

Review

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Acetylenic antifolates as anticancer agents

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Abstract: Folates are crucial cofactors involved in the *de novo* generation of purine and deoxythymidine monophosphate, which are essential for DNA synthesis. Antifolates are structural analogues of folate derivatives that act as inhibitors of folate-dependent enzymes and constitute the oldest antimetabolite class of anticancer agents. This review focuses on antifolates with remarkable anticancer activities that include a terminal alkyne function in their molecular structure. The properties of CB3717, a tremendous inhibitor of thymidylate synthase, are described, and the development of raltitrexed and pralatrexate, a dihydrofolate reductase inhibitor approved by the U.S. Food and Drug Administration (FDA) as the first drug for the treatment of relapsed and refractory peripheral T cell lymphoma are presented.

Keywords: antifolate; cancer; CB3717; pralatrexate; raltitrexed; terminal alkyne.

Introduction

Folates, composed of a pterin ring coupled with *p*-aminobenzoate and glutamate moieties (Figure 1A), are cofactors of enzymes involved in DNA/RNA syntheses and methylation processes. The endogenous synthesis of these molecules is only possible in bacteria; thus they are provided to humans by food intake. Given the vital

importance of folates in cellular mechanisms, health authorities have underlined their absolute necessity of absorption, issuing notifications for recommended daily intake ratios [1–3]. This family of compounds can exist in either an oxidized form or a reduced dihydrofolate (DHF) or tetrahydrofolate (THF) form (Figure 1B). THF and its derivatives include two chiral centers (C6 and C α), and natural THF and its 5-substituted derivatives consist of the (6S, α S) diastereomers, and due to nomenclature rules the natural N10-substituted reduced folates are designated as (6S, α S) diastereomers. (6S, α S) is the only active form of folates in cells and bears one-carbon groups with different oxidation states (Figure 1C). It can bear a methyl group on its N-5 position (most reduced form), methylene or methenyl moieties on positions 5 and 10 (intermediate), a formyl group on position 5 or 10 or a formimino group on position 5. These one-carbon groups are further transferred to specific substrates, as one-carbon transfer mechanisms are the main cellular processes in which folates are involved (Figure 2) [4–6].

Polyglutamated THF constitutes the major folate form of ingested folate. Once hydrolyzed in the gut lumen into monoglutamate by glutamate carboxypeptidase II [7], its transport into enterocytes is ensured mainly by proton-coupled folate transporter (PCFT) proteins. After internalization, the THF is converted into the 5-methylated form that circulates in peripheral blood vessels [8]. Another source of folate comes from the absorption of synthetic folic acid, which is provided by food fortification or as a nutritional supplement. Folic acid is also internalized in enterocytes either by active transport processes using PCFT proteins or simply by passive diffusion. To play its cofactor role, folic acid is then converted by the dihydrofolate reductase enzyme (DHFR) into THF and transformed, via several processes, to the plasma-circulating 5-methyl tetrahydrofolate monoglutamate. This latter molecule, resulting either from diet or synthetic source, is further internalized by RFC proteins or folate receptors in other somatic cells where it is polyglutamated by folyl-polyglutamate synthetase (FPGS) to finally enter the one-carbon metabolic network [9–12].

Folate-mediated pathways are interdependent and are essential for many cellular biosyntheses. In

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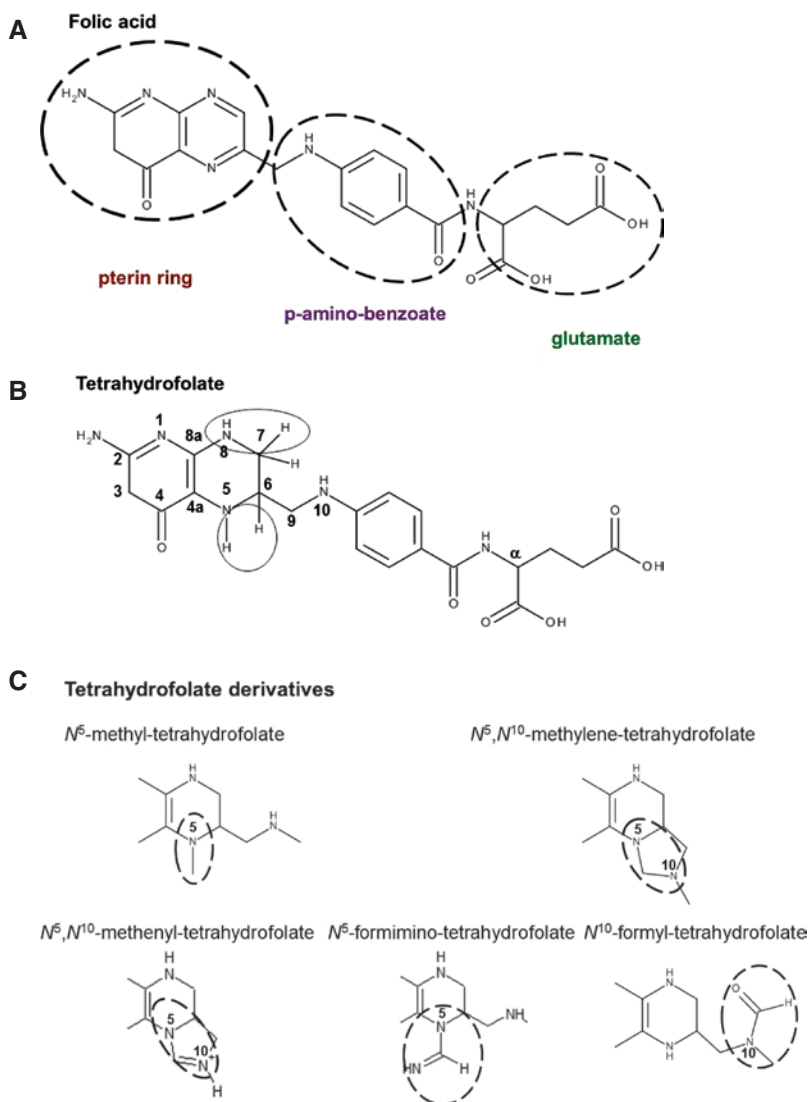


Figure 1: Chemical structures of folic acid (A), tetrahydrofolate (B) and its derivatives (C).

DNA synthesis, for instance, the de novo generation of purine heterocycles is mediated by β -glycinamide ribonucleotide transformylase (GARFT) and 5'-amino-4'-imidazolecarboxamide ribonucleotide transformylase (AICARFT), two key enzymes for which 10-formyl THF acts as a cofactor. Moreover, thymidylate synthase (TYMS) requires 5,10-methylene THF as a cofactor when converting deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP) for the de novo synthesis of deoxythymidine triphosphate, one of the four building blocks of DNA [4–6].

Antifolates are structural analogues of folate derivatives that tend to act as inhibitors of folate-dependent enzymes. These compounds constitute the oldest antimetabolite class of anticancer agents [13, 14]. In this review, the main emphasis will be placed on antifolates with a

terminal alkyne function in their molecular structure showing remarkable anticancer activities.

Acetylenic antifolate as a TYMS inhibitor: CB3717, the forerunner of raltitrexed

Thymidylate synthase activation depends on the formation of a ternary complex composed of the protein, its substrate (dUMP), and 5,10-methylene THF, its cofactor. dTMP synthesis, which is essential for DNA synthesis and repair, is obtained via the reductive methylation of dUMP when 5,10-methylene THF is oxidized to 7,8-DHF.

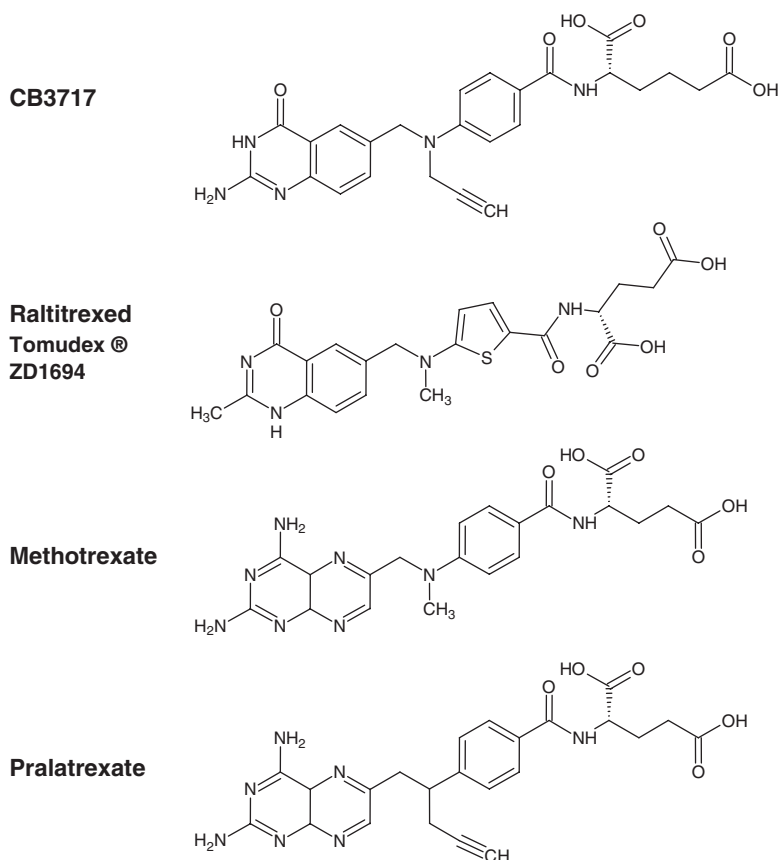


Figure 3: Chemical structures of THF and antifolate anticancer agents.

metastatic colorectal cancer, although its administration is often limited to specific cases only [46–50].

Acetylenic antifolate as a DHFR inhibitor: pralatrexate

DHFR that catalyzes the reduction of DHF to THF also constitutes a key target in anticancer therapy as its inhibition blocks the THF synthesis and thus leads to a depletion in purine and pyrimidine precursors, which are essential for DNA, RNA and hence protein synthesis. The DHFR inhibitor methotrexate (MTX, 4-amino-10-methylpteroyl-glutamic acid) was first demonstrated to exhibit an anti-neoplastic effect in 1948 [51] (Figure 3). This anticancer agent is now commonly used for the treatment of a wide range of cancers including leukemia, lymphoma, breast, lung, bladder carcinomas, head and neck cancer, and osteogenic carcinoma [52]. In the early 1980s, Sirotnak et al. [53, 54] developed a new family of folate analogues composed of a series of 10-deazaaminopterins (with a carbon atom instead of a nitrogen atom present at position 10) that were

found to be more potent than MTX. Their exalted activity were attributed to an enhanced capacity of internalization coupled with a higher degree of glutamation, as these molecules were shown to have notable affinity for the RFC proteins [53] and the FPGS enzyme [55, 56].

Also, with the development of CB3717, the role of propargylic moiety for the generation of anticancer properties was explored by several research groups. While Jackman et al. [57, 58] were investigating the activity of the propargylic group of 2-amino-deficient folate analogues, Piper et al. [59] investigated the potency of the 10-*N*-propargylaminopterins, which was shown to be more active than MTX, although requiring a high-dose injection to be effective *in vivo*. Based on these results, DeGraw et al. [60] synthesized the 10-propargyl-10-deazaaminopterins molecule or pralatrexate (PDX) (Figure 3) and evaluated its anticancer properties in *in vitro* and *in vivo* studies. Even if the structure was found to inhibit DHFR less efficiently than MTX ($K_i=3$ -fold higher), it exhibited an outstanding cytotoxicity, being five times more potent than MTX in cell growth inhibition ($IC_{50}^{MTX}=9.50$ nmol/L, $IC_{50}^{PDX}=2.0$ nmol/L) and quite more effective on murine mammary models *in vivo* [60].

Given these promising results, PDX was then evaluated on human cancer cell lines *in vitro* and investigated on human tumor xenografted mice *in vivo*. Breast and non-small cell lung cancer (NSCLC) constituted the first human cell lines on which PDX demonstrated its efficacy, being up to 30-fold more cytotoxic than MTX. As the most sensitive cell line was detected to be the adenocarcinoma cell line, NSCLC tumor xenografts (LX-1 and A549 cell lines) were chosen to be studied *in vivo*. Whereas tumors of MTX-treated mice did not yield in any healing, PDX led to a complete regression of the tumors in 75% of the animals [56]. The superior antitumor activity of PDX on NSCLC tumor models was also further confirmed by Izbicka et al. [61].

The antitumor efficacy of PDX was also examined on human lymphoma, whether used as a single agent or combined with other cytotoxic agents such as the nucleoside analogue gemcitabine or the proteasome inhibitor bortezomib. The IC_{50} values obtained with all of the Hodgkin or non-Hodgkin lymphoma cell lines treated with PDX were ten-fold smaller than the values obtained with MTX-treated cells. In addition, no or very slight regression of tumors was observed in MTX-treated lymphoma xenografted mice (RL- and SKI-DLBCL), whereas PDX treatment led to complete regression in 30% of RL- and 56% of SKI-DLBCL-xenografted mice [62]. Toner et al. [63] have shown the synergic effect of PDX and gemcitabine especially in treatments occurring in a scheduled manner (gemcitabine administration 24 h after the PDX treatment) as they demonstrated the significantly superior activity of this combination when compared with the MTX→cytarabine combination on animals with SKI-DLBCL xenografts (3/5 complete remission for PDX→gemcitabine, whereas no remission for MTX→cytarabine). A similar synergetic efficacy was also noticed by Marchi et al. [64] when they tested PDX in combination with bortezomib in *in vitro* and *in vivo* models of T-cell lymphoid malignancies.

To understand the molecular basis underlying the enhancement of the cytotoxic effect with the propargylic molecule, studies evaluating the expression levels of the genes coding for proteins involved in one-carbon metabolisms were conducted. As the RFC1 that codes for the RFC protein was found to be more expressed in PDX-sensitive cell lines (diffuse large B cell and HT cell line), it was suggested that a better internalization of the drug could lead to the enhancement of its antiproliferative activity [63]. A similar investigation was further carried out on a wide panel of cancer cells including colon, breast, melanoma, NSCLC, ovarian, prostate and head and neck cell lines by Serova et al. [65]. The authors analyzed the expression

levels of genes coding for DHFR, FPGS, RFC, TYMS, GARFT, SLC25A32 (mitochondrial folate transporter/carrier) and ATP-binding cassette transporters (ABCB1) and did not notice any correlation between PDX sensitivity and the expression levels of TYMS, GARFT, SLC25A32 or ABCB1, nor of RFC1, and thus they could not support the findings previously established. However, they determined a significant increase in the levels of FPGS genes, indicating again the important role of glutamation in acquiring cytotoxicity. A moderate but not statistically significant increase in the expression of DHFR was also reported. To better characterize the molecular mechanisms implied in PDX-sensitive cell lines, the group also generated PDX- and MTX-resistant cells from cells detected to be the most sensitive to the corresponding drugs. The examination of gene expression levels in DU-PDX and HEP-PDX cell lines, both being PDX resistant, revealed a considerable decrease in the expression of RFC1, indicating a decisive role of the folate transport protein for PDX to be active, as previously suggested [53, 63]. Furthermore, an increase in the ABCB1 gene was also observed. Modification in ABCB1 expression levels did not show any correlation with PDX sensitivity though, as the inhibition of the ABC proteins did not restore sensitivity to the drug. Interestingly, even though MTX-resistant cells exhibited an extensive increase in DHFR levels as expected since DHFR over-regulation constitutes the main mechanism for the generation of MTX resistance [66], the increase observed for PDX-resistant cells was not statistically significant, suggesting different molecular mechanisms of action for these two antifolates. This hypothesis is actually in agreement with Zain and O'Connor's [67] findings, which indicated that modifications in gene expression levels in MTX-treated cells were mainly occurring in genes involved in folate metabolism, whereas the gene expression of PDX-treated cells was essentially disrupted for genes implied in pathways regulating immunomodulation and transcription factors.

Based on the encouraging results obtained during the first preclinical studies, the clinical evaluation of PDX began with NSCLC patients. The phase I studies revealed mucositis as the drug's dose-limiting toxicity. Its antitumor activity was confirmed as two of 33 patients with stage IV NSCLC responded favorably to the treatment and the conditions of five of 33 patients were stabilized [68]. The phase II study of 38 NSCLC patients provided satisfactory results, with 10% of objective responses and 31% of disease stabilization. Stomatitis and mucositis constituted the main toxicities associated with the treatment [69]. When examining the efficacy of the drug in combination with probenecid on patients presenting

solid tumors, Fury et al. [70] mentioned the possibility of supplementation with vitamin B₁₂ and folate in order to prevent mucositis, hence allowing a dose escalation during treatments. Azzoli et al. [71] evaluated PDX in combination with taxanes, incorporating to their protocol the co-administration of vitamin B₁₂ and folic acid. This supplementation allowed the patients to tolerate safely higher doses of the drug [71], confirming the predictions of Fury et al. [70]. From then on, investigators always incorporated vitamin B₁₂ and folic acid supplementation into their protocols.

PDX was further evaluated on other carcinomas. Although the drug did not show any activity on patients with malignant pleural mesothelioma [72], it demonstrated successful activity on lymphoma, with achievement of complete regression in all of the patients with T-cell lymphoma in a study carried out on 20 patients, 16 of whom presenting B-cell and four presenting T-cell lymphomas [73]. The authors tested two different doses for the treatment of T-cell lymphoma: the first treatment at the recommended dose (135 mg/m² every other week) resulted in the development of severe mucositis, whereas the administration of 30 mg/m² of PDX weekly for 6 weeks was well tolerated by three other patients. These findings led to a new phase I clinical study that redefined the maximum-tolerated dose of PDX, which decreased the ratio of patients developing stomatitis from almost 100%–17% [74]. Based on these results, a multicenter phase II study, carried out with patients with relapsed or refractory peripheral T-cell lymphoma, demonstrated the drug's outstanding efficacy, as on 109 evaluable patients 29% experienced objective responses and 38% achieved complete remission. These data led to the approval of PDX by the U.S. FDA in 2009 for the treatment of peripheral T-cell lymphoma on patients who have relapsed or have not responded to other chemotherapy drugs [75–77]. PDX is the first drug approved for this disease [78].

To conclude, this review focused on antifolates with a terminal alkyne function. Antifolates constitute the first class of anticancer antimetabolites, and the development of folate antagonists bearing a propargyl group led to the generation of the first clinically evaluated folate-based TYMS inhibitor, CB3717. Yet, the clinical studies of this highly potent molecule revealed a serious nephrotoxicity of the structure due to its poor solubility. CB3717 structure optimization studies contributed to the development of two marketed anticancer agents: raltitrexed, a TYMS inhibitor used for the treatment of colorectal cancer, and pralatrexate, a propargylic DHFR inhibitor, which is the first drug approved for relapsed or refractory peripheral T-cell lymphoma. These results highlight the potential of

the propargyl group for the development of novel potent anticancer molecules.

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