Review

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Thymidylate synthase inhibitors for thoracic tumors

Abstract: Thymidylate synthase (TS) is a valid target for treatment of non-small cell lung cancer (NSCLC) and mesothelioma (MPM). The TS inhibitor pemetrexed (PMX) is now commonly used in combination with cisplatin (CDDP) or carboplatin, in the first-line setting for both of these lethal diseases. A combination of another TS inhibitor, Tomudex with CDDP, demonstrated similar activity in MPM patients. However, the efficacy of TS inhibitors is limited by uptake, metabolism and target affinity. While uptake of most antifolates is mediated by the reduced folate carrier, PMX is also a good substrate for the proton-coupled folate transporter (PCFT), which displays optimal activity at the acidic pH of the tumor microenvironment. NSCLC and MPM have a variable expression of PCFT, which might be caused by the differential methylation status of the PCFT promoter, resulting in decreased anticancer activity. PMX and TDX demonstrated similar activity also depends on polyglutamylation, catalyzed by folylpolyglutamate synthetase (FPGS), which results in intracellular retention and thus enhanced cytotoxicity. We demonstrated that resistance to the classical antifolate methotrexate in human leukemia cells with a marked loss of FPGS activity was associated with impaired splicing of FPGS pre-mRNA. Moreover, we recently showed that the sensitivity of MPM patients to PMX-carboplatin is related to tumor TS expression. Patients with low TS mRNA levels had a significantly longer overall survival (20 vs. 7 months) compared with patients with high expression. Intriguingly, recent trials demonstrated that histology plays an important role in the sensitivity of NSCLC patients, and PMX-CDDP is now approved only for adenocarcinoma patients. These results might be partly explained by the higher TS expression detected in lung tumors of squamous histology. In summary, the clinical success of TS inhibitors may depend on biomarker-driven patient selection, and further studies should evaluate the genetic/epigenetic factors involved in the modulation of these biomarkers in the preclinical and clinical setting.

Keywords: mesothelioma; non-small cell lung cancer; pemetrexed; pharmacogenetics; thymidylate synthase.

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Introduction

Thymidylate synthase (TS) inhibitors such as pemetrexed changed the current practice of treatment of advanced non-squamous non-small cell lung cancer (NSCLC) as well as mesothelioma. In both diseases, its combination with cisplatin has become first-line treatment [1–3]. In this review, we highlight molecular determinants of TS inhibitors in NSCLC, as well as new pharmacodynamics and pharmacogenetic biomarkers. The evaluation of these markers could help in the selection of the optimal clinical dose, as well as markers of resistance, which might be used to tailor TS inhibitor-based chemotherapy. This review is an updated, shortened version of a review published previously [4].

Lung cancer represents the leading cause of cancer mortality worldwide with a 5-year survival rate at 10%–15% and <7% of patients alive 10 years after diagnosis. Improvement of treatment remains a major challenge [5]. Surgery is curative only in early stage (I–II) NSCLC, while radiotherapy is an option in some patients. Platinum-doublet treatment is considered the front-line standard therapy in advanced NSCLC [6], improving both disease-free survival and 5-year overall survival (OS) [7] of locally advanced or stage IIIA disease, as well as stages IIIB and IV [8]. Platinum-based doublet therapy is the standard of care against these tumors. In patients with good performance status, different combinations with cisplatin, gemcitabine, taxanes, irinotecan, vinorelbine,
ifosfamide, mitomycin C, vindesine or vinblastine reached better results than single agent treatment both in terms of response and survival [9]. Three-drug combinations were better than two-drug combinations only in terms of response [10], while single-agent chemotherapy is only applied in patients with poor performance status. Because of the lower incidence of side effects, carboplatin has often replaced cisplatin, although several meta-analyses showed higher response rates for cisplatin compared with carboplatin combinations [11]. Third-generation drugs, such as gemcitabine, taxanes, irinotecan, pemetrexed and vinorelbine, in combination with platinum compounds, now constitute the core of chemotherapy in advanced NSCLC [12]. Non-platinum based combinations of some of these drugs is now considered as an alternative first-line treatment when platinum therapy is contraindicated [13].

In patients with non-squamous NSCLC, the combination of pemetrexed with cisplatin [1] was recently approved as first-line therapy. Only for a small group of NSCLC patients with activating mutations in the epidermal growth factor receptor (EGFR), tyrosine kinase inhibitors such as erlotinib and gefitinib are approved as first-line treatment [14]. However, larger studies are needed to examine the efficacy and safety of different potential third-line regimens in an effort to prolong survival while maintaining quality of life of patients with advanced NSCLC.

Role of thymidylate synthase as therapeutic target

TS is a folate-dependent enzyme that catalyzes the methylation of deoxyuridine-5′-monophosphate (dUMP) using 5,10-methylene-tetrahydrofolate (5,10-CH$_2$-THF) as the methyl donor to form deoxothymidine-5′-monophosphate (dTMP) [15]. This reaction is the only de novo source for dTMP, which is critical for DNA replication and repair, which in turn is essential for cell proliferation (Figure 1). Therefore, inhibition of DNA synthesis through TS is an established successful antimetabolite approach controlling tumor cell growth. Hence, TS inhibitors can be considered one of the first “targeted” drugs because they were specifically designed in order to interfere with a very well-defined molecular target, DNA with essential functions in cell growth and proliferation. Like all “targeted” drugs, the target has essential functions in normal cells. Regarding TS inhibitors, proliferating tissues, such as bone marrow and gastrointestinal mucosa, are also dependent on DNA synthesis and thus sensitive to TS inhibition, leading to myelosuppression, mucositis and diarrhea.

As TS has two substrates, dUMP and 5,10-CH$_2$-THF, inhibition can be mediated by a dUMP or a folate analog (Figures 1 and 2). 5-Fluorouracil (5-FU) was introduced into the clinical setting more than 50 years ago [16]. 5-FU is a precursor of fluoro-dUMP (FdUMP), which inhibits TS by the formation of a covalent ternary complex with TS and CH2-THF, resulting in a depletion of dTMP and inhibition of DNA synthesis leading to thymine-less death. TS inhibition also results in an increase in 2′-deoxyuridine-5′-triphosphate (dUTP), leading to misincorporation of dUTP into DNA: its excision, catalyzed by uracil-DNA glycosylase, results in DNA damage. Moreover, a specific interaction exists between oncogenes and TS, by binding of TS protein to p53 and c-myc RNA [17, 18], while wild-type p53 can also inhibit TS promoter activity [19].

At transcriptional level, TS expression is activated by the transcription factor E2F1, which plays a key role in cell cycle progression [20]. In addition, TS protein functions as an RNA-binding protein repressing the translation of its own mRNA [21, 22]. When TS inhibitors bind to TS, the complex cannot interact with its cognate mRNA, which results in increased TS protein expression [23]. Hence, elevated expression of TS after exposure to TS inhibitors was, in part, attributable to increased protein stability [24, 25].

Thymidylate synthase inhibitors in NSCLC

Pemetrexed

As pemetrexed not only inhibits TS but also dihydrofolate reductase (DHFR) and the purine de novo pathway, it is often referred to as a multitargeted antifolate. Pemetrexed was first registered for the treatment of malignant pleural mesothelioma, based on a randomized, Phase III, single-blind, multicenter trial which compared cisplatin alone versus cisplatin plus pemetrexed. In that trial, the addition of pemetrexed to cisplatin significantly improved both the survival, by 3 months, and the overall response rate [26]. Because pemetrexed was also active as a single agent in Phase II and Phase III trials with NSCLC patients previously treated with chemotherapy, it was also approved for second-line treatment of NSCLC [27]. In that trial, pemetrexed (500 mg/m$^2$ every 3 weeks, with vitamin B12 and folic acid supplementation) was equally as effective as docetaxel (75 mg/m$^2$ every 3 weeks), with a median survival of 8.3
Figure 1  Uptake and metabolism of antifolates.
RTX and MTX are both taken up by the reduced folate carrier (RFC), while PMX is also a substrate for the proton-coupled folate transporter (PCFT). Both RTX and PMX are activated to their polyglutamate forms by folylpolyglutamate synthetase (FPGS); both the monoglutamate and polyglutamate (Glu_n) forms inhibit dihydrofolate reductase (DHFR) and thymidylate synthase (TS), which converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) for which 5,10-methylene-tetrahydrofolate (5,10-CH_2-THF) is the methyl donor. Especially PMX-Glu_n is a more effective inhibitor of TS. 5-Fluoro-deoxyuridine monophosphate (FdUMP) forms a ternary stable covalent complex with TS and 5,10-CH_2-THF. PMX and PMX-Glu_n also inhibit the purine de novo enzymes aminoimidazole carboxamide ribonucleotide transformylase (AICARFT) and glycinamide ribonucleotide formyl transferase (GARFT), for which 10-formyl-tetrahydrofolate (10-CHO-THF) donates the formyl group. Phosphoribosyl pyrophosphate (PRPP) is the initial phosphoribose ring donor for phosphoribosyl pyrophosphate amidotransferase (PPAT) in the first step of the purine de novo pathway, leading to the synthesis of IMP, precursor for the purine (deoxy) nucleotides (d)AMP and (d)GMP, substrates for RNA and DNA synthesis. UFT and S-1 are oral drug formulations which contain flotefur (FT), which is a prodrug for 5-fluorouracil (5-FU), which inhibits TS via its metabolite FdUMP. 5-FU can also be formed via several enzymatic steps from another 5-FU prodrug capecitabine (Xeloda, Xel). Abbreviations: DHF, dihydrofolate; THF, tetrahydrofolate; Gln, glutamine.

vs. 7.9 months, respectively. However, side effects of pemetrexed were significantly lower than with docetaxel.

In first-line therapy of advanced NSCLC patients, the combinations of pemetrexed plus cisplatin or carboplatin were equally as effective as gemcitabine plus cisplatin or carboplatin [2], while carboplatin plus pemetrexed or gemcitabine were also equally effective. In this study with 436 patients, the overall median survival was 7.3 months.

Figure 2  Structural formulas.
5-Methyl-tetrahydrofolate (5-CH_3-THF), the physiological precursor for 5,10-CH_2-THF in a reaction catalyzed by methylene tetrahydrofolate (MTHFR), methotrexate (MTX), pemetrexed (PMX, ALIMTA) and Raltitrexed (RTX, Tomudex).
for the pemetrexed/carboplatin arm and 7.0 months for the gemcitabine/carboplatin arm, but pemetrexed/carboplatin caused less toxicity [28]. In a randomized Phase III trial enrolling 1725 chemotherapy-naive patients, cisplatin plus gemcitabine or cisplatin plus pemetrexed showed similar efficacy with better tolerability and more convenient administration than cisplatin/gemcitabine [1], with an OS of 10.3 months in both arms and a response rate of 30.6% in the pemetrexed/cisplatin arm and 28.2% in the gemcitabine/cisplatin arm. For cisplatin/pemetrexed, the rates of grade 3 or 4 neutropenia, anemia and thrombocytopenia (p ≤ 0.001), febrile neutropenia (p = 0.002) and alopecia (p < 0.001) were significantly lower [1].

In the same study, patients with non-squamous NSCLC performed statistically superior in the cisplatin/pemetrexed treatment arm, whereas patients with squamous cell histology performed better with cisplatin/gemcitabine [1]. Other Phase II studies and retrospective review of two large, randomized, Phase III trials supported the predictive role of histology for pemetrexed [2], which was confirmed in a multicenter, double-blind Phase III trial evaluating pemetrexed versus placebo in stage IIIB/IV NSCLC patients who did not progress after four cycles of first-line platinum-based chemotherapy [29]. Pemetrexed showed a statistically significant superior activity in non-squamous (n=482; 4.5 months vs. 2.6 months) than in squamous histology (n=182; 2.8 months vs. 2.6 months). Therefore, pemetrexed was approved for use in first-line treatment of advanced non-squamous NSCLC.

Thymidylate synthase expression as determinant of pemetrexed activity

One potential explanation of the differential activity with pemetrexed treatment was related to the baseline expression of TS gene and protein, which were significantly higher in squamous cell carcinoma compared with adenocarcinoma patients (p<0.0001) [29].

Pemetrexed is a multitargeted agent that enters the cell via the reduced folate carrier (RFC) and is converted by folypolyglutamate synthetase (FPGS) to a series of active polyglutamate derivatives able to inhibit several folate-dependent enzymes such as TS, DHFR, glycinate ribonucleotide formyl transferase (GARFT) and, to a lesser extent, aminoimidazole carboxamide ribonucleotide transformylase (AICARFT) [30] (Figure 1). This mechanism of action leads to depletion of fully reduced folates and results in disruption of both purine and pyrimidine nucleotides. These polyglutamates are retained intracellularly longer than the parent compound, resulting in more prolonged cytotoxic effects. Whereas GARFT is inhibited just weakly by pemetrexed, TS represents the main target of this drug and its inhibition leads to the interruption of tetrahydrofolate oxidation with consequential lack of DHFR activity [31]. However, GARFT inhibition may become important in the case of TS overexpression or mutation, and the multitargeted effects of pemetrexed explain its broader spectrum of cytotoxic activity when compared in preclinical studies with other antimetabolites such as 5-FU and antifolates such as methotrexate or raltitrexed [32, 33].

Preclinical data showed a significant correlation between overexpression of TS mRNA or protein with reduced sensitivity to pemetrexed in NSCLC cell lines [34, 35]. The inhibition of DNA synthesis and induction of apoptotic cell death by pemetrexed were also significantly reduced by overexpression of TS [36]. Messenger RNA expression of genes involved in the mechanism of action of pemetrexed was correlated with in vitro sensitivity of 61 freshly explanted human tumor specimens, and low gene expression levels of TS, as well as of GARFT and DHFR, significantly correlated with chemosensitivity to pemetrexed [37]. Moreover, xenografts with TS overexpression were more resistant to pemetrexed [36]. Hence, patients with “low” baseline TS were more likely to respond to pemetrexed than patients with “high” baseline TS [38]. Two recent studies on mesothelioma samples reported that TS protein and mRNA expression significantly correlated with survival and response rates in patients treated with pemetrexed-based regimens [39, 40]. Similarly, the immunohistochemical analysis of TS expression in 24 NSCLC specimens from pemetrexed non-responding patients was significantly higher than that in those of responders [36]. In another study, the analysis of 49 tumor specimens from NSCLC patients treated with pemetrexed showed that low TS expression was correlated with longer median PFS (4.8 vs. 3.4 months; p=0.01), whereas in patients with adenocarcinoma, the low TS patient group also had a longer median survival as compared with patients with high TS expression [41].

However, there may be a difference in the potential prognostic and predictive role of TS. Although several studies suggested that TS levels may be associated with stage of disease, lymph node metastasis, tumor differentiation, prognosis and tumor cell proliferation, many results were controversial [42–44]. A high cytoplasmic tumoral TS analyzed by automated in situ protein quantification (AQUA) was associated with improved survival for NSCLC patients [44], but in colon cancer improved survival was found for patients with low tumoral TS expression as detected by immunohistochemistry [45]. However, standard immunohistochemistry with visual scoring in
an attempt to quantify protein expression has significant technical limitations, including the non-quantitative chemistry of routine immunoperoxidase stains and the subjective light intensity perception of the human eye [45]. Additional studies, with consistent methodology, and broader validation cohorts are needed to define the precise value of TS.

Although overexpression of TS is the most studied mechanism involved in resistance to pemetrexed, multiple other mechanisms of antifolate resistance might reduce pemetrexed activity in vitro and in vivo, such as (i) impaired uptake due to loss of transporter function of either the RFC or the proton-coupled folate transporter (PCFT); (ii) overexpression of ATP-driven multidrug resistance (MDR) transporters which increase drug efflux; (iii) defective antifolate polyglutamylation due to decreased FPGS expression, inactivating mutations or altered splicing; (iv) increased expression of γ-glutamyl hydrolase (GGH); (v) overexpression of DHFR and mutations that decrease its affinity; and (vi) expansion of intracellular 5,6,7,8-tetrahydrofolate (THF) cofactor pools [46].

Previous preclinical findings showed that multidrug resistance protein 1 (MRP1) – MRP5 and BCRP can transport folates and antifolates including methotrexate, raltitrexed and pemetrexed. Polymerase chain reaction (PCR) analysis in NSCLC samples showed an increased MRP4 (significant) or MRP5 (trend) in resistant tumors [37], as well as a higher gene and protein expression of ABCC11/MRP8 [47]. However, in 13 lung adenocarcinoma cells there was no correlation between ABCC11 gene expression and pemetrexed sensitivity, whereas in most antifolate-resistant selected cell lines no overexpression of any of the MRPs or BCRP has been reported [48]. By contrast, 14 human leukemia cells were made resistant against various polyglutamatable inhibitors of DHFR, TS and GARFT, using a repeated high-dose intermittent schedule mimicking the chemotherapeutic treatment. The vast majority of these antifolate-resistant cell lines had a 90%–99% loss of FPGS activity, but without any substantial decrease in FPGS mRNA levels. Cross-resistance was observed in polyglutamylation-dependent antifolates including pemetrexed up to five orders of magnitude, while retaining sensitivity to polyglutamylating-independent antifolates [49]. Although inactivating FPGS mutations exist, they are not a frequent cause for the loss of FPGS function [50]. However, multiple antifolate-resistant tumor cell lines showed a marked suppression of FPGS activity in the absence of decreased FPGS mRNA levels [51]. An alternative explanation for the post-transcriptional loss of FPGS activity in the absence of decreased FPGS mRNA levels is the alternative splicing of FPGS, as demonstrated in leukemia cells [52].

The impact of an increase in the intracellular THF cofactor pool size on the cytotoxic activity of multiple antifolates was shown by a marked increase of the 50% inhibitory concentrations for the polyglutamatable antifolates pemetrexed and raltitrexed. The high intracellular folate pool resulted in a significant suppression of the formation of antifolate polyglutamates, whereas folate depletion resulted in enhanced pemetrexed inhibition of purine synthesis [53, 54]. Of note, impaired RFC function is an important mechanism of resistance to methotrexate and other antifolates in vitro [55], and may be associated with clinical resistance to this agent in the treatment of acute lymphoblastic leukemia [56]. The transport for pemetrexed is very different, because an RFC-independent, pH-dependent pathway serves as an alternative transport route for this drug and is mediated by the PCFT [57]. A high PCFT increased the growth inhibitory activity of pemetrexed, illustrating its unique role in the transport and pharmacological activity of pemetrexed. Because of the ubiquitous expression of PCFT in human tumors, and the ability of PCFT to sustain pemetrexed activity even in the absence of RFC, tumor cells are unlikely to become resistant to pemetrexed as a result of impaired transport because of the redundancy of these genetically distinct routes [58].

However, the promoter of the PCFT is highly methylated [59], leading to reduced activity in cancer cell systems. Promoter silencing through methylation and gene copy loss accounted for the loss of PCFT activity in antifolate-resistant HeLa R1-11 cells [60]. A plausible modality to overcome antifolate resistance in cells that are devoid of PCFT activity due to promoter methylation might be the combination with relatively well-tolerated demethylating agents, such as 5-Aza-dC [59]. Therefore, data about the possible association of PCFT promoter methylation and clinical outcome in NSCLC patients treated with pemetrexed can be very useful to offer alternative therapies to resistant patients.

In conclusion, resistance to pemetrexed in NSCLC is likely to be related to TS and a transporter, but other mechanisms need further clarification. However, the clinical safety profile of pemetrexed makes this drug an attractive agent for polychemotherapy regimens, because it can overcome tumor resistance and improve clinical outcome.

**UFT and S-1**

As primary lung cancers are characterized by high expression and activity of dihydroprymidinedehydrogenase (DPD), which is the key enzyme of 5-FU degradation,
studies on the activity of oral 5-FU derivatives preventing 5-FU degradation appeared of interest for NSCLC treatment. The first oral 5-FU derivatives were tegafur [61], and 5′-deoxy-5-fluorouridine (5-DFUR), which yielded a response rate lower than 15% [62]. Other rationally engineered and metabolically activated DPD inhibiting fluoropyrimidines (DIFs), such as UFT [63] and S-1 [64], proved to be effective in several randomized controlled studies in NSCLC patients.

UFT combines uracil, a competitive inhibitor of DPD, with the 5-FU prodrug tegafur in a 4:1 molar ratio (Figure 1). In Japan, UFT is approved for several tumors, including NSCLC [65], while in the UK, the combination of UFT and folinic acid is approved as first-line treatment for metastatic colorectal cancer and was active in second- or third-line therapy of Caucasian NSCLC patients [66], and because of its favorable toxicity profile warranted further investigation. Another more promising oral 5-FU prodrug formulation is S-1, in which a more potent DPD inhibitor is combined. S-1 combines the 5-FU prodrug tegafur (florafur, FT) with two enzyme inhibitors, CDHP (5-chloro-2,4-dihydroxypyridine) and OXO (potassium oxonate), in a molar ratio of 1(FT):0.4(CDHP):1(OXO). CDHP is a reversible potent competitive inhibitor of DPD, preventing the degradation of FT-derived 5-FU. Therefore, 5-FU remains in plasma and tumor tissue longer and at higher levels than when low dose 5-FU is continuously infused intravenously. OXO is a reversible competitive inhibitor of orotate phosphoribosyltransferase, which is expressed at high levels in the gastrointestinal tract. After oral S-1 administration, OXO will prevent activation of 5-FU in gastrointestinal endothelial cells, resulting in a reduction in diarrhea and mucositis caused by 5-FU. A recent Phase III trial showed that treatment with carboplatin plus S-1 was non-inferior than carboplatin plus paclitaxel with regard to OS in 564 chemotherapy-naive Japanese patients [67]. Median OS was 15.2 months in patients in the carboplatin and S-1 arm and 13.3 months in the carboplatin/paclitaxel arm, respectively, suggesting that S-1 might be a valid treatment option in patients with advanced NSCLC.

Preclinical studies showed a synergistic antitumor effect of S-1 with the EGFR tyrosine kinase inhibitor (TKI) gefitinib in NSCLC cell lines. Importantly, gefitinib reduced the expression of the transcription factor E2F1, down-regulating TS at both mRNA and protein levels, favoring the activity of S-1 [68]. Furthermore, the combination of S-1 and gefitinib synergistically inhibited the growth of NSCLC gefitinib-resistant NSCLC cells and xenografts with MET amplification, suggesting that the addition of S-1 to EGFR TKIs is a promising strategy to overcome EGFR TKI resistance in NSCLC with MET amplification [69].

Although a recent study showed that tumor expression levels of TS and DPD were predictive of response to S-1-carboplatin chemotherapy [70], a pooled analysis of S-1 trials in NSCLC demonstrated that the activity of S-1 was independent of tumor histology [71].

Other thymidylate synthase inhibitors

Other TS inhibitors in NSCLC include the quinazoline folate analog raltitrexed (Figures 1 and 2), which is a direct and specific TS inhibitor. In a Phase I trial, the dose-escalation study of the combination of raltitrexed and cisplatin was studied in 19 patients with previously untreated metastatic NSCLC. In this trial, 3 patients achieved a partial response, 13 had stable disease and 3 progressed [72]. However, withdrawal of the sponsor’s support has left raltitrexed (i.e., Tomudex) without the level of continuing investigation afforded to pemetrexed.

Raltitrexed was also evaluated in the treatment of mesothelioma in combination with cisplatin versus cisplatin alone, reaching an overall survival of 11.4 and 8.8 months, respectively [73]. Evaluation of raltitrexed as an alternative for pemetrexed in mesothelioma is ongoing.

Capecitabine is another oral 5-FU prodrug, for which thymidine phosphorylase (TP) mediates the final step of its activation pathway. A high TP expression could provide a rationale for the use of this drug in NSCLC. High TP expression in lung cancer cells and stroma was associated with response to capecitabine/docetaxel chemotherapy [74], and might be a useful predictor of NSCLC response to capecitabine-based chemotherapy. Moreover, the high response rate observed in a Phase II trial of docetaxel plus capecitabine in pretreated NSCLC patients [75] encourage further evaluation of capecitabine in NSCLC. However, the prognostic significance of TP expression in NSCLC as well as the possible role of capecitabine in NSCLC treatment need further evaluation.

Conclusions and future perspectives

TS is a validated target in NSCLC and mesothelioma, resulting in the widespread use of the TS inhibitor pemetrexed as first/second-line and maintenance therapy for both non-squamous NSCLC and mesothelioma. However, benefits from conventional chemotherapy in NSCLC have plateaued, while much more cost-effective results should be obtained with individualized patient treatment.
Accordingly, the clinical success for TS inhibitors may ultimately be dependent upon our ability to correctly administer these agents following appropriate biomarker-driven patient selection, including TS genotype and expression, and using the right combination therapy.

The levels of TS expression have been correlated with resistance to therapy with pemetrexed, and a prospective validation on the role of TS for pemetrexed-based chemotherapy is ongoing in a Phase III randomized study [4]. Further studies should also evaluate the genetic and epigenetic factors involved in the regulation of TS. The promoter of the human TS gene contains a 28-bp tandem repeat near the initiation codon in its 5′-untranslated region. This tandem repeat region functions as an enhancer element for the TS gene promoter, the TS enhancer region (TSER). TSER has been shown to be polymorphic: (i) a variable number of the tandem repeat of a 28-bp sequence (2R/3R) and (ii) a G>C single nucleotide substitution within the repeats, altering the E-box sequence binding an upstream stimulatory factor (USF-1) [76]. In vitro expression studies have suggested an association between the number of repeats and TS expression: the presence of a triple repeat (3R/3R) results in 2.6-fold greater TS expression than a double repeat [77]. However, the relationship between these polymorphisms, regulation of TS expression and patient response to fluoropyrimidine treatment has been inconsistent, and no correlation between TS polymorphisms and survival was observed in patients enrolled in a randomized Phase II study of pemetrexed compared with pemetrexed plus carboplatin in pretreated patients with advanced NSCLC [78].

Negative results were also reported in a study on TS gene copy number, which was increased more frequently in squamous cell carcinomas, but was not associated with prognosis. These results are consistent with studies in colorectal cancer which showed that tandem repeats were associated with TS activity in normal colon tissue, but not in tumors [4]. Therefore, it seems unlikely that

![Figure 3](image-url)
the TS gene copy number will predict clinical benefit from treatment with pemetrexed, although this can only be validated in prospective clinical studies. Another promising genetic marker consists of a polymorphism in methylene tetrahydrofolate reductase (MTHFR), for which a number of polymorphisms have been described. In a combined analysis of 206 patients treated with either pemetrexed or pemetrexed plus carboplatin, an increased progression-free and overall survival were observed for patients harboring the TT genotype [79]. This genotype results in an altered MTHFR activity affecting the reduced folate pools, which is important for sensitivity to pemetrexed.

A recent study evaluated two microRNA targeting TSs, miR-192 and miR-215. However, downregulation of TS by miR-192/215 did not increase 5-FU sensitivity. By contrast, overexpression of both microRNAs resulted in a reduction of cell proliferation and therefore diminished the effectiveness of 5-phase specific drugs such as 5-FU, suggesting that miR-192 and miR-215 can still play a role in resistance to TS inhibitors [80]. Therefore, further studies should evaluate whether miRNA expressed in NSCLC cells might regulate resistance mechanisms towards pemetrexed, as reported for other chemotherapeutic agents [81]. Additional information will also be gained with the integration of data from “next-generation” or “massively parallel” sequencing platforms.

Regarding drug combinations (Figure 3), another intriguing observation is the downregulation of TS by several targeted drugs, such as the EGFR TKI erlotinib which decreased TS expression and activity, showing synergistic interaction with pemetrexed in NSCLC cells [40]. Similar results were observed with the novel EGFR TKI BIBW2992, providing a scientific rationale for testing the combination of BIBW2992 with pemetrexed in patients who develop acquired resistance after harboring the EGFR T790M mutation [82].

Other targeted agents modulating TS included the PKCβ inhibitor enzastaurin and the Src inhibitor dasatinib, which synergistically enhanced pemetrexed cytotoxicity in NSCLC cells [83, 84]. In mesothelioma cells, the multiple targeted drug vandetanib also enhanced sensitivity to pemetrexed [85].

These data suggest innovative combinations improving the efficacy of TS-inhibiting agents for the treatment of NSCLC, which should be tested and validated in the clinical setting.

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