Antifolate Inhibitors of Thymidylate Synthase as Anticancer Drugs

A. Jarmuła*

Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur St., 02-093 Warszawa, Poland

Abstract: Inhibitors of thymidylate synthase (TS) play an essential role in the pharmacological management of several tumors. Two antifolates, Raltitrexed and Pemetrexed, are licensed anticancer drugs, with Pemetrexed, unlike Raltitrexed, undergoing further intense clinical development. Other antifolate TS inhibitors, recently/currently tested in clinical studies, that show encouraging anticancer activities are Plevitrexed, GW7904L and Nolatrexed. A new prospect among antifolates, demonstrating a very desirable pattern of pharmacological properties, is BGC 945 that showed promising antitumor activities and has been nominated for clinical development. In this paper, apart from reviewing their biochemical and pharmacological properties, up-to-date characteristics of clinical development of all the mentioned agents are presented. In addition, trends and perspectives for developing improved antifolate inhibitors of TS and future drugs are discussed. Drug resistance is the main barrier to more effective treatment of cancers with antifolates; therefore, mechanisms of antifolate resistance and currently applied approaches to overcome it are also pointed out in the review.

Keywords: Thymidylate synthase (TS), inhibitors, antifolates, anticancer drugs.

INTRODUCTION

Thymidylate synthase (TS) ThyA (EC 2.1.1.45) is an enzyme crucial for DNA synthesis. TS ThyA (later denoted shortly as TS) catalyzes the conversion of deoxyuridylate (dUMP) and 5,10-methylene-5,6,7,8-tetrahydrofolate (mTHF) to thymidylate (dTMP) and 7,8-dihydrofolate (DHF) by reductive methylation, with mTHF serving as both methyl donor and reducing agent [1-3]. The reaction catalyzed by TS is a pivotal step in the *de novo* biosynthetic pathway leading to dTTP (one of the four building blocks of DNA). Inhibition of TS blocks, in the absence of preformed thymidine, DNA synthesis and eventually leads to cell death, a phenomenon termed "thymineless death" [4]. Therefore, targeting of TS for inactivation in TS-overexpressing cells, such as tumor cells, constitutes an important strategy in the development of drugs for chemotherapy [5,6]. Several classes of agents have been examined, either experimentally or through clinical testing, for their inhibitory effect against TS. The most widely utilized agent, 5-fluorouracil (FUra), was first developed in 1957 based on the observation that uracil may be preferentially used in DNA synthesis in tumors compared to normal cells [7-8]. FUra and its several pro-drugs (e.g., capecitabine, ftorafur, 5'-deoxy-5-fluorouridine) remain to these days essential in the treatment of various types of cancer, including gastrointestinal neoplasia, breast, head and neck, ovary and pancreas cancers [9-11]. FUra is anabolized to a nucleotide analog of dUMP, 5-fluoro-2'-deoxyuridylate (FdUMP), which is a tight-binding inhibitor of TS. The mechanism-based inhibition of TS by FdUMP involves a time-dependent formation of the ternary complex between TS, FdUMP and the folate cofactor, mTHF, upon which the reaction stops as the fluorine substituent fails to dissociate from the pyrimidine ring, resulting in a slowly reversible inactivation of the enzyme [12]. FUra is by no means an optimal drug because it is inefficiently converted to FdUMP, with part of it catabolized to toxic metabolites. Moreover, tumors may exhibit acquired or intrinsic resistance to FUra [13,14]. Both the aforementioned factors and the lack of selectivity of FUra for TS cause that the search for effective and specific inhibitors of TS continues, aiming mainly at analogues of the enzyme substrate, dUMP, and folate cofactor, mTHF (mTHF analogues belong to so called antifolates).

The most studied analogues of dUMP through the years were those substituted in the pyrimidine ring, either at the C(5) (most frequently) or C(2), C(4) or C(6) atoms [15-20]. The C(5)-analogues of dUMP were shown to span a very broad spectrum of inhibitory activities against TS, depending on the physicochemical properties of the substituent. The latter relationship was quantitatively interpreted in the QSAR models, showing the activity of the inhibitors to be directly proportional to the electron withdrawing, while inversely proportional to the steric, effects of the substituents [21-23]. The recent QSAR model reported by Jarmuła *et al.* [23] suggested a set of new, potentially very strong inhibitors of TS in this group, but no follow-up studies were conducted so far. A strong, mechanism-based inhibitory effect, exhibited by a C(4)-substituted analogue of dUMP, N(4)-hydroxy-dCMP, was potentiated in the 5-fluoro congener, N(4)-OH-FdCMP [24,25]. The studies aimed at explanation of the inhibitory mechanism of both agents are ongoing [26], including the recently performed high-resolution crystallographic experiments (Jarmuła *et al.*, in preparation). Altogether, while TS-targeted analogues of dUMP and inhibitory strategies pertaining to them, such as the enzyme-catalyzed therapeutic activation (ECTA) [27,28] or the approach utilizing FdUMP(N) oligodeoxynucleotides (ODNs) [6,29], remain the subject of continuous investigation, till present only the classical, "old-aged" FUra and its several pro-drugs succeeded to become anticancer drugs.

*Address correspondence to this author at the Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur St., 02-093 Warszawa, Poland; Tel: (4822) 5892297; Fax: (4822) 8225342;
E-mail: a.jarmula@nencki.gov.pl

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An important factor reducing the effectiveness of FdUMP as TS inhibitor is metabolic resistance, a phenomenon consisting of the protection of enzyme against drugs by accumulation of substrate to the level sufficient to compete efficiently with the inhibitor, leading to the restoration of the full activity of the metabolic pathway involving the target enzyme [30,31]. It was one of the reasons causing the researchers to focus their interest on analogues of the folate cofactor of TS, leading ultimately to the development of inhibitors pertaining to this group. This review concentrates on antifolates targeted at TS that are currently/were recently on clinical trials or are on the market as drugs in anticancer therapy. These agents, shown in Fig. (**1**), are discussed in subsequent sections, and their selected characteristics are summarized in Table **1**. In addition to reviewing the current state of the antifolate group of TS inhibitors, trends and perspectives are discussed for developing improved drugs in this group in the future.

RALTITREXED

Raltitrexed (Tomudex, ZD1694, ICI D1694) was developed as a direct (it does not require to be modulated by other agents) and specific inhibitor of TS [32-34]. It is an analog of CB3717 (PDDF), a quinazoline-based antifolate analogue that was first investigated in the 1980s and proved to be a potent inhibitor of TS [35,36]. Unfortunately, the evaluation of CB3717 as an anticancer drug demonstrated severe life-

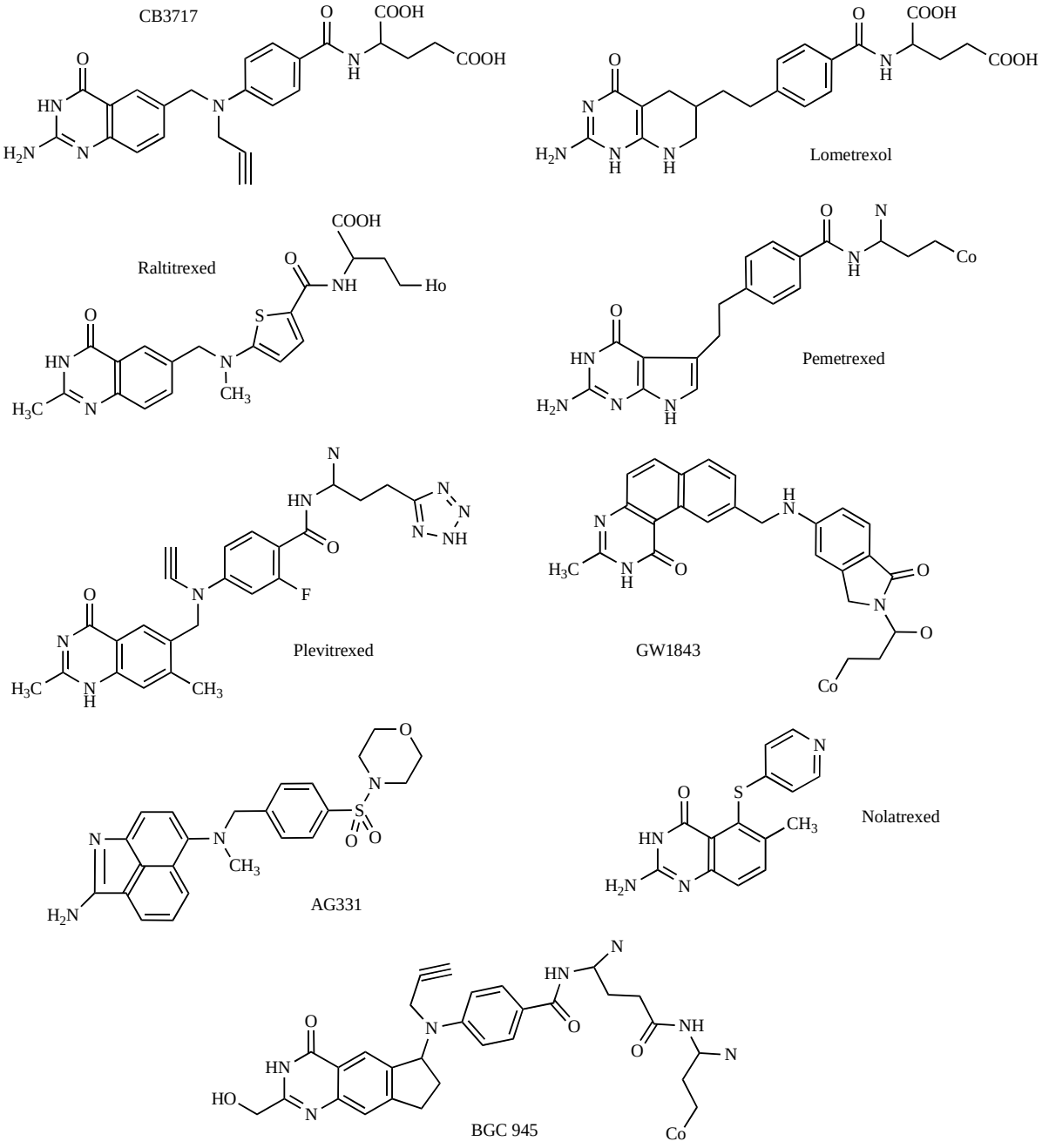

Fig. (1). Antifolate inhibitors of TS.

Table 1. Selected Characteristics of Important Antifolate Inhibitors of TS

Agent	Uptake	Polyglutamation	Crystal structures	Clinical status***
Raltitrexed	RFC	+	1HZW,** 1I00, 1HVY, 1RTS, 2TSR, 2KCE	approved as drug (CRC), 4 active trials in MPM and CRC
Pemetrexed	RFC, PCFT, several other transporters	+	1JU6, 1JUJ	approved as drug (MPM, NSCLC), 196 active trials in a range of cancers
Plevitrexed	RFC, α-FR	–	–	no active trials
GW1843*	RFC	+	2VF0, 1F28, 1SYN, 1TSD, 1TLC	1 active trial in CRC
Nolatrexed	passive diffusion	–	–	no active trials
BGC 945	α-FR	–	–	2 active trials in ovarian cancer

*Now reformulated as GS7904L.
**Identification codes in the Protein Data Bank.
***According to the search on *ClinicalTrials.gov* as of June 2010.

threatening renal and hepatic toxicity associated with its administration, thereby ruling out its further clinical development [37-39]. Studies on CB3717 were, and still are, important for the drug design process. Obtaining the crystal structures of TS ternary complexes with dUMP/FdUMP and CB3717 [40-50] allowed to explore the binding mode of CB3717 to TSs from various organisms and therefore was, and still is, of help in the process of rational design of new folate analogs as potent TS inhibitors. Raltitrexed was modeled by replacing the CH_2-C≡CH group and the benzene ring in CB3717 with methyl group and thiophene ring, respectively, rendering it a water-soluble, non-nephrotoxic, and more potent than CB3717, TS-inhibitory agent [51,32]. Being an analog of mTHF, raltitrexed cannot be incorporated into DNA, and its inhibitory effect is independent on dUMP accumulation caused by TS inhibition. The drug is efficiently transported by the reduced folate carrier (RFC) into cells, where it undergoes an extensive polyglutamation by the folylpolyglutamate synthase (FPGS) [52]. Once polyglutamated, it becomes over 100-fold more inhibitory active and retains within cells for a considerably prolonged time in comparison to its monoglutamated form [53]. Raltitrexed binds to *E. coli* TS in similar manner as CB3717, with the differences between the two inhibitors being accommodated by the enzyme with small shifts in the positions of the key active site residues [54]. The ability of Raltitrexed to compete with the mTHF cofactor at the enzyme active site does not depend on the formation of a covalent bond between the catalytic cysteine and the pyrimidine ring of the dUMP substrate, which, once realized, was taken as a possible indication for further TS-targeted drug design [55].

Early clinical evaluation of Raltitrexed demonstrated the therapeutic effectiveness of the drug against metastatic colorectal cancer (mCRC) [37]. In 1995 it was approved in several countries for the treatment of mCRC, thus becoming the first new drug against colorectal cancer (CRC) in about 35 years [56]. At that time, the development of Raltitrexed was considered a marked success in the rational drug design [57]. However, since then the reputation of the drug has been on the downswing. The AstraZeneca company stopped prematurely the phase III Pan European Trial in Adjuvant Colon Cancer-1 (PETACC-1) due to excess of drug-related deaths and serious adverse events among patients treated with Raltitrexed [58]. Raltitrexed-related mortality of 4 %, compared with 0.5 % for the 2 infusional regimens of FUra in combination with leucovorin (LV), was observed in the major phase III trial of the Medical Research Council (MRC) [59,60]. Although several other trials, in particular those combining Raltitrexed with oxaliplatin, offered good response rates and tolerance when used as first- or second-line therapy in patients with CRC [61-67], the disappointing results of the PETACC-1 and MRC trials were decisive for virtual discontinuation of further clinical development of Raltitrexed in CRC. In accordance, in the 2005 (reviewed in 2008) TA93 Guidance, the National Institute for Health and Clinical Excellence (NICE) in England recommended against the use of Raltitrexed as the therapy for CRC, except for appropriately designed clinical studies [68]. The drug has never been approved for the treatment of CRC in the United States. While it remains approved in several countries (e.g., Canada, Australia, Japan, numerous European countries), its usage is basically limited to patients intolerant to FUra [56]. The latter restriction corresponds with the published phase III PETACC-1 results, which failed to demonstrate non-inferiority of Raltitrexed against FUra in combination with LV with respect to relapse-free and overall survivals [69]. Hence, further "career" of Raltitrexed is uncertain, especially as the industry support for its investigation has been withdrawn after the MRC and PETACC-1 trials [56].

Besides its activity against CRC, the drug was shown to be active, both as a single agent and in combination with either cisplatin or oxaliplatin, in phase II/III trials in malignant pleural mesothelioma (MPM) [70-73], but these studies need to be pursued further. Yet as of June 2010, only 11 clinical trials (including 4 active with 1 study recruiting new patients) involving Raltitrexed were registered with the clinical trials database, *ClinicalTrials.gov* (compare with the number of trials involving Pemetrexed; see Pemetrexed section), reflecting a distinctly lowered rate of current research on the drug. On the positive end, experiences collected dur-

ing investigation of Raltitrexed were/will be of benefit to the clinical development of new agents targeted against TS.

PEMETREXED

Pemetrexed (ALIMTA, MTA, LY231514), a new-generation antifolate, is a multi-targeted drug. In addition to its primary target, TS, Pemetrexed inhibits other enzymes involved in pyrimidine synthesis such as dihydrofolate reductase (DHFR) and aminoimidazole carboxamide ribonucleotide formyltransferase (AICARFT), as well as the purine synthesis enzyme glycinamide ribonucleotide formyltransferase (GARFT) [74,75]. The drug was developed by replacing the 5-dezapteridine ring in lometrexol [76], an antifolate inhibitor of *de novo* purine synthesis designed in a collaborative effort of Princeton, Yale, the University of Southern California, and Eli Lilly and Company, with a pyrrolopyrimidine ring yielding unexpectedly a potent inhibitor of TS (in contrast to lometrexol which does not inhibit TS) [77]. One of major advantages of Pemetrexed is its high affinity for several folate transporters. In particular, one important pathway for the uptake of the drug into cells is an RFC-independent route mediated by the proton-coupled folate transporter (PCFT) [78]. Therefore, the activity of Pemetrexed is preserved even in case of lost or impaired activity of RFC [79,80] that favorably distinguishes the drug from Raltitrexed. Another advantage of the drug is its very extensive polyglutamation by FPGS, allowing the drug to persist for a markedly prolonged time within the cells [81,78]. The latter causes marked accumulation of Pemetrexed polyglutamates within the cells and facilitates a prolonged and potent inhibition of the drug's major target, TS, as well as a similarly prolonged but 50-times less potent inhibition of GARFT and even weaker inhibition of the drug's remaining enzyme targets [78]. Besides, the level of activity of the drug is markedly sensitive to the level of cellular folates in that the concurrent contraction in the folate pool supports the preservation of a high-level activity of the drug [82,78]. Yet another advantageous feature of Pemetrexed (and, in this case, of other antifolates as well) is that its inhibitory effect against TS is insensitive to dUMP accumulation (occurring upon exposure of cancer cells to Pemetrexed [83]), as dUMP and Pemetrexed bind to the enzyme at different sites. Indeed, the excess of dUMP may even enhance the binding of the polyglutamated forms of Pemetrexed to TS [78]. From the structural standpoint based on relevant crystal structures [84], binding of Pemetrexed induces TS active site closure and leads to formation of a covalent adduct between the enzyme and the substrate, thus differing from the binding of Raltitrexed. A large, almost 100-times increase in affinity for TS of the polyglutamated, relative to monoglutamated, form of the drug seems to originate from specific interactions between the polyglutamyl moiety and a positively charged groove on the surface of the enzyme [84].

Clinical evaluation of Pemetrexed demonstrated its usefulness as the anticancer agent [66 and references therein]. Accordingly, it was approved in February 2004 by the United States Food and Drug Administration (FDA) for use in combination with cisplatin in MPM [85] and later that year as a second-line single agent for the treatment of locally advanced or metastatic non-small cell lung cancer (NSCLC) with non-squamous histology [86]. A few years later, in September 2008, FDA approved Pemetrexed in combination with cisplatin as a first-line treatment for NSCLC [87] and in the next year as a cost-effective maintenance therapy for this cancer [88]. In accordance, the NICE in England recommended Pemetrexed in combination with cisplatin as an option for the first-line treatment of NSCLC [89] and, most recently, as the maintenance therapy for this cancer [90]. The drug has also been recommended by NICE as a treatment option for MPM [91]. Beside the approvals in United States and England, the drug has been licensed for the treatment of NSCLC in more than 85 countries across the World, including, e.g., European Union, Canada, Japan, China, India, South Africa and Australia. Pemetrexed alone or in combination with other chemotherapeutic agents shows also activity in a number of other tumors including head and neck, breast, bladder, cervical, gastric, pancreatic, ovarian and colorectal cancers [92-95]. The most common adverse events associated with the administration of Pemetrexed involve neutropenia, leukopenia, fatigue, nausea, dyspnea, anemia, anorexia, edema, rashes and vomiting. Severe toxicities, however, are reduced by low-level supplementation of folic acid and vitamin B-12 [93,96-98]. On the other hand, increasing the levels of folates diminishes the activity of Pemetrexed; hence, the optimization of folic acid supplementation to the level which decreases toxicities without significantly compromising the antitumor effect of the drug, remains an issue of importance [78].

Unlike in the case of Raltitrexed, there is little concern about the future of Pemetrexed, as the drug spreads itself steadily on the pharmacological market and is being constantly investigated for its possible inductions/extensions of application in several types of cancer [94,95,99-109]. At the time of this work, the search on *ClinicalTrials.gov* yielded altogether 330 clinical studies involving Pemetrexed, 117 of which were currently recruiting new patients. The main issues that remain to be resolved to "catalyze" further progress in the development of this drug involve: improved selection of patients that are likely to respond to the drug, identification of better markers of sensitivity to the drug, choice of most effective doses when using Pemetrexed in combination with other agents or supplemented with vitamin B-12 or folic acid, and finding a way to combine the drug with the radiation therapy for optimal effect [60].

PLEVITREXED

Plevitrexed (ZD9331, BGC9331) is a classical antifolate that is a potent and specific inhibitor of TS [110-113]. The drug was designed to be active without necessity of polyglutamation. The double rationale behind this was to overcome the resistance due to reduced expression of FPGS whilst reduce the toxicities associated with polyglutamation and drug retention in normal tissues. The drug molecule was developed by structural modifications of the ICI 198583 molecule (2-desamino-2-methyl analogue of CB3717) by incorporation of 7-methyl and 2'-fluoro groups and substitution of the γ-carboxyl of the glutamate chain with tetrazole ring [6,110]. Plevitrexed can be transported into cells *via* RFC, as well as the α-folate receptor (α-FR), the latter overexpressed in most of the epithelial ovarian tumors [112,114,115]. The drug demonstrated promising phase I and II activities and manageable toxicities in ovarian, pancreatic, gastric, colorectal,

non-small-cell lung and other solid cancers [116-125]. The multi-center, randomized, phase II/III study showed that in pancreatic cancer Plevitrexed is equivalent to an anticancer drug, gemcitabine, and may offer an alternative to current therapies [126]. In 2007, the drug was granted US orphan drug designation for gastric and ovarian cancer indications. BTG International, the life sciences company that developed Plevitrexed, has announced to be planning to take the drug further through development and is seeking opportunities to evaluate an improved clinical protocol in advanced gastric cancer [127]. However, as of June 2010, the search on *ClinicalTrials.gov* showed no active clinical trials involving the treatment with Plevitrexed. The true anticancer potential of the drug still remains to be explored.

GW1843

GW1843 (OSI-7904, BW1843, 1843U89) is, most probably, the most potent known folate-based inhibitor of TS [37,128,129]. It enters cells *via* RFC and, being a good substrate of FPGS, undergoes conversion by this enzyme, predominantly to the diglutamate that is accumulated intracellularly [128,130]. The high potency of GW1843, combined with its high substrate activity for FPGS, enables GW1843 cytotoxicity to cells with reduced FPGS activity, even those resistant to Plevitrexed [131]. The drug does not need further glutamylation (beyond diglutamate), as it does not cause further increase of the drug affinity for TS binding that is anyway high [128,130,132]. However, due to the absence of higher glutamylated forms, the drug is secreted out of cells with relative ease, contributing to the loss of GW1843 cytotoxic potency, concurrent with a decrease in the drug exposure time [133]. Compared to CB3717, GW1843 has an extra ring attached to the quinazoline portion of the molecule, making the molecule of GW1843 more bulky and about 4 Å longer than that of CB3717. As shown in the crystal structure of the ternary complex between *E.coli* TS, dUMP and GW1843, the extra bulk of GW1843 is accommodated by the enzyme by means of a local distortion of the active site that results in several "novel" interactions between the drug and active site groups [134-136]. The covalent bond between the catalytic cysteine and dUMP does not form. GW1843 has an interesting property of inactivating TS at a stoichiometric ratio of one molecule per TS dimer. In accomplishing this, the drug acts as a competitive inhibitor of TS at the binding site and a noncompetitive inhibitor at the other site [134].

Assessed in preclinical *in vivo* studies, GW1843 demonstrated antitumor effects in a broad range of human tumor xenografts implanted in mice [128,137-140]. Treatment-associated toxicities were reduced and/or eliminated by co-administration of folic acid without compromising the antitumor activities [139]. Phase I clinical trial in patients with advanced solid malignancies determined the maximal doses of the drug at a given schedule with or without supplementation with folic acid [141]. In December 2000, the drug was reformulated by encapsulation into liposomes (renamed as GS7904L), in order to improve efficacy and convenience of dose schedule. Examined using human tumor xenografts in mice, GS7904L showed an improved efficacy over GW1843 [142]. GS7904L has also better bioavailability (through decreased clearance and volume of distribution) than GW1843, enhancing the tumor localization of active drug and potentially simplifying the drug administration schedule [142-144]. GS7904L, alone or in combinations with other agents (cisplatin, oxaliplatin), is currently in phase I and II clinical trials against a range of solid tumors, including recently completed trials against gastric or gastroesophageal (GEJ), biliary tract and head and neck cancers, as well as an ongoing trial against advanced CRC [143-148]. To date, the drug showed some promises in gastric and CRC tumors.

NOLATREXED

Nolatrexed (AG337, Thymitaq) and AG331 are two lipophilic TS inhibitors that lack the glutamate side chain and thus do not undergo polyglutamation. For this reason, the drugs are capable of effective TS inhibition in FPGS-deficient cells. However, due to the lack of polyglutamated forms, prolonged intracellular retention of the drugs and, in turn, prolonged TS inhibition, are impossible, therefore administration *via* continuous infusion is indicated [132,53]. The drugs - structurally unrelated to mTHF - were developed using an iterative computer-assisted drug design approach, which combined high-resolution X-ray crystallography, molecular modeling and synthesis [149-153]. Both drugs lack the requirement for active cellular uptake, entering the cells by passive diffusion rather than being transported by RFC or *via* other specific transport systems, thereby circumventing possible resistance due to lowered RFC transport. The drugs are potent noncompetitive inhibitors of human TS [150,154] and much less potent inhibitors of cancer growth [150,154-157]. The noncompetitive character of inhibition is unexpected, considering that the drugs bind at the folate binding site; the mechanism behind this is not clear [133]. Preclinical data on AG331 in rat and human hepatoma cells suggested an alternate mode of action, proposed to result from biotransformation of the drug into unidentified toxic metabolites [155]. Severe liver toxicity observed in phase I clinical studies, together with more rapid progress of studies on Nolatrexed, resulted in the termination of further clinical development of AG331 [158,159].

Nolatrexed was synthesized shortly after AG331 [152]. Unlike with AG331, TS was suggested as the sole locus of action [154,157]. However, sensitivity to Nolatrexed showed no correlation with TS activity, TS protein or TS mRNA levels in a panel of human colorectal carcinoma and leukaemia cell lines, suggesting additional sensitivity determinants to be involved [160]. Clinical evaluation of the drug started with several phase I clinical trials, testing a number of dose and schedule regimens [161-165]. In phase II clinical trials, antitumor activity was observed primarily in head and neck cancer and hepatocellular carcinoma (HCC) [166,167]; some activity was also apparent in patients with pancreatic cancer, CRC and NSCLC [133, reviewed in 168]. As a result, multicenter phase III study was conducted in North America, Europe and South Africa, in order to compare Nolatrexed to another anticancer drug, doxorubicin (the control arm) in 445 patients with unresectable HCC. The study, known as Evaluation of Thymitaq in Hepatocellular Carcinoma (ETHECC), failed to achieve its primary endpoint, which was a survival benefit for patients treated with Nolatrexed [169]. Similar result was observed in a small phase II study comparing Nolatrexed to doxorubicin in 54 Chinese patients with advanced HCC [170]. In parallel, yet another small

phase II study of Nolatrexed in 48 patients with advanced HCC demonstrated minimal activity and serious stomatitis, thus further questioning the usefulness of the drug as a single agent for HCC [171]. Two randomized trials comparing Nolatrexed to methotrexate in patients with recurrent head and neck cancer were also unsuccessful, showing no efficacy advantage in the Nolatrexed arm [172]. A search of the two public clinical trials databases (*ClinicalTrials.gov* and *Current Controlled Trials*) showed no further trials for Nolatrexed as of June 2010.

BGC 945

BGC 945 (ONX 0801) is a very potent, novel class TS inhibitor, targeted specifically to α-FR, with low affinity for RFC. The drug exerts selective toxicity against α-FR-overexpressing tumors where it is retained for a prolonged time and at much higher concentrations compared to plasma or normal tissues [173,174]. The drug needs no polyglutamation for its full effect to be exerted. This is due to the presence of a modified glutamate ligand, in the form of L-Glu-γ-D-Glu dipeptide, with the D-enantiomer stabilizing the BGC 945 molecule (as well as molecules of other antifolates with dipeptide ligands [175,176]) against enzymatic hydrolysis *in vivo* [173]. These desirable properties of BGC 945 may potentially account for a high efficacy and low toxicity of the drug in patients with tumors expressing α-FR, such as most ovarian cancers and many other epithelial tumors [174]. Accordingly, the drug has been nominated for clinical development and is expected to enter phase I studies in the near future. Currently, two "preparatory" clinical trials to measure the levels of α-FR in stored plasma and blood samples from patients with ovarian cancer are ongoing.

DISCUSSION AND CONCLUSIONS

Since the approval of Raltitrexed in 1995 for the treatment of CRC [56], a variety of antifolates have been developed as TS inhibitors and tested for anticancer activity and cytotoxicity. Both ligand- and, later on, receptor-based design paths have contributed to the development arsenal of the scientists and researchers engaged in the process. Some of the most promising agents developed with either approach, including Pemetrexed, Plevitrexed, GW1843, AG331 and Nolatrexed, have advanced to the clinical trial stage, giving rise to hopeful expectations. However, until these days, only one of these agents, Pemetrexed, had joined Raltitrexed on the approved list, becoming the licensed drug for the treatment of MPM and NSCLC [85-91]. Other candidates, such as Plevitrexed and GW1843 (now reformulated as GS7904L), have yet not emerged successful and, at the best, need further thorough clinical evaluation before having the chance to be considered for possible therapeutic use in patients. Development of Nolatrexed has practically ceased after the unsatisfactory results of the pivotal phase III ETHECC trial [169]. BGC 945, a new prospect among antifolates, has yet to be validated through extensive full-stage clinical tests.

The two cases of Raltitrexed and Pemetrexed, being in a clear contrast, deserve an additional word of comment. The Raltitrexed-related deaths observed in the MRC and PETACC-1 trials, which drastically slowed down further drug development, were later attributed in majority to violations in protocols and a failure to adjust doses with respect to toxicity or to the presence of renal impairment [56,177,178]. Optimization of the administration and supplementation of the drug was afterward never a subject of a comprehensive investigation. Therefore, even with the continuous use of Raltitrexed in CRC, it may be considered that the therapeutic potential of the drug has not been exploited properly. Moreover, the current limited pace of research on Raltitrexed does not promise well in this regard for the future. This is in contrast with Pemetrexed, which, regardless of having a natural, advantageous therapeutic profile, was developed consistently and dependably, with the current, rapid pace of research on the drug offering good chances for further optimization of its efficacy and tolerability in patients.

One very important obstacle that limits the efficacy of antifolates is the drug resistance. As discussed extensively in a recent review by Assaraf [179], the well recognized mechanisms of antifolate resistance with respect to TS inhibition include: (1) impaired uptake due to lost or reduced expression of RFC, (2) decreased retention inside the cells due to (i) decreased activity of FPGS and hence defective polyglutamation, or (ii) increased activity of γ-glutamyl hydrolase (GGH) and hence enhanced hydrolysis of the polyglutamate tails, (3) increased efflux due to overexpression of ATP-driven multidrug resistance (MDR) efflux transporters, (4) increased expression of the enzyme; mutations in the enzyme protein lowering affinity for antifolates, and (5) augmentation of folate cofactor pools in tumor cells. Strategies aimed at overcoming antifolate resistance are developed, with certain antifolates discussed in our review (BGC 945, Plevitrexed, Nolatrexed) designed to circumvent the (1) and/or (2) mechanisms (see Table **1**). Currently, the most important approaches targeted against antifolate resistance include: restoration of RFC expression in tumor cells, targeting of α-FR or PCFT for antifolate uptake, induction of α-FR and β-FR expressions, use of antifolates with improved transport and polyglutamation characteristics [179 and references therein]. Further identification and characterization of molecular mechanisms underlying antifolate resistance will aid in devising the most suitable strategies to overcome/circumvent drug-resistance effects and thus improve therapeutic efficacies of the antifolate drugs.

The future of TS antifolate inhibitors seems to depend on advances in the receptor-based design, allowing better accounting for the structural features of the molecular targets/resistance determinants, such as TS itself, RFC, α-FR and FPGS, directly contributing to the activity and specificity of TS-targeted antifolates. In a recent 2-part review by Gangjee *et al.* [180,181], the structure-activity relationships (SAR) of various classes of antifolates are discussed that delineate preliminary structural features responsible for the interaction of agents from each class with the aforementioned targets. Such observations, combined into comprehensive 3D-QSAR models, will eventually elucidate structural dependencies that should be adhered to in the design of improved, specific antifolate inhibitors of TS. The approach itself does not guarantee success (in the form of new drugs), as the drug development and testing is a multi-issue, multi-step and costly process, where the research does not necessarily synergize but often competes with economic issues,

but it increases the chances of a marked progress in the up-coming years.

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ABBREVIATIONS

TS = thymidylate synthase

dUMP = deoxyuridylate

mTHF = 5,10-methylene-5,6,7,8-tetrahydrofolate

FUra = 5-fluorouracil

FdUMP = 5-fluoro-2'-deoxyuridylate

RFC = reduced folate carrier

FPGS = folylpolyglutamate synthase

CRC = colorectal cancer

NICE = National Institute for Health and Clinical Excellence

MPM = malignant pleural mesothelioma

PCFT = proton-coupled folate transporter

FDA = Food and Drug Administration

NSCLC = non-small cell lung cancer

α-FR = α-folate receptor

HCC = hepatocellular carcinoma

REFERENCES

[1] Carreras, C.W.; Santi, D.V. The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.*, **1995**, *64*, 721-762.

[2] Stroud, R.M.; Finer-Moore, J.S. Conformational dynamics along an enzymatic reaction pathway: thymidylate synthase, "the movie". *Biochemistry*, **2003**, *42*, 239-247.

[3] Finer-Moore, J.S.; Santi, D.V.; Stroud, R.M. Lessons and conclusions from dissecting the mechanism of a bisubstrate enzyme: thymidylate synthase mutagenesis, function and structure. *Biochemistry*, **2003**, *42*, 248-256.

[4] Cohen, S.S. On the nature of thymineless death. *Ann. NY Acad. Sci.*, **1971**, *186*, 292-301.

[5] Costi, M.P.; Ferrari, S.; Venturelli, A.; Calo, S.; Tondi, D.; Barlocco, D. Thymidylate synthase structure, function and implication in drug discovery. *Curr. Med. Chem.*, **2005**, *12*, 763-771.

[6] Gmeiner, W.H. Novel chemical strategies for thymidylate synthase inhibition. *Curr. Med. Chem.*, **2005**, *12*, 191-202.

[7] Heidelberger, C.; Chaudhuri, N.K.; Dannenberg, P.; Mooren, D.; Griesbach, L.; Duschinsky, R.; Schnitzer, R.J.; Pleven, E.; Scheiner, J. Fluorinated pyrimidines, a new class of tumor-inhibitory compounds. *Nature*, **1957**, *179*, 663-666.

[8] Ackland, S.P.; Clarke, S.J.; Beale, P.; Peters, G.J. Thymidylate synthase inhibitors. In *Cancer Chemotherapy and Biological Response Modifiers*, Annual 20, Giaccone, G.; Schilsky, R.; Sondel, P., Eds.; Elsevier Science B.V.: Amsterdam, **2002**, pp. 1-36.

[9] Malet-Martino, M.; Martino, R. Clinical studies of three oral prodrugs of 5-fluorouracil (capecitabine, UFT, S-1): a review. *Oncologist*, **2002**, *7*, 288-323.

[10] Petty, R.D.; Cassidy, J. Novel fluoropyrimidines: improving the efficacy and tolerability of cytotoxic therapy. *Curr. Cancer Drug Targets*, **2004**, *4*, 191-204.

[11] Berger, F.G.; Berger, S.H. Thymidylate synthase as a chemotherapeutic drug target. Where are we after fifty years? *Cancer Biol. Ther.*, **2006**, *5*, 1238-1241.

[12] Santi, D.V.; McHenry, C.S.; Raines, R.T.; Ivanetich, K.M. Kinetics and thermodynamics of the interaction of 5-fluoro-2'-deoxyuridylate with thymidylate synthase. *Biochemistry*, **1987**, *26*, 8606-8613.

[13] Washtien, W.L. Increased levels of thymidylate synthase in cells exposed to 5- fluorouracil. *Mol. Pharmacol.*, **1984**, *25*, 171-177.

[14] Berger, S.H.; Jenh, C.H.; Johnson, L.F.; Berger, F.G. Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Mol. Pharmacol.*, **1985**, *28*, 461-467.

[15] De Clercq, E.; Balzarini, J.; Torrence, P.F.; Mertes, M.P.; Schmidt, C.L.; Shugar, D.; Barr, P.J.; Jones, A.S.; Verhelst, G.; Walker, R.T. Thymidylate synthetase as target enzyme for the inhibitory activity of 5-substituted-2'-deoxyuridines on mouse leukemia L1210 cell growth. *Mol. Pharmacol.*, **1981**, *19*, 321-330.

[16] Balzarini, J.; De Clercq, E.; Mertes, M.P.; Shugar, D.; Torrence, P.F. 5-substituted 2'-deoxyuridines: correlation between inhibition of tumor cell growth and inhibition of thymidine kinase and thymidylate synthetase. *Biochem. Pharmacol.*, **1982**, *31*, 3673-3682.

[17] Dzik, J.M.; Bretner, M.; Kulikowski, T.; Gołos, B.; Jarmuła, A.; Poznański, J.; Rode, W.; Shugar, D. Synthesis and interactions with thymidylate synthase of 2,4-dithio analogues of dUMP and 5-fluoro-dUMP. *Biochim. Biophys. Acta*, **1996**, *1293*, 1-8.

[18] Jarmuła, A.; Anulewicz, R.; Leś, A.; Cyrański, M.K.; Adamowicz, L.; Bretner, M.; Felczak, K.; Kulikowski, T.; Krygowski, T.M.; Rode, W. Crystal structures of 5-fluoro- dUrd and its 2 and/or 4-thio analogues: models of substituted dUMP pyrimidine ring interacting with thymidylate synthase. *Biochim. Biophys. Acta*, **1998**, *1382*, 277-286.

[19] Jarmuła, A.; Cyrański, M.K.; Leś, A.; Krygowski, T.M.; Rode, W. Interaction of thymidylate synthase with 5-fluoro-substituted dUMP analogues in view of the pyrimidine ring structure. *Pol. J. Chem.*, **1998**, *72*, 1958-1962.

[20] Douglas, K.T. The thymidylate synthesis cycle and anticancer drugs. *Med. Res. Rev.*, **1987**, *7*, 441-475.

[21] Wataya, Y.; Santi, D.V.; Hansch, C. Inhibition of Lactobacillus casei thymidylate synthetase by 5-substituted 2'-deoxyuridylates. Preliminary quantitative structure-activity relationship. *J. Med. Chem.*, **1977**, *20*, 1469-1473.

[22] Jarmuła, A.; Leś, A.; Rode, W. Different activities of 5-hydroxy-dUMP and 5-hydroxymethyl-dUMP in thymidylate synthase-catalyzed reaction in view of molecular modeling and structural studies. *Bioorg. Chem.*, **2000**, *28*, 156-162.

[23] Jarmuła, A.; Cieplak, P.; Krygowski, T.M.; Rode, W. The effect of 5-substitution in the pyrimidine ring of dUMP on the interaction with thymidylate synthase: molecular modeling and QSAR. *Bioorg. Med. Chem.*, **2007**, *15*, 2346-2358.

[24] Rode, W.; Zieliński, Z.; Dzik, J.M.; Kulikowski, T.; Bretner, M.; Kierdaszuk, B.; Cieśla, J.; Shugar, D. Mechanism of inhibition of mammalian tumor and other thymidylate synthases by N4-hydroxy-dCMP, N4-hydroxy-5-fluoro-dCMP, and related analogs. *Biochemistry*, **1990**, *29*, 10835-10842.

[25] Felczak, K.; Miazga, A.; Poznański, J.; Bretner, M.; Kulikowski, T.; Dzik, J.M.; Gołos, B.; Zieliński, Z.; Cieśla, J.; Rode, W. 5-substituted N4-hydroxy-2'-deoxycytydines and their 5'-monophosphates: synthesis, conformation, interaction with tumor thymidylate synthase, and *in vitro* antitumour activity. *J. Med. Chem.*, **2000**, *43*, 4647-4656.

[26] Jarmuła, A.; Rypniewski, W.R.; Felczak, K.; Rode, W. X-ray crystal and *ab initio* structures of 3',5'-di-O-acetyl-N(4)-hydroxy-2'-deoxycytidine and its 5-fluoro analogue: models of the N(4)-OH-dCMP and N(4)-OH-FdCMP molecules interacting with thymidylate synthase. *Struct. Chem.*, **2005**, *16*, 541-549.

[27] Li, Q.; Boyer, C.; Lee, J.Y.; Shepard, H.M. A novel approach to thymidylate synthase as a target for cancer chemotherapy. *Mol. Pharmacol.*, **2001**, *59*, 446-452.

[28] Lackey, D.B.; Groziak, M.P.; Sergeeva, M.; Beryt, M.; Boyer, C.; Stroud, R.M.; Sayre, P.; Park, J.W.; Johnston, P.; Slamon, D.; Shepard, H.M.; Pegram, M. Enzyme-catalyzed therapeutic agent (ECTA) design: activation of the antitumour ECTA compound NB1011 by thymidylate synthase. *Biochem. Pharmacol.*, **2001**, *61*, 179-189.

[29] Bijnsdorp, I.V.; Comijn, E.M.; Padron, J.M.; Gmeiner, W.H.; Peters, G.J. Mechanisms of action of FdUMP[10]: metabolite activa-

tion and thymidylate synthase inhibition. *Oncol. Rep.*, **2007**, *18*, 287-291.

[30] Christopherson, R.I.; Duggleby, R.G. Metabolic resistance: the protection of enzymes against drugs which are tight-binding inhibitors by the accumulation of substrate. *Eur. J. Biochem.*, **1983**, *134*, 331-335.

[31] Duggleby, R.G.; Christopherson, R.I. Metabolic resistance to tight-binding inhibitors of enzymes involved in the *de novo* pyrimidine pathway. *Eur. J. Biochem.*, **1984**, *143*, 221- 226.

[32] Jackman, A.L.; Taylor, G.A.; Gibson, W.; Kimbell, R.; Brown, M.; Calvert, A.H.; Judson, I.R.; Hughes, L.R. ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth *in vitro* and *in vivo*: a new agent for clinical study. *Cancer Res.*, **1991**, *51*, 5579-5586.

[33] Hughes, L.R.; Stephens, T.C.; Boyle, F.T.; Jackman, A.L. Raltitrexed (Tomudex), a highly polyglutamatable antifolate thymidylate synthase inhibitor: design and preclinical activity. In *Antifolate drugs in cancer therapy*, Jackman, A.L., Ed.; Humana Press: Totowa, NJ, **1999**, pp. 147-165.

[34] Beale, P.; Clarke, S. Tomudex: clinical development. In *Antifolate drugs in cancer therapy*, Jackman, A.L., Ed.; Humana Press: Totowa, NJ, **1999**, pp. 167-181.

[35] Jones, T.R.; Calvert, A.H.; Jackman, A.L.; Brown, S.J.; Jones, M.; Harrap, K.R. A potent antitumor quinazoline inhibitor of thymidylate synthase: synthesis, biological properties and therapeutic results in mice. *Eur. J. Cancer*, **1981**, *17*, 11-19.

[36] Clarke, S.J.; Jackman, A.L.; Judson, I.R. The history of the development and clinical use of CB3717 and ICI D1694. *Adv. Exp. Med. Biol.*, **1993**, *339*, 277-287.

[37] Jackman, A.L.; Calvert, A.H. Folate-based thymidylate synthase inhibitors as anticancer drugs. *Ann. Oncol.*, **1995**, *6*, 871-881.

[38] Cantwell, B.M.J.; Macaulay, V.; Harris, A.L.; Kaye, S.B.; Smith, I.E.; Milsted, R.A.V.; Calvert, A.H. Phase II study of the antifolate N10-propargyl-5,8-dideazafolic acid (CB3717) in advanced breast cancer. *Eur. J. Cancer Clin. Oncol.*, **1988**, *24*, 733-736.

[39] Calvert, A.H.; Alison, D.L.; Harland, S.J.; Robinson, B.A.; Jackman, A.L.; Jones, T.R.; Newell, D.R.; Siddik, Z.H.; Wiltshaw, E.; McElwain, T.J. A phase I evaluation of the quinazoline antifolate thymidylate synthase inhibitor, N10-propargyl-5,8-dideazafolic acid, CB3717. *J. Clin. Oncol.*, **1986**, *4*, 1245-1252.

[40] Newby, Z.; Lee T.L.; Morse, R.J.; Liu, L.; Liu, Y.; Venkatraman, P.; Santi, D.V.; Finer- Moore, J.S.; Stroud, R.M. The role of protein dynamics in thymidylate synthase catalysis. Variants of conserved dUMP-binding Tyr-261. *Biochemistry*, **2006**, *45*, 7415-7428.

[41] Birdsall, D.L.; Finer-Moore, J.; Stroud, R.M. The only active mutant of thymidylate synthase D169, a residue far from the site of methyl transfer, demonstrates the exquisite nature of enzyme specificity. *Protein Eng.*, **2003**, *16*, 229-240.

[42] Anderson, A.C.; O'Neil, R.H.; DeLano, W.L.; Stroud, R.M. The structural mechanism for half-the-sites reactivity in an enzyme, thymidylate synthase, involves a relay of changes between subunits. *Biochemistry*, **1999**, *38*, 13829-13836.

[43] Reyes, C.L.; Sage, C.R.; Rutenber, E.E.; Nissen, R.M.; Finer-Moore, J.S.; Stroud, R.M. Inactivity of N229A thymidylate synthase due to water-mediated effects: isolating a late stage in methyl transfer. *J. Mol. Biol.*, **1998**, *284*, 699-712.

[44] Stout, T.J.; Sage, C.R.; Stroud, R.M. The additivity of substrate fragments in enzyme- ligand binding. *Structure*, **1998**, *6*, 839-848.

[45] Sage, C.R.; Rutenber, E.E.; Stout, T.J.; Stroud, R.M. An essential role for water in an enzyme reaction mechanism: the crystal structure of the thymidylate synthase mutant E58Q. *Biochemistry*, **1996**, *35*, 16270-16281.

[46] Birdsall, D.L.; Huang, W.; Santi, D.V.; Stroud, R.M.; Finer-Moore, J. The separate effects of E60Q in Lactobacillus casei thymidylate synthase delineate between mechanisms for formation of intermediates in catalysis. *Protein Eng.*, **1998**, *11*, 171-183.

[47] Finer-Moore, J.; Fauman, E.B.; Foster, P.G.; Perry, K.M.; Santi, D.V.; Stroud, R.M. Refined structures of substrate-bound and phosphate-bound thymidylate synthase from Lactobacillus casei. *J. Mol. Biol.*, **1993**, *232*, 1101-1116.

[48] Kamb, A.; Finer-Moore, J.; Calvert, A.H.; Stroud, R.M. Structural basis for recognition of polyglutamyl folates by thymidylate synthase. *Biochemistry*, **1992**, *31*, 9883-9890.

[49] Montfort, W.R.; Perry, K.M.; Fauman, E.B.; Finer-Moore, J.S.; Maley, G.F.; Hardy, L.; Maley, F.; Stroud, R.M. Structure, multiple site binding, and segmental accommodation in thymidylate synthase on binding dUMP and anti-folate. *Biochemistry*, **1990**, *29*, 6964- 6977.

[50] Matthews, D.A.; Appelt, K.; Oatley, S.J.; Xuong, N.H. Crystal structure of Escherichia coli thymidylate synthase containing bound 5-fluoro-2'-deoxyuridylate and 10-propargyl- 5,8-dideazafolate. *J. Mol. Biol.*, **1990a**, *214*, 923-936.

[51] Marsham, P.R.; Hughes, L.R.; Jackman, A.L.; Hayter, A.J.; Oldfield, J.; Wardleworth, J.M.; Bishop, J.A.; O'Connor, B.M.; Calvert, A.H. Quinazoline antifolate thymidylate synthase inhibitors: heterocyclic benzoyl ring modifications. *J. Med. Chem.*, **1991**, *34*, 1594-1605.

[52] Jackman, A.L.; Gibson, W.; Brown, M.; Kimbell, R.; Boyle, F.T. The role of the reduced folate carrier and metabolism to intracellular polyglutamates for the activity of ICI D1694. *Adv. Exp. Med. Biol.*, **1993**, *339*, 265-276.

[53] Chu, E.; Callender, M.A.; Farrell, M.P.; Schmitz, J.C. Thymidylate synthase inhibitors as anticancer agents: from bench to bedside. *Cancer Chemother. Pharmacol.*, **2003**, *52*(Suppl. 1), S80-S89.

[54] Rutenber, E.E.; Stroud, R.M. Binding of the anticancer drug ZD1694 to *E. coli* thymidylate synthase: assessing specificity and affinity. *Structure*, **1996**, *4*, 1317-1324.

[55] Almog, R.; Waddling, C.A.; Maley, F.; Maley, G.F.; Van Roey, P. Crystal structure of a deletion mutant of human thymidylate synthase delta (7-29) and its ternary complex with Tomudex and dUMP. *Protein Sci.*, **2001**, *10*, 988-996.

[56] Wilson, K.S.; Taylor, S.C.M. Raltitrexed: optimism and reality. *Expert Opin. Drug Metab. Toxicol.*, **2009**, *5*, 1447-1454.

[57] Jackman, A.L.; Boyle, E.T.; Harrap, K.R. Tomudex (ZD1694): from concept to care, a programme in rational drug discovery. *Invest. New Drugs*, **1996**, *14*, 305-316.

[58] Editorial; Drug-company decision to end cancer trial. *Lancet*, **1999**, *354*, 1045.

[59] Maughan, T.S.; James, R.D.; Kerr, D.J.; Ledermann, J.A.; McArdle, C.; Seymour, M.T.; Cohen, D.; Hopwood, P.; Johnston, C.; Stephens, R.J.; British MRC Colorectal Cancer Working Party; Comparison of survival, palliation, and quality of life with three chemotherapy regimens in metastatic colorectal cancer: a multicentre randomised trial. *Lancet*, **2002**, *359*, 1555-1563.

[60] Ackland, S.P.; Clarke, S.J.; Beale, P.; Peters, G.J. Thymidylate synthase inhibitors. *Updat. Cancer Ther.*, **2006**, *1*, 403-427.

[61] Van Cutsem, E.; Cunningham, D.; Maroun, J.; Cervantes, A.; Glimelius, B. Raltitrexed: current clinical status and future directions. *Ann. Oncol.*, **2002**, *13*, 513-522.

[62] Cascinu, S.; Graziano, F.; Ferrau, F.; Catalano, V.; Massacesi, C.; Santini, D.; Silva, R.R.; Barni, S.; Zaniboni, A.; Battelli, N.; Siena, S.; Giordani, P.; Mari, D.; Baldelli, A.M.; Antognoli, S.; Maisano, R.; Priolo, D.; Pessi, M.A.; Tonini, G.; Rota, S.; Labianca, R. Raltitrexed plus oxaliplatin (TOMOX) as first-line chemotherapy for metastatic colorectal cancer. A phase II study of the Italian Group for the Study of Gastrointestinal Tract Carcinomas (GISCAD). *Ann. Oncol.*, **2002**, *13*, 716-720.

[63] Seitz, J.F.; Bennouna, J.; Paillot, B.; Gamelin, E.; Francois, E.; Conroy, T.; Raoul, J.L.; Becouarn, Y.; Bertheault-Cvitkovic, F.; Ychou, M.; Nasca, S.; Fandi, A.; Barthelemy, P.; Douillard, J.Y. Multicentre non-randomized phase II study of raltitrexed (Tomudex) and oxaliplatin in non-pretreated metastatic colorectal cancer patients. *Ann. Oncol.*, **2002**, *13*, 1072-1079.

[64] Neri, B.; Doni, L.; Fulignati, C.; Perfetto, F.; Turrini, M.; Andreoli, F.; Pantalone, D.; Pernice, L.M.; Taruffi, F.; Martini, V.; Poma, A.; Valeri, A.; Bacci, G.; Sancez, L.; Moretti, R. Raltitrexed plus oxaliplatin as first-line chemotherapy in metastatic colorectal carcinoma: a multicentric phase II trial. *Anticancer Drugs*, **2002**, *13*, 719-724.

[65] Martoni, A.; Mini, E.; Pinto, C.; Gentile, A.L.; Nobili, S.; Dentico, P.; Marino, A.; Scicolone, S.; Angelelli, B.; Mazzei, T. Oxaliplatin plus raltitrexed in the treatment of patients with advanced colorectal cancer: a phase II study. *Anticancer Res.*, **2003**, *23*, 687-691.

[66] Santini, D.; Massacesi, C.; D'Angelillo, R.M.; Marcucci, F.; Campisi, C.; Vincenzi, B.; Pilone, A.; Bianco, V.; Bonsignori, M.; Tonini, G. Raltitrexed plus weekly oxaliplatin as first-line chemotherapy in metastatic colorectal cancer: a multicenter non-randomized phase II study. *Med. Oncol.*, **2004**, *21*, 59-66.

[67] Laudani, A.; Gebbia, V.; Leonardi, V.; Savio, G.; Borsellino, N.; Cusimano, M.P.; Calabria, C.; Stefano, R.; Agostara, B. Activity and toxicity of oxaliplatin plus raltitrexed in 5-fluorouracil refractory metastatic colorectal adeno-carcinoma. *Anticancer Res.*, **2004**, *24*, 1139-1142.

[68] Hind, D.; Tappenden, P.; Tumur, I.; Eggington, S.; Sutcliffe, P.; Ryan, A. The use of irinotecan, oxaliplatin and raltitrexed for the treatment of advanced colorectal cancer: systematic review and economic evaluation (review of NICE Guidance No. 33). *Health Technol. Assess.*, **2008**, *12*, 1-182.

[69] Popov, I.; Carrato, A.; Sobrero, A.; Vincent, M.; Kerr, D.; Labianca, R.; Bianco, A.R.; El-Serafi, M.; Bedenne, L.; Paillot, B.; Mini, E.; Sanches, E.; Welch, J.; Collette, L.; Praet, M.; Wils, J. Raltitrexed (Tomudex) versus standard leucovorin-modulated bolus 5-fluorouracil: results from the randomised phase III Pan-European Trial in Adjuvant Colon Cancer 01 (PETACC-1). *Eur. J. Cancer*, **2008**, *44*, 2204-2211.

[70] Fizazi, K.; Doubre, H.; Le Chevalier, T.; Riviere, A.; Viala, J.; Daniel, C.; Robert, L.; Barthelemy, P.; Fandi, A.; Ruffie, P. Combination of raltitrexed and oxaliplatin is an active regimen in malignant mesothelioma: results of a phase II study. *J. Clin. Oncol.*, **2003**, *21*, 349-354.

[71] Baas. P.; Ardizzoni, A.; Grossi, F.; Nackaerts, K.; Numico, G.; Van Marck. E.; van de Vijver, M.; Monetti, F.; Smid-Geinaerdt, M.J.; van Zandwijk, N.; Debruyne, C.; Legrand, C.; Giaccone, G.; EORTC Lung Cancer Group. The activity of raltitrexed (Tomudex) in malignant pleural mesothelioma: an EORTC phase II study (08992). *Eur. J. Cancer*, **2003**, *39*, 353-357.

[72] van Meerbeeck, J.P.; Gaafar, R.; Manegold, C.; Van Klaveren, R.J.; Van Marck, E.A.; Vincent, M.; Legrand, C.; Bottomley, A.; Debruyne, C.; Giaccone, G.; European Organisation for Research and Treatment of Cancer Lung Cancer Group; National Cancer Institute of Canada. Randomized phase III study of cisplatin with or without raltitrexed in patients with malignant pleural mesothelioma: an intergroup study of the European Organisation for Research and Treatment of Cancer Lung Cancer Group and the National Cancer Institute of Canada. *J. Clin. Oncol.*, **2005**, *23*, 6881-6889.

[73] Porta, C. Adding raltitrexed to cisplatin improves overall survival in people with malignant pleural mesothelioma. *Cancer Treat. Rev.*, **2006**, *32*, 229-233.

[74] Mendelsohn, L.G.; Shih, C.; Chen, V.J.; Habeck, L.L.; Gates, S.B.; Shackelford, K.A. Enzyme inhibition, polyglutamation and the effect of LY231514 (MTA) on purine biosynthesis. *Semin. Oncol.*, **1999**, *26*, 42-47.

[75] Shih, C.; Chen, V.J.; Gossett, L.S.; Gates, S.B.; MacKellar, W.C.; Habeck, L.L.; Shackelford, K.A.; Mendelsohn, L.G.; Soose, D.J.; Patel, V.F.; Andis, S.L.; Bewley, J.R.; Rayl, E.A.; Moroson, B.A.; Beardsley, G.P.; Kohler, W.; Ratnam, M.; Schultz, R.M. LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate- requiring enzymes. *Cancer Res.*, **1997**, *57*, 1116-1123.

[76] Taylor, E.C.; Harrington, P.J.; Fletcher, S.R.; Beardsley, G.P.; Moran, R.G. Synthesis of the antileukemic agents 5,10-dideazaaminopterin and 5,10-dideaza-5,6,7,8- tetrahydroaminopterin. *J. Med. Chem.*, **1985**, *28*, 914-921.

[77] Taylor, E.C.; Kuhnt, D.; Shih, C.; Rinzel, S.M.; Grindey, G.B.; Barredo, J.; Jannatipour, M.; Moran, R.G. A dideazatetrahydrofolate analogue lacking a chiral center at C-6, N-[4- 2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl[benzoyl]-L-glutamic acid, is an inhibitor of thymidylate synthase. *J. Med. Chem.*, **1992**, *35*, 4450- 4454.

[78] Chattopadhyay, S.; Moran, R.G.; Goldman, I.D. Pemetrexed: biochemical and cellular pharmacology, mechanisms, and clinical applications. *Mol. Cancer Ther.*, **2007**, *6*, 404- 417.

[79] Zhao, R.; Hanscom, M.; Chattopadhyay, S.; Goldman, I.D. Selective preservation of pemetrexed pharmacological activity in HeLa cells lacking the reduced folate carrier; association with the presence of a secondary transport pathway. *Cancer Res.*, **2004**, *64*, 3313-3319.

[80] Chattopadhyay, S.; Wang, Y.; Zhao, R.; Goldman, I.D. Lack of impact of the loss of constitutive folate receptor alpha expression, achieved by RNA interference, on the activity of the new generation antifolate Pemetrexed in HeLa cells. *Clin. Cancer Res.*, **2004**, *10*, 7986-7993.

[81] Habeck, L.L.; Mendelsohn, L.G.; Shih, C.; Taylor, E.C.; Colman, P.D.; Gossett, L.S.; Leitner, T.A.; Schultz, R.M.; Andis, S.L.; Moran, R.G. Substrate specificity of mammalian folylpolyglutamate synthetase for 5,10-dideazatetrahydrofolate analogs. *Mol. Pharmacol.*, **1995**, *48*, 326-333.

[82] Zhao, R.; Gao, F.; Goldman, I.D. Marked suppression of the activity of some, but not all, antifolate compounds by augmentation of folate cofactor pools within tumor cells. *Biochem. Pharmacol.*, **2001**, *61*, 857-865.

[83] Chen, V.J.; Bewley, J.R.; Andis, S.L.; Schultz, R.M.; Iversen, P.W.; Shih, C.; Mendelsohn, L.G.; Seitz, D.E.; Tonkinson, J.L. Preclinical cellular pharmacology of LY231514 (MTA): a comparison with methotrexate, LY309887 and raltitrexed for their effects on intracellular folate and nucleoside triphosphate pools in CCRF-CEM cells. *Br. J. Cancer*, **1998**, *78*(Suppl. 3), 27-34.

[84] Sayre, P.H.; Finer-Moore, J.S.; Fritz, T.A.; Biermann, D.; Gates, S.B.; MacKellar, W.C.; Patel, V.F.; Stroud, R.M. Multi-targeted antifolates aimed at avoiding drug resistance from covalent closed inhibitory complexes with human and Escherichia coli thymidylate synthases. *J. Mol. Biol.*, **2001**, *313*, 813-829.

[85] Hazarika, M.; White, R.M.; Johnson, J.R.; Pazdur, R. FDA drug approval summaries: Pemetrexed (ALIMTA). *Oncologist*, **2004**, *9*, 482-488.

[86] Muhsin, M.; Gricks, C.; Kirkpatrick, P. Pemetrexed disodium. *Nat. Rev. Drug Discov.*, **2004**, *3*, 825-826.

[87] Hsu, J.Y.; Wakelee, H. Pemetrexed disodium for the treatment of NSCLC: an update. *Drugs Today (Barc)*, **2008**, *44*, 669-678.

[88] Owonikoko, T.K.; Ramalingam, S.S.; Belani, C.P. Maintenance therapy for advanced non-small cell lung cancer: current status, controversies, and emerging consensus. *Clin. Cancer Res.*, **2010**, *16*, 2496-2504.

[89] National Institute for Health and Clinical Excellence (NICE) Pemetrexed for the first- line treatment of non-small-cell lung cancer. Technology appraisal guidance 181; London, UK, September **2009**.

[90] National Institute for Health and Clinical Excellence (NICE) Pemetrexed for the maintenance treatment of non-small-cell lung cancer. Final appraisal determination; London, UK, March **2010**.

[91] National Institute for Health and Clinical Excellence (NICE) Pemetrexed for the treatment of malignant pleural mesothelioma. Technology appraisal guidance 135; London, UK, January **2008**.

[92] Adjei, A.A. Clinical studies of Pemetrexed and gemcitabine combinations. *Ann. Oncol.*, **2006**, *17*(Suppl. 5), v29-v32.

[93] Adjei, A.A. Pemetrexed (ALIMTA), a novel multitargeted antineoplastic agent. *Clin. Cancer Res.*, **2004**, *10*, 4276s-4280s.

[94] Editorial; Positioning Pemetrexed in the treatment of ovarian cancer. *Eur. J. Cancer*, **2009**, *45*, 1330-1332, and references therein.

[95] Tomao, F.; Panici, P.B.; Frati, L.; Tomao, S. Emerging role of Pemetrexed in ovarian cancer. *Exp. Rev. Anticancer Ther.*, **2009**, *9*, 1727-1735.

[96] Adjei, A.A. Pharmacology and mechanism of action of Pemetrexed. *Clin. Lung Cancer*, **2004**, *5*(Suppl. 2), S51-S55.

[97] Scagliotti, G.V.; Shin, D.M.; Kindler, H.L.; Vasconcelles, M.J.; Keppler, U.; Manegold, C.; Burris, H.; Gatzemeier, U.; Blatter, J.; Symanowski, J.T.; Rusthoven, J.J. Phase II study of Pemetrexed with and without folic acid and vitamin B12 as front-line therapy in malignant pleural mesothelioma. *J. Clin. Oncol.*, **2003**, *21*, 1556-1561.

[98] Vogelzang, N.J.; Rusthoven, J.J.; Symanowski, J.; Denham, C.; Kaukel, E.; Ruffie, P.; Gatzemeier, U.; Boyer, M.; Emri, S.; Manegold, C.; Niyikiza, C.; Paoletti, P. Phase III study of Pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. *J. Clin. Oncol.*, **2003**, *21*, 2636-2644.

[99] Green, J.; Dundar, Y.; Dodd, S.; Dickson, R.; Walley, T. Pemetrexed disodium in combination with cisplatin versus other cytotoxic agents or supportive care for the treatment of malignant pleural mesothelioma. *Cochrane Database Syst. Rev.*, **2007**, *1*, CD005574.

[100] Lorusso, D.; Ferrandina, G.; Pignata, S.; Ludovisi, M.; Vigano, R.; Scalone, S.; Scollo, P.; Breda, E.; Pietragalla, A.; Scambia, G. Evaluation of Pemetrexed (ALIMTA, LY231514) as second-line chemotherapy in persistent or recurrent carcinoma of the cervix: the CERVIX 1 study of the MITO (Multicentre Italian Trials in Ovarian Cancer and Gynecologic Malignancies) Group. *Ann. Oncol.*, **2010**, *21*, 61-66.

[101] Miller, D.S.; Blessing, J.A.; Drake, R.D.; Higgins, R.; McMeekin, D.S.; Puneky, L.V.; Krasner, C.N. A phase II evaluation of Pemetrexed (ALIMTA, LY231514, IND #40061) in the treatment of recurrent or persistent endometrial carcinoma: a phase II study of the gynecologic oncology. *Gynecol. Oncol.*, **2009**, *115*, 443-446.

[102] Miller, D.S.; Blessing, J.A.; Krasner, C.N.; Mannel, R.S.; Hanjani, P.; Pearl, M.L.; Waggoner, S.E.; Boardman, C.H. Phase II evaluation of Pemetrexed in the treatment of recurrent or persistent platinum-resistant ovarian or primary peritoneal carcinoma: a study of the gynecologic oncology group. *J. Clin. Oncol.*, **2009**, *27*, 2686-2691.

[103] Kim, Y.H.; Chung, H.C.; Kang, W.K.; Park, S.R.; Kim, C.S.; Kim, T.Y.; Shin, S.W.; Park, B.J.; Cha, S.J.; Bang, Y.J. Pemetrexed and cisplatin in patients with advanced gastric cancer: a Korean cancer study group multicentre phase II study. *Cancer Chemother. Pharmacol.*, **2008**, *62*, 263-270.

[104] Celio, L.; Sternberg, C.N.; Labianca, R.; La Torre, I.; Amoroso, V.; Barone, C.; Pinotti, G.; Cascinu, S.; Di Costanzo, F.; Cetto, G.L.; Bajetta, E. Pemetrexed in combination with oxaliplatin as a first-line therapy for advanced gastric cancer: a multi-institutional phase II study. *Ann. Oncol.*, **2009**, *20*, 1062-1067.

[105] Dreicer, R.; Li, H.; Cooney, M.M.; Wilding, G.; Roth, B.J. Phase II trial of Pemetrexed disodium and gemcitabine in advanced urothelial cancer (E4802): a trial of the Eastern Cooperative Oncology Group. *Cancer*, **2008**, *112*, 2671-2675.

[106] Miller, D.S.; Blessing, J.A.; Bodurka, D.C.; Bonebrake, A.J.; Schorge, J.O.; Gynecologic Oncology Group. Evaluation of Pemetrexed (ALIMTA, LY231514) as second line chemotherapy in persistent or recurrent carcinoma of the cervix: a phase II study of the Gynecologic Oncology Group. *Gynecol. Oncol.*, **2008**, *110*, 65-70.

[107] Dean-Colomb, W.; Esteva, F.J. Emerging agents in the treatment of anthracycline- and taxane-refractory metastatic breast cancer. *Semin. Oncol.*, **2008**,. *35*(2 Suppl. 2), S31- S38.

[108] Scagliotti, G.; Hanna, N.; Fossella, F.; Sugarman, K.; Blatter, J.; Peterson, P.; Simms, L.; Shepherd, F.A. The differential efficacy of Pemetrexed according to NSCLC histology: a review of two phase III studies. *Oncologist*, **2009**, *14*, 253-263.

[109] Gronberg, B.H.; Bremnes, R.M.; Flotten, O.; Amundsen, T.; Brunsvig, P.Fr.; Hjelde, H.H.; Kaasa, S.; von Plessen, C.; Stornes, F.; Tollali, T.; Wammer, F.; Aasebo, U.; Sundstrom, S. Phase III study by the Norwegian Lung Cancer Study Group: Pemetrexed plus carboplatin compared with gemcitabine plus carboplatin as first-line chemotherapy in advanced non-small-cell lung cancer. *J. Clin. Oncol.*, **2009**, *27*, 3217-3224.

[110] Jackman, A.L.; Kimbell, R.; Aherne, G.W.; Brunton, L.; Jansen, G.; Stephens, T.C.; Smith, M.N.; Wardleworth, J.M.; Boyle, F.T. Cellular pharmacology and *in vivo* activity of a new anticancer agent, ZD9331: a water-soluble, nonpolyglutamatable, quinazoline-based inhibitor of thymidylate synthase. *Clin. Cancer Res.*, **1997**, *3*, 911-921.

[111] Jackman, A.L.; Kimbell, R.; Brown, M.; Brunton, L.; Harrap, K.R.; Wardleworth, J.M.; Boyle, F.T. The antitumour activity of ZD9331, a non-polyglutamatable quinazoline thymidylate synthase inhibitor. *Adv. Exp. Med. Biol.*, **1994**, *370*, 185-188.

[112] Benepal, T.S.; Judson, I. ZD9331: discovery to clinical development. *Anticancer Drugs*, **2005**, *16*, 1-9.

[113] Niculescu-Duvaz, I. ZD9331 AstraZeneca. *Curr. Opin. Investig. Drugs*, **2000**, *1*, 141- 149.

[114] Theti, D.S.; Jackman, A.L. The role of α-folate receptor-mediated transport in the antitumor activity of antifolate drugs. *Clin. Cancer Res.*, **2004**, *10*, 1080-1089.

[115] Jackman, A.L.; Theti, D.S.; Gibbs, D.D. Antifolates targeted specifically to the folate receptor. *Adv. Drug Deliv. Rev.*, **2004**, *56*, 1111-1125.

[116] Rader, J.S.; Clarke-Pearson, D.; Moore, M.; Carson, L.; Holloway, R.; Kao, M.-S.; Wiznitzer, I.; Douglass, E.C. A phase II study to determine the efficacy and tolerability of intravenous ZD9331 in heavily pretreated patients with ovarian cancer. *Gynecol. Oncol.*, **2003**, *91*, 318-325.

[117] Benson, A.; Poplin, E.; Vergote, I. ZD9331 in combination with topotecan: phase I and II experience. *Anticancer Drugs*, **2003**, *14 Suppl. 1*, S21-S27.

[118] Plummer, R.; Rees, C.; Hughes, A.; Beale, P.; Highley, M.; Trigo, J.; Sathyarathnarn, G.; Judson, I.; Calvert, H.; Jackman, A.; Mitchell, F.; Smith, R.; Douglass, E. A phase I trial of ZD9331, a water-soluble, nonpolyglutamatable, thymidylate synthase inhibitor. *Clin. Cancer Res.*, **2003**, *9*, 1313-1322.

[119] de Jonge, M.J.A.; Punt, C.J.A.; Sparreboom, A.; Planting, A.S.T.; Peters, M.E.W.J.; van de Schraaf, J.; Jackman, A.; Smith, R.; de Mulder, P.H.M.; Verweij, J. Phase I and pharmacologic study of oral ZD9331, a novel nonpolyglutamated thymidylate synthase inhibitor, in adult patients with solid tumors. *J. Clin. Oncol.*, **2002**, *20*, 1923-1931.

[120] Bilenker, J.H.; Stevenson, J.P.; Flaherty, K.T.; Algazy, K.; McLaughlin, K.; Haller, D.G.; Giantonio, B.J.; Koehler, M.; Garcia-Vargas, J.E.; O'Dwyer, P.J. Phase I trial of the antifolate ZD9331 in combination with cisplatin in patients with refractory solid malignancies. *Cancer Chemother. Pharmacol.*, **2004**, *53*, 357-360.

[121] Petruzelka, L. Phase II multicentre trial of ZD9331 monotherapy as first-line treatment for gastric cancer. *Anticancer Drugs*, **2003**, *14 Suppl. 1*, S7-S12.

[122] Hainsworth, J.; Vergote, I.; Janssens, J. A review of phase II studies of ZD9331 treatment for relapsed or refractory solid tumors. *Anticancers Drugs*, **2003**, *14*(Suppl. 1), S13-S19.

[123] Koizumi, W.; Aiba, K.; Sasaki, T.; Sato, A.; Horikoshi, N. Phase I dose-escalation trial of ZD9331 in Japanese patients with refractory, solid malignancies. *Anticancer Drugs*, **2003**, *14*(Suppl. 1), S1-S5.

[124] Schwartz, G.H.; Jones, C.B.; Garrison, M.; Patnaik, A.; Takimoto, C.; McCreery, H.; Skinner, M.; Tolcher, A.W.; Rowinsky, E.K. A phase I and pharmacokinetic study of the nonpolyglutamatable thymidylate synthase inhibitor ZD9331 plus docetaxel in patients with advanced solid malignancies. *Invest. New Drugs*, **2004**, *22*, 437-448.

[125] Benepal, T.; Jackman, A.; Pyle, L.; Bate, S.; Hardcastle, A.; Aherne, W.; Mitchell, F.; Simmons, L.; Ruddle, R.; Raynaud, F.; Gore, M. A phase I pharmacokinetic and pharmacodynamic study of BGC9331 and carboplatin in relapsed gynaecological malignancies. *Br. J. Cancer*, **2005**, *93*, 868-875.

[126] Smith, D.; Gallagher, N. A phase II/III study comparing intravenous ZD9331 with gemcitabine in patients with pancreatic cancer. *Eur. J. Cancer*, **2003**, *39*, 1377-1383.

[127] Taylor, J. New FDA Orphan Drugs: Leukocyte Interleukin, Pafuramidine Maleate, Plevitrexed. *Medscape Today*, **2007**, http://www.medscape.com/viewarticle/558340

[128] Duch, D.S.; Banks, S.; Dev, I.K.; Dickerson, S.H.; Ferone, R.; Heath, L.S.; Humphreys, J.; Knick, V.; Pendergast, W.; Singer, S.; Smith, G.K.; Waters, K.; Wilson, H.R. Biochemical and cellular pharmacology of 1843U89, a novel benzoquinazoline inhibitor of thymidylate synthase. *Cancer Res.*, **1993**, *53*, 810-818.

[129] Dev, I.K.; Dallas, W.S.; Ferone, R.; Hanlon, M.; McKee, D.D.; Yates, B.B. Mode of binding of folate analogs to thymidylate synthase. *J. Biol. Chem.*, **1994**, *269*, 1873-1882.

[130] Hanlon, M.H.; Ferone, R. *In vitro* uptake, and cellular retention of 1843U89 and other benzoquinazoline inhibitors of thymidylate synthase. *Cancer Res.*, **1996**, *56*, 3301-3306.

[131] Kisliuk, R.L. Deaza analogs of folic acid as antitumor agents. *Curr. Pharm. Des.*, **2003**, *9*, 2615-2625.

[132] Touroutoglou, N.; Pazdur, R. Thymidylate synthase inhibitors. *Clin. Cancer Res.*, **1996**, *2*, 227-243.

[133] McGuire, J.J. Anticancer antifolates: current status and future directions. *Curr. Pharm. Des.*, **2003**, *9*, 2593-2613.

[134] Stout, T.J.; Stroud, R.M. The complex of the anticancer therapeutic, BW1843U89, with thymidylate synthase at 2.0 Å resolution: implications for a new mode of inhibition. *Structure*, **1996**, *4*, 67-77.

[135] Weichsel, A.; Montfort, W.R. Ligand-induced distortion of an active site in thymidylate synthase upon binding anticancer drug 1843U89. *Nat. Struct. Biol.*, **1995**, *2*, 1095- 1101.

[136] Montfort, W.R.; Weichsel, A. Thymidylate synthase: structure, inhibition, and strained conformation during catalysis. *Pharmacol. Ther.*, **1997**, *76*, 29-43.

[137] Banks, S.D.; Waters, K.A.; Barrett, L.L.; Dickerson, S.; Pendergast, W.; Smith, G.K. Destruction of WiDr multicellular tumor spheroids with the novel thymidylate synthase inhibitor 1843U89 at physiological thymidine concentrations. *Cancer Chemother. Pharmacol.*, **1994**, *33*, 455-459.

[138] Boytos, C.M.; Ferone, R.; Jones, W.A.; Rudolph, S.K.; Franklin, S.L.; Houle, C.D. *In vivo* antitumor activity of 1843U89 against experimental liver metastases in the nude mouse. *Proc. Am. Assoc. Cancer Res.*, **1995**, *36*: 376.

[139] Smith, G.K.; Amyx, H.; Boytos, C.M.; Duch, D.S.; Ferone, R.; Wilson, H.R. Enhanced antitumor activity for the thymidylate synthase inhibitor 1843U89 through decreased host toxicity with oral folic acid. *Cancer Res.*, **1995**, *55*, 6117-6125.

[140] Smith, G.K.; Bigley, J.W.; Dev, I.K.; Duch, D.S.; Ferone, R.; Pendergast, W. GW1843, a potent, noncompetitive thymidylate synthase inhibitor – preclinical and preliminary clinical studies. In *Antifolate drugs in cancer Therapy*, Jackman, A.L., Ed.; Humana Press, Inc.: Totowa, NJ, **1999**, pp. 203-227.

[141] Schwartz, G.; Johnson, T.R.; Goetz, A.; Burris, H.; Smetzer, L.; Lampkin, T.; Sailstad, J.; Hohneker, J.A.; Von Hoff, D.D.; Rowinsky, E.K. A phase I and pharmacokinetic study of 1843U89, a noncompetitive inhibitor of thymidylate synthase, in patients with advanced solid malignancies. *Clin. Cancer Res.*, **2001**, *7*, 1901-1911.

[142] Desjardins, J.; Emerson, D.L.; Colagiovanni, D.B.; Abbott, E.; Brown, E.N.; Drolet, D.W. Pharmacokinetics, safety, and efficacy of a liposome encapsulated thymidylate synthase inhibitor, OSI-7904L [(S)-2-[5-[(1,2-dihydro-3-methyl-1- oxobenzo[f]quinazoline-9-yl)methyl]amino-1-oxo-2-isoindolynl]-glutaric acid] in mice. *J. Pharmacol. Exp. Ther.*, **2004**, *309*, 894-902.

[143] Beutel, G.; Glen, H.; Schöffski, P.; Chick, J.; Gill, S.; Cassidy, J.; Twelves, C. Phase I study of OSI-7904L, a novel liposomal thymidylate synthase inhibitor in patients with refractory solid tumors. *Clin. Cancer Res.*, **2005**, *11*, 5487-5495.

[144] Ricart, A.D.; Berlin, J.D.; Papadopoulos, K.P.; Syed, S.; Drolet, D.W.; Quaratino- Baker, C.; Horan, J.; Chick, J.; Vermeulen, W.; Tolcher, A.W.; Rowinsky, E.K.; Rothenberg, M.L. Phase I, pharmacokinetic and biological correlative study of OSI- 7904L, a novel liposomal thymidylate synthase inhibitor, and cisplatin in patients with solid tumors. *Clin. Cancer Res.*, **2008**, *14*, 7947-7955.

[145] Falk, S.; Anthoney, A.; Eatock, M.; Van Cutsem, E.; Chick, J.; Glen, H.; Valle, J.W.; Drolet, D.W.; Albert, D.; Ferry, D.; Ajani, J. Multicentre phase II pharmacokinetic and pharmacodynamic study of OSI-7904L in previously untreated patients with advanced gastric or gastroesophageal junction adenocarcinoma. *Br. J. Cancer*, **2006**, *95*, 450-456.

[146] Ciuleanu, T.; Diculescu, M.; Hoepffner, N.M.; Trojan, J.; Sailer, V.; Zalupski, M.; Herrmann, T.; Roth, A.; Chick, J.; Brock, K.; Albert, D.; Philip, P.A. A randomised phase II study of OSI-7904L versus 5-fluorouracil (FU)/leucovorin (LV) as first-line treatment in patients with advanced biliary cancers. *Invest. New Drugs*, **2007**, *25*, 385- 390.

[147] Clamp, A.R.; Schöffski, P.; Valle, J.W.; Wilson, R.H.; Marreaud, S.; Govaerts, A.S.; Debois, M.; Lacombe, D.; Twelves, C.; Chick, J.; Jayson, G.C.; EORTC New Drug Development Group. A phase I and pharmacokinetic study of OSI-7904L, a liposomal thymidylate synthase inhibitor in combination with oxaliplatin in patients with advanced colorectal cancer. *Cancer Chemother. Pharmacol.*, **2008**, *61*, 579-585.

[148] Hough, B.; Posner, M.; Chung, C.; Hainsworth, J.; Horan, J.; Chick, J.; Gibson, M.K.; University of Pittsburgh Medical Center, Pittsburgh, PA; Dana-Farber Cancer Institute, Boston, MA; Vanderbilt University, Nashville, TN; Sarah Cannon Research Institute, Nashville, TN; OSI Pharmaceuticals, Oxford, United Kingdom. A phase II study of single agent OSI-7904L in patients with metastatic or recurrent squamous cell carcinoma of the head and neck (SCCHN). *J. Clin. Oncol.*, **2009**, *27 (suppl; abstr e17005)*.

[149] Appelt, K.; Bacquet, R.J.; Bartlett, C.A.; Booth, C.L.J.; Freer, S.T.; Fuhry, M.A.M.; Gehring, M.R.; Herrmann, S.M.; Howland, E.F.; Janson, C.A.; Jones, T.R.; Kan, C.-C.; Kathardekar, V.; Lewis, K.K.; Marzoni, G.P.; Matthews, D.A.; Mohr, C.; Moomaw, E.W.; Morse, C.A.; Oatley, S.J.; Ogden, R.C.; Rami Reddy, M.; Reich, S.H.; Schoettlin, W.S.; Smith, W.W.; Varney, M.D.; Villafranca, J.E.; Ward, R.W.; Webber, S.; Webber, S.E.; Welsh, K.M.; White, J. Design of enzyme inhibitors using iterative protein crystallographic analysis. *J. Med. Chem.*, **1991**, *34*, 1925-1934.

[150] Varney, M.D.; Marzoni, G.P.; Palmer, C.L.; Deal, J.G.; Webber, S.; Welsh, K.M.; Bacquet, R.J.; Bartlett, C.A.; Morse, C.A.; Booth, C.L.J.; Herrmann, S.M.; Howland, E.F.; Ward, R.W.; White, J. Crystal-structure-based design and synthesis of benz[cd]indole-containing inhibitors of thymidylate synthase. *J. Med. Chem.*, **1992**, *35*, 663-676.

[151] Reich, S.H.; Fuhry, M.A.M.; Nguyen, D.; Pino, M.J.; Welsh, K.M.; Webber, S.; Janson, C.A.; Jordan, S.R.; Matthews, D.A.; Smith, W.W.; Bartlett, C.A.; Booth, C.L.J.; Herrmann, S.M.; Howland, E.F.; Morse, C.A.; Ward, R.W.; White, J. Design and synthesis of novel 6,7-imidazotetrahydroquinoline inhibitors of thymidylate synthase using iterative protein crystal structure analysis. *J. Med. Chem.*, **1992**, *35*, 847-858.

[152] Webber, S.E.; Bleckman, T.M.; Attard, J.; Deal, J.G.; Kathardekar, V.; Welsh, K.M.; Webber, S.; Janson, C.A.; Matthews, D.A.; Smith, W.W.; Freer, S.T.; Jordan, S.R.; Bacquet, R.J.; Howland, E.F.; Booth, C.L.J.; Ward, R.W.; Herrmann, S.M.; White, J.; Morse, C.A.; Hilliard, J.A.; Bartlett, C.A. Design of thymidylate synthase inhibitors using protein crystal structures: the synthesis and biological evaluation of a novel class of 5-substituted quinazolinones. *J. Med. Chem.*, **1993**, *36*, 733-746.

[153] Jones, T.R.; Varney, M.D.; Webber, S.E.; Lewis, K.K.; Marzoni, G.P.; Palmer, C.L.; Kathardekar, V.; Welsh, K.M.; Webber, S.; Matthews, D.A.; Appelt, K.; Smith, W.W.; Janson, C.A.; Villafranca, J.E.; Bacquet, R.J.; Howland, E.F.; Booth, C.L.J.; Herrmann, S.M.; Ward, R.W.; White, J.; Moomaw, E.W.; Bartlett, C.A.; Morse, C.A. Structure- based design of lipophilic quinazoline inhibitors of thymidylate synthase. *J. Med. Chem.*, **1996**, *39*, 904-917.

[154] Webber, S.; Bartlett, C.A.; Boritzki, T.J.; Hilliard, J.A.; Howland, E.F.; Johnston, A.L.; Kosa, M.; Margosiak, S.A.; Morse, C.A.; Shetty, B.V. AG337, a novel lipophilic thymidylate synthase inhibitor: *in vitro* and *in vivo* preclinical studies. *Cancer Chemother. Pharmacol.*, **1996**, *37*, 509-517.

[155] O'Connor, B.M.; Webber, S.; Jackson, R.C.; Galivan, J.; Rhee, M.S. Biological activity of a novel rationally designed lipophilic thymidylate synthase inhibitor. *Cancer Chemother. Pharmacol.*, **1994**, *34*, 225-229.

[156] McGuire, J.J.; Magee, K.J.; Russell, C.A.; Canestrari, J.M. Thymidylate synthase as a target for growth inhibition in methotrexate-sensitive and -resistant human head and neck cancer and leukemia cell lines. *Oncol. Res.*, **1997**, *9*, 139-147.

[157] McGuire, J.J.; Canestrari, J.G.; Nagel, G.S. Characterization of the effect of AG337, a novel lipophilic thymidylate synthase inhibitor, on human head and neck and human leukemia cell lines. *Int. J. Oncol.*, **1999**, *15*, 1245-1250.

[158] O'Dwyer, P.J.; Laub, P.B.; DeMaria, D.; Qian, M.; Reilly, D.; Giantonio, B.; Johnston, A.L.; Wu, E.Y.; Bauman, L.; Clendeninn, N.J.; Gallo, J.M. Phase I trial of the thymidylate synthase inhibitor AG331 as a 5-day continuous infusion. *Clin. Cancer Res.*, **1996**, *2*, 1685-1692.

[159] Koda, R.T.; Garcia, A.A.; Chatterjee, D.J.; Li, W.Y.; Parimoo, D.; Jeffers, S.; Rogers, M.; Leichman, C.G.; Leichman, L.; Wu, E.Y.; Shetty, B.V.; Webber, S.; Clendinnin, N.; Muggia, F.M. Phase I study of AG331, a novel thymidylate synthase inhibitor, in patients with refractory solid tumors. *Cancer Chemother. Pharmacol.*, **1999**, *43*, 489- 496.

[160] Estlin, E.J.; Balmanno, K.; Calvert, A.H.; Hall, A.G.; Lunec, J.; Newell, D.R.; Pearson, A.D.; Taylor, G.A. The relationship between intrinsic thymidylate synthase expression and sensitivity to THYMITAQ in human leukaemia and colorectal carcinoma cell lines. *Br. J. Cancer*, **1997**, *76*, 1579-1585.

[161] Rafi, I.; Taylor, G.A.; Calvete, J.A.; Boddy, A.V.; Balmanno, K.; Bailey, N.; Lind, M.; Calvert, A.H.; Webber, S.; Jackson, R.C. Clinical pharmacokinetic and pharmacodynamic studies with the non classical antifolate thymidylate synthase inhibitor 3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)-quinazolone dihydrochloride (AG337) given by 24-hour continuous intravenous infusion. *Clin. Cancer Res.*, **1995**, *1*, 1275-1284.

[162] Rafi, I.; Boddy, A.V.; Calvete, J.A.; Taylor, G.A.; Newell, D.R.; Bailey, N.P.; Lind, M.J.; Green, M.; Hines, J.; Johnstone, A.; Clendeninn, N.; Calvert, A.H. Preclinical and phase I clinical studies with the non-classical antifolate thymidylate synthase inhibitor nolatrexed dihydrochloride given by prolonged administration in patients with solid tumors. *J. Clin. Oncol.*, **1998**, *16*, 1131-1141.

[163] Creaven, P.J.; Pendyala, L.; Meropol, N.J.; Clendeninn, N.J.; Wu, E.Y.; Loewen, G.M.; Proefrock, A.; Johnston, A.; Dixon, M. Initial clinical trial and pharmacokinetics of Thymitaq (AG337) by 10-day

continuous infusion in patients with advanced solid tumors. *Cancer Chemother. Pharmacol.*, **1998**, *41*, 167-170.

[164] Hughes, A.N.; Rafi, I.; Griffin, A.J.; Calvert, A.H.; Newell, D.R.; Calvete, J.A.; Johnston, A.; Clendeninn, N.J; Boddy, A.V. Phase I studies with the non-classical antifolate nolatrexed dihydrochloride (AG337, Thymitaq) administered orally for 5 days. *Clin. Cancer Res.*, **1999**, *5*, 111-118.

[165] Jodrell, D.I.; Bowman, A.; Rye, R.; Byrne, B.; Boddy, A.V.; Rafi, I.; Taylor, G.A.; Johnston, A.; Clendeninn, N.J. A phase I study of the lipophilic thymidylate synthase inhibitor Thymitaq (nolatrexed dihydrochloride) given by 10-day oral administration. *Br. J. Cancer*, **1999**, *79*, 915-920.

[166] Belani, C.P.; Agarwala, S.; Johnson, J.; Cohn, A.; Bernstein, J.; Langer, C.; Jones, V.; White, C.; Loh, K.; Whiter, D.; Chew, T.; Johnston, A.; Clendeninn, N. A phase II trial with Thymitaq (AG337) in patients with squamous cell carcinoma of the head and neck. *Proc. Am. Soc. Clin. Oncol.*, **1997a**, *16*, 387a.

[167] Stuart, K.; Tessitore, J.; Rudy, J.; Clendeninn, N.; Johnston, A. A phase II trial of nolatrexed dihydrochloride in patients with advanced hepatocellular carcinoma. *Cancer*, **1999**, *86*, 410-414.

[168] Hughes, A.; Calvert, A.H. Preclinical and clinical studies with the novel thymidylate synthase inhibitor nolatrexed dihydrochloride (Thymitaq, AG337). In *Anticancer Drug Development Guide: Antifolate Drugs in Cancer Therapy*, Jackman, A.L., Ed.; Humana Press, **1999**, pp. 229-241.

[169] Gish, R.G.; Porta, C.; Lazar, L.; Ruff, P.; Feld, R.; Croitoru, A.; Feun, L.; Jeziorski, K.; Leighton, J.; Knox, J.; Gallo, J.; Kennealey, G.T. Phase III randomized controlled trial comparing the survival of patients with unresectable hepatocellular carcinoma treated with Nolatrexed or Doxorubicin. *J. Clin. Oncol.*, **2007**, *25*, 3069-3075.

[170] Mok, T.S.; Leung, T.W.; Lee, S.D.; Chao, Y.; Chan, A.T.; Huang, A.; Lui, M.C.; Yeo, W.; Chak, K.; Johnston, A.; Johnson, P. A multi-centre randomized phase II study of nolatrexed versus doxorubicin in treatment of Chinese patients with advanced hepatocellular carcinoma. *Cancer Chemother. Pharmacol.*, **1999**, *44*, 307-311.

[171] Jhaver, M.; Rosen, L.; Dancey, J.; Hochster, H.; Hamburg, S.; Tempero, M.; Clendeninn, N.; Mani, S. Phase II trial of nolatrexed dihydrochloride (Thymitaq, AG337) in patients with advanced hepatocellular carcinoma. *Invest. New Drugs*, **2006**, *25*, 85-94.

[172] Pivot, X.; Wadler, S.; Kelly, C.; Ruxer, R.; Tortochaux, J.; Stern, J.; Belpomme, D.; Humblet, Y.; Domenge, C.; Clendeninn, N.; Johnston, A.; Penning, C.; Schneider, M. Results of two randomized trials comparing nolatrexed (Thymitaq) *versus* methotrexate in patients with recurrent head and neck cancer. *Ann. Oncol.*, **2001**, *12*, 1595-1599.

[173] Gibbs, D.D.; Theti, D.S.; Wood, N.; Green, M.; Raynaud, F.; Valenti, M.; Forster, M.D.; Mitchell, F.; Bavetsias, V.; Henderson, E.; Jackman, A.L. BGC 945, a novel tumor-selective thymidylate synthase inhibitor targeted to α-folate receptor- overexpressing tumors. *Cancer Res.*, **2005**, *65*, 11721-11728.

[174] Pillai, R.G.; Forster, M.; Perumal, M.; Mitchell, F.; Leyton, J.; Aibgirhio, F.I.; Golovko, O.; Jackman, A.L.; Aboagye, E.O. Imaging pharmacodynamics of the α-folate receptor- targeted thymidylate synthase inhibitor BGC 945. *Cancer Res.*, **2008**, *68*, 3827-3834.

[175] Bavetsias, V.; Jackman, A.L.; Kimbell, R.; Gibson, W.; Boyle, F.T.; Bisset, G.M.F. Quinazoline antifolate thymidylate synthase inhibitors: γ-linked L-D, D-D and D-L dipeptide analogues of 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI 198583). *J. Med. Chem.*, **1996**, *39*, 73-85.

[176] Jodrell, D.I.; Gibson, W.; Bissett, G.M.F.; Boyle, F.T.; Judson, I.R.; Jackman, A.L. The *in vivo* metabolic stability of dipeptide analogues of the quinazoline antifolate, ICI 198583, in mice. *Biochem. Pharmacol.*, **1993**, *46*, 2229-2234.

[177] Thomas, R.J.; Williams, M.; Garcia-Vargas, J. Lessons learned from raltitrexed – quality assurance, patient education and intensive supportive drugs to optimize tolerability. *Clin. Oncol.*, **2003**, *15*, 227-232.

[178] Walling, J. From methotrexate to pemetrexed and beyond. A review of the pharmacodynamic and clinical properties of antifolates. *Invest. New Drugs*, **2006**, *24*, 37-77.

[179] Assaraf, Y.G. Molecular basis of antifolate resistance. *Cancer Metastasis Rev.*, **2007**, *26*, 153-181.

[180] Gangjee, A.; Jain, H.D.; Kurup, S. Recent advances in classical and non-classical antifolates as antitumor and antiopportunistic infection agents: part 1. *Anticancer Agents Med. Chem.*, **2007**, *7*, 524-542.

[181] Gangjee, A.; Jain, H.D.; Kurup, S. Recent advances in classical and non-classical antifolates as antitumor and antiopportunistic infection agents: part 2. *Anticancer Agents Med. Chem.*, **2008**, *8*, 205-231.

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