogs such as ZD1694. Thus, this model provides a rationale for the acute induction of TS that arises in direct response to exposure to the class of TS inhibitor compounds. Abrogation of this normal TS translational autoregulatory process may represent a biologically relevant mechanism to maintain normal cellular synthetic function in the setting of an acute cellular stress such as exposure to an antineoplastic agent. Finally, it offers a novel mechanism for the development of acute drug resistance to compounds that specifically target TS.

Discussion

Since the initial synthesis of 5-FU some 46 years ago, significant advances have been made in the development of TS inhibitor compounds. However, many questions remain as to which factors are critical in identifying the optimal TS inhibitor compound for clinical application. The first generation of TS inhibitors, including 5-FU, were directed against colorectal and breast cancer as well as other gastrointestinal malignancies. However, the

newer generation of inhibitors have activity against a different spectrum of tumors including head and neck cancer, non-small-cell lung cancer, mesothelioma, and hepatoma. How and why has this change occurred? An understanding of the mechanisms mediating this potential shift in tumor specificity of TS inhibitors may provide new insights into tumor cell biology and specific mechanisms of cytotoxicity of the different compounds.

Before acting at the target tumor tissue, each TS inhibitor must enter the systemic circulation through either absorption from the gastrointestinal tract and bioavailability or direct intravenous injection. The next step that these compounds must face is drug metabolism and pharmacokinetic elimination through hepatobiliary and/or renal processes. Thus, issues relating to bioavailability, biodistribution, and drug pharmacokinetics play an important role as initial steps in determining the final antitumor activity of a given TS inhibitor. Once delivered to the target tumor cell, the drug must enter cells through either specific carrier-mediated systems or passive diffusion. It is conceivable that the different spectrum of antitumor activity of the various antifolate TS inhibitors may be due in part to inherent differences in their respective abilities to be effectively transported into malignant cells. Moreover, the levels of normal reduced folates as well as thymidine in the general circulation, the tumor tissue microenvironment, and within the tumor cell itself, may need to be considered. Finally, the potential selective nature of these compounds may depend on the differential level of expression of the various transport systems among various normal host tissues as well as altered levels of cellular reduced folates and thymidine nucleoside within the normal cell milieu.

Once inside malignant cells, 5-FU and the various antifolate analogs such as raltitrexed, pemetrexed, and ZD9331 require metabolic activation to either nucleotide metabolites or higher polyglutamate forms to exert their cytotoxic effects. It is reasonable to assume that malignant cells of diverse origins will express different levels of the critical activating enzymes. With regard to the antifolate class of compounds, the balance between the level of expression of the activating enzyme FPGS and the breakdown enzyme γ-glutamyl hydrolase is critical in mediating the final polyglutamate status. Another important factor to consider is the level of expression of the target enzyme, namely TS, in various tumor types. There is growing evidence that the level of TS expression, at either the RNA or the protein level, may be dramatically different in certain types of human malignancies. This differential level of expression may help to explain why a given antifolate analog is effective against one tumor type but not another. It is possible that the cellular localization of TS may also play an important role in determining whether a specific TS inhibitor is able to exert its inhibitory effect.

For well over 40 years, the long-held view was that TS functions as a critical cellular catalytic enzyme to provide the essential nucleotide precursors for DNA biosynthesis. However, our laboratory has shown that TS, in its capacity as an RNA-binding protein, functions as an important regulator of certain key aspects of cellular metabolism, including cell-cycle progression, apoptosis, and perhaps even chemosensitivity [18, 25, 48, 49]. Thus, the design and development of future TS inhibitors must take into account the potential downstream cellular signaling consequences of inhibition of TS. The rapid advances in biotechnology with DNA microarray systems, tissue microarrays, high-throughput screening, and advanced bioinformatics will be especially important in this regard. In particular, the availability of rapid, relatively inexpensive, valid, and reliable biotechnology for molecular profiling of a patient's tumor will allow the direct and specific application of a given TS inhibitor to individual patients. Finally, research efforts must continue to focus on understanding the potential mechanisms by which cellular resistance develops to these agents. Such studies will be particularly important in serving as the basis for the rational design of novel therapeutic strategies to prevent and/or overcome the development of drug resistance and enhance the therapeutic activity of this important class of anticancer compounds.

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