New Antifolates in Clinical Development

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Numerous new antifolate drugs have been developed in an attempt to overcome the potential mechanisms of tumor cell resistance to methotrexate, which can include decreased drug transport into cells; decreased

**Introduction**

Antifolates, such as methotrexate, were one of the earliest classes of drugs developed for clinical use in cancer chemotherapy. Today, methotrexate is still used extensively in the treatment of human leukemia, breast cancer, head and neck cancer, choriocarcinoma, osteosarcoma, and lymphoma. Antifolate compounds also have important clinical utility outside the realm of oncology, in the treatment of such diverse diseases as rheumatoid arthritis, psoriasis, bacterial and plasmodial infections, and opportunistic infections associated with AIDS.

Currently, numerous promising antineoplastic antifolate drugs are in clinical development, and many of these agents have interesting and unique mechanisms of action. An understanding of the cellular pharmacology of these agents will help the practicing clinician effectively use these new drugs as they become available for the treatment of human malignancies.

**Methotrexate**

**Mechanism of Action**

The most widely used and best understood antifolate in cancer therapy is methotrexate, which differs from the essential vitamin, folic acid, by having an amino group substituted for a hydroxyl at the 4-position on the pteridine ring (Figure 1). This change transforms the enzyme substrate into a tight-binding inhibitor of dihydrofolate reductase (DHFR), a key enzyme required to maintain adequate intracellular levels of reduced folates [1].

Dihydrofolate reductase is critically important because folate molecules are biochemically active only in their fully reduced form as tetrahydrofolates. The tetrahydrofolates are essential cofactors that donate one-carbon groups in the enzymatic biosynthesis of thymidylate and purine nucleotide precursors for DNA synthesis (Figure 2). One reduced folate, 5,10-methylenetetrahydrofolate, participates in the reaction catalyzed by the enzyme thymidylate synthase (TS), which converts deoxyuridylate (dUMP) into thymidylate (dTMP). As a consequence of this reaction, 5,10-methylenetetrahydrofolate undergoes oxidation to dihydrofolate, which must then be reduced by DHFR back to tetrahydrofolate in order to replenish the intracellular reduced-folate pools. Another reduced folate, 10-formyltetrahydrofolate, serves as a one-carbon donor for the reactions catalyzed by glycaminid e ribonucleotide (GAR) and aminimidazole carboxamide ribonucleotide (AICAR) transformylases. These enzymes are involved in the de novo biosynthesis of purine nucleotides. Thus, inhibition of DHFR by antifolates can lead ultimately to the decreased production of several essential precursors for DNA synthesis.

Both methotrexate and naturally occurring folate compounds can undergo intracellular metabolism to polyglutamate derivatives. These reactions, catalyzed by the enzyme polyglutamyl synthase (FPGS), attach up to six glutamate residues to the pteridine ring, which help trap these molecules within the cell by decreasing their efflux. Methotrexate polyglutamates are also potent direct inhibitors of DHFR, as well as other folate-dependent enzymes, such as TS and GAR and AICAR transformylases. Furthermore, DHFR inhibition leads to the accumulation of dihydrofolate polyglutamates within the cell, which can directly inhibit the folate-dependent enzymes involved in the synthesis of thymidylate and purine nucleotides [2,3]. Thus, inhibition of DNA synthesis by the antifolates is a multifactorial process, resulting from the partial depletion of the intracellular reduced folate pool and from the direct inhibition of folate-dependent enzymes. Administration of exogenous reduced folates, such as leucovorin calcium, to methotrexate-treated nonmalignant cells efficiently replenishes the reduced folate pool and directly competes with the
drug-induced inhibition of folate-dependent enzymes. This is the biochemical rationale for the clinical use of leucovorin rescue to prevent severe toxicity in high-dose methotrexate chemotherapy regimens.

Ultimately, the depletion of thymidylate and purine nucleotide cofactors for DNA synthesis leads to a cessation of DNA synthesis, but it is not clear whether this action alone is enough to induce cell death. Lethal DNA damage resulting from a drug-induced lack of essential nucleotides may occur because of ineffective DNA repair, or because of misincorporation of uracil deoxynucleotides into DNA. Additional studies on this important issue are clearly necessary. Furthermore, the relative importance of the inhibition of thymidylate or purine nucleotide synthesis in the generation of methotrexate-induced cytotoxicity has yet to be defined.

**Cellular Pharmacology**

**Influx into Cells**—At least two distinct, energy-dependent, carrier-mediated transport systems are responsible for the uptake of methotrexate into mammalian cells [4]. The classic reduced-folate carrier system, which transports reduced folates, such as 5-methyltetrahydrofolate, and antifolates, such as methotrexate, has affinity constants in the micromolar range, and it is a relatively less efficient transporter of folic acid. A second carrier system that utilizes the hydrophobic membrane-associated binding protein, the human folate receptor (hFR), has a higher affinity (nanomolar range) for folic acid and reduced folates than it does for methotrexate. Some tumors, such as human ovarian cancers, overexpress the hFR on their cell surface.

The exact contribution of these two transport pathways to the uptake of methotrexate in clinical cancer chemotherapy is an area of intensive research. However, methotrexate resistance resulting from the decreased activity of one or both of these transport systems has been demonstrated in vitro, suggesting that transport deficiencies may be clinically important [1]. More lipophilic antifolates, such as trimetrexate and piritrexim, are not substrates for these carrier-mediated folate transport systems, and can enter cells by either passive diffusion or by other transport mechanisms. Cell lines that are resistant to methotrexate because of decreased transport generally retain their sensitivity to these more lipophilic antifolate agents [5].

**Efflux from Cells**—Efflux of methotrexate from the cell is also mediated by several different transport systems, some of which are clearly distinct from the influx systems. Methotrexate efflux is not associated with the P-glycoprotein, multidrug resistance (MDR) system that has been described for numerous other antineoplastic agents. However, drug efflux is heavily influenced by the degree of methotrexate polyglutamation. As mentioned previously, both normal and malignant cells contain the enzyme FPGS, which can add glutamyl groups in a gamma peptide linkage to the pteridine ring in naturally occurring folates and in some antifolate drugs as well. This reaction serves two important functions: First, it facilitates the accumulation of intracellular folates by converting them into large natural polyglutamates, and may account for the chronic hepatotoxicity associated with methotrexate therapy [1]. In addition, the selective nature of methotrexate cytotoxicity may result, in part, from the increased polyglutamation of methotrexate in cancer cells compared to normal tissues. The decreased ability to polyglutamate antifolates also appears to be an important mechanism of clinical drug resistance [6].

**Binding to DHFR**—Methotrexate is a competitive inhibitor of DHFR that binds noncovalently to this enzyme at the same binding site as the normal substrate, dihydrofolate. This interaction also depends on the intracellular concentration of reduced nicotinamide dinucleotide phosphate (NADPH), which is a normal cofactor for DHFR. Point mutations both inside and outside of the enzyme's active site have been identified that decrease the binding affinity of DHFR for methotrexate [1]. Thus, mutations in the DHFR enzyme are another potential mechanism of resistance to antifolates.

Sensitivity to methotrexate cytotoxicity is highly dependent on the absolute amount of DHFR enzyme within the cell. Both human tumors and cancer cell lines that have increased levels of DHFR due to gene amplification are relatively resistant methotrexate [7]. More subtle mechanisms may also exist that allow cells to acutely increase DHFR expression in response to antifolate treatment. The expression of DHFR appears to be controlled at least partially by the binding of the DHFR protein...
to its own mRNA (Figure 3) [8]. This binding of DHFR prevents further synthesis of the DHFR protein, resulting in an autoregulatory negative feedback loop. Excess normal substrate, such as dihydrofolate, or inhibitors, such as methotrexate, interfere with the binding of the DHFR protein to its mRNA and block this negative feedback loop, thereby allowing the synthesis of DHFR protein to continue unimpeded. This permits normal cellular function to be maintained, even in the presence of inhibitors such as methotrexate or other antifolates. The importance of this mechanism in the development of clinical antifolate resistance must be defined.

For a detailed discussion of the pharmacokinetics of methotrexate, including specific dosage and scheduling information, readers are referred to reference 1.

New Antifolates

Advances in our understanding of the cellular pharmacology of methotrexate have led to the rational design of several new antifolate compounds with unique pharmacologic properties. Many of these newer agents have been developed in an attempt to overcome the potential mechanisms of methotrexate resistance that have been observed in mammalian cells, including decreased drug transport, decreased polyglutamation, mutations in the DHFR enzyme, and increased expression of the DHFR protein. As a consequence, many of the newer antifolates have greater lipid solubility and/or better transport characteristics than methotrexate, as well as an improved ability to undergo polyglutamation. Furthermore, several of the new antifolates uniquely target specific folate-dependent enzymes, such as TS or GAR transformylase.

Trimetrexate

Although trimetrexate (Neutrexin) was originally developed to treat malaria, it recently was approved by the FDA for the treatment of Pneumocystis carinii pneumonia. It has also undergone extensive clinical testing as an anticancer agent. Structurally, trimetrexate is a nonclassic, quinazoline-derived, lipophilic antifolate, with greater DHFR inhibitory activity than methotrexate [5]. Like methotrexate, trimetrexate results in at least a partial depletion of the intracellular reduced folate pool and inhibits de novo purine synthesis by causing an accumulation of dihydrofolate polyglutamates. Although trimetrexate is not a substrate for the folate transport systems, it rapidly enters cells by a non-energy-dependent pathway, achieving high intracellular concentrations [9]. Most cell lines resistant to methotrexate because of decreased active transport are still sensitive to trimetrexate. However, unlike methotrexate, trimetrexate is a potential substrate for the P-glycoprotein, MDR efflux pump, and thus, it may show cross resistance to natural product anticancer agents [10]. Because trimetrexate does not contain a terminal glutamyl residue, it cannot be polyglutamated, and consequently, it is not selectively retained within cells for prolonged periods like methotrexate. This probably explains why preclinical studies have shown that more prolonged exposures to trimetrexate are necessary for optimal antitumor activity [9].

Clinical development of trimetrexate as an antitumor agent proceeded rapidly after it was shown that the drug was effective against cell lines that were resistant to methotrexate as a result of decreased folate transport or decreased polyglutamation. In vitro resistance to trimetrexate can occur as a result of increased expression of the MDR phenotype, increased DHFR expression, or decreased uptake into cells by an as yet uncharacterized mechanism [11]. In preclinical testing against various cell lines, trimetrexate has shown a broader range of clinical activity than methotrexate [9].

Dosing and Pharmacokinetics--Trimetrexate has been administered by various different schedules, ranging from short intravenous infusions every few weeks to 5-day continuous infusions. The most commonly used regimen has been an intravenous bolus of 8 to 12 mg/m² daily for 5 days every 3 weeks. Oral administration has been used less commonly in anticancer trials, although the mean bioavailability of trimetrexate in humans after an oral dose of 60 mg/m² is about 44% [12]. Compared with methotrexate, trimetrexate is highly protein bound (> 90%), with elimination occurring primarily via hepatic metabolism [5]. Some of the resulting metabolites are active against DHFR; however, most of these have not been well characterized. Renal clearance of the parent molecule is < 5%, and the terminal half-life ranges from 15 to 20 hours [5].

Clinical Toxicity--The primary dose-limiting toxicity of trimetrexate has been predictable, noncumulative myelosuppression, predominantly leukopenia. Other clinical toxicities include reversible elevation of liver transaminases, skin rash, mild nausea and vomiting, mucositis, hypersensitivity reactions, and fever. Several different skin lesions have been associated with trimetrexate therapy; the most common of these is erythroderma appearing over the neck and trunk [13]. Relatively rare hypersensitivity reactions may be related to the drug's ability to inhibit enzymes
involved in histamine metabolism, which results in a corresponding increase in tissue histamine levels [14].

**Antitumor Activity**—Trimetrexate has undergone extensive phase II testing as a single agent in a broad range of histologic tumor types (!-Table 1) [15-35]. Objective response rates of 26% have been reported in head and neck cancer, [15] 14% to 19% in non-small-cell lung cancer, [16,18] 17% in urothelial cancer, [20] and 5% to 15% in breast cancer. [24,25] However, the overall activity of single-agent trimetrexate in clinical trials has been disappointing, with no responses seen in pancreatic cancer, [31] colorectal cancer, [32] or melanoma [33].

Preclinical testing of the combination of trimetrexate and fluorouracil plus leucovorin has shown in vitro activity comparable to or better than methotrexate plus fluorouracil/leucovorin [36]. The trimetrexate combination has a theoretical advantage of avoiding the potential competition between methotrexate and leucovorin for uptake by the cell's active transport mechanisms [5]. Clinically, this regimen produced response rates of 20% in previously treated patients with gastrointestinal cancer [37]. Other active combinations include trimetrexate plus either cisplatin (Platinol) or cyclophosphamide (Cytoxan, Neosar) [38,39]. Presently, clinical testing of trimetrexate combinations is continuing.

**Treatment of Opportunistic Infections**—Both *P carinii* and *Toxoplasma gondii* are important pathogens in AIDS that lack an active folate transport system and thus are resistant to methotrexate. However, the more lipophilic trimetrexate penetrates these organisms and inhibits their DHFR. Trimetrexate with leucovorin rescue can effectively treat *Pneumocystis pneumonia* in AIDS patients [40]. It is currently recommended as second-line therapy for this opportunistic infection in patients who are either resistant to or who cannot tolerate initial therapy with trimethoprim-sulfamethoxazole.

**Edatrexate**
Edatrexate (10-ethyl-10-deaza-aminopterin [EdAM]) is a classic antifolate DHFR inhibitor currently in phase II testing. Structurally, edatrexate is similar to methotrexate, differing only by the substitution of an alkylated carbon at the N10 position. Like methotrexate, edatrexate is actively transported into cells by the reduced folate transport system and is readily polyglutamated by FPGS. However, compared with methotrexate, edatrexate is taken up and selectively retained to a greater extent in tumor cells than in normal cells [41]. This may account for edatrexate's better therapeutic index in preclinical models. Edatrexate polyglutamates are more potent inhibitors of DHFR than methotrexate polyglutamates, but they are somewhat less active against TS [41]. Because of edatrexate's broad range of antitumor activity in animal models, the drug entered clinical testing with high expectations.

**Dosage and Pharmacokinetics**—The most common dosage schedule for edatrexate has been 80 mg/m² intravenously every week. In rats, about 30% of edatrexate is protein bound, with most of the elimination of the drug occurring in the liver via nonsaturable biliary excretion [41]. Renal excretion of unchanged drug ranges from 13% to 55%. In humans, elimination is best described by a triexponential model with a terminal half-life of 11.9 hours [41].

**Clinical toxicity** of edatrexate is similar to that of methotrexate. Mucositis and stomatitis have been the dose-limiting side effects in most studies. Also observed were myelosuppression, nausea and vomiting, reversible elevation of liver transaminases, diarrhea, rash, fatigue, mild alopecia, and pneumonitis.

**Antitumor Activity**—In phase II testing, edatrexate has shown promising activity in non-small-cell lung cancer, head and neck cancer, metastatic breast cancer, and soft tissue sarcomas (!-Table 2) [42-59]. While these results are encouraging, it still must be proven whether edatrexate has better activity than methotrexate. Edatrexate is also undergoing testing in combination with other agents, including cisplatin, carboplatin (Paraplatin), paclitaxel (Taxol), mitomycin (Mutamycin), vinblastine, and doxorubicin [41].

Because the addition of leucovorin appears to protect normal tissues from edatrexate-associated toxicity, phase I trials have been initiated using high doses (up to 3,000 mg/m²) in combination with leucovorin rescue [60,61]. Most of the high-dose regimens involve monitoring the plasma levels of edatrexate to guide the duration of leucovorin rescue in a manner analogous to that used with high-dose methotrexate. Toxicities of high-dose edatrexate observed in preliminary phase I testing include gastrointestinal symptoms, elevated liver transaminases, and skin rashes. The efficacy of high-dose approaches is currently being examined.

**Piritrexim**
Piritrexim (BW301U) is a quinazoline-derived lipophilic inhibitor of DHFR that is related to trimetrexate. Like that drug, piritrexim can rapidly enter cells by passive diffusion, resulting in high
intracellular drug concentrations. It is also effective in killing transport-deficient cell lines that are resistant to methotrexate.

Because piritrexim is not a substrate for polyglutamation, the drug is not selectively retained within cells for prolonged periods. It is a potent inhibitor of DHFR and, like methotrexate and trimetrexate, it can inhibit de novo purine biosynthesis by causing an accumulation of dihydrofolate polyglutamates. A theoretical advantage of piritrexim over trimetrexate is a lack of any known effects on histamine metabolism, which may lower the risk of hypersensitivity reactions [9].

**Dosage and Pharmacokinetics**—Piritrexim has a reliably high oral bioavailability of about 75%, [62] which has led to its development as an oral lipophilic antifolate. Most commonly, it has been administered in oral daily doses of 75 to 150 mg bid or tid every 5 days, with cycles repeated every 3 weeks. Oral absorption is rapid, with peak plasma levels appearing at 1.5 hours after ingestion [63]. Elimination occurs primarily via hepatic metabolism of the drug to active metabolites, and the terminal half-life of the parent compound is about 1.5 to 4.5 hours [63]. The dose-limiting toxicity of oral piritrexim observed in most studies has been predictable leukopenia. Mucositis, stomatitis, nausea and vomiting, diarrhea, fatigue, anorexia, elevated liver transaminases, and skin rash have also been reported.

**Clinical Activity**—Single-agent oral piritrexim has clinical activity in melanoma, urothelial cancers, and head and neck cancers ([Table 3] [64-69]). Tolerable combinations of piritrexim with cisplatin, fluorouracil, and leucovorin have been tested, with promising results achieved in head and neck cancer [70]. An interesting attempt to alternate piritrexim with methotrexate did not have any greater activity than methotrexate alone [71]. The potential for oral administration has also led to studies of piritrexim in the treatment of non-neoplastic diseases, including psoriasis and opportunistic AIDS infections, such as P carinii pneumonia [9]. However, the therapeutic advantages of this new antifolate over FDA-approved agents, such as methotrexate or trimetrexate, must still be proven.

**Tomudex**

Tomudex (ZD1694) is a quinazoline antifolate that inhibits TS by occupying the folate-binding pocket on the protein. Tomudex enters the cell via the reduced-folate active transport system, and it is a substrate for polyglutamation by FPGS. Because the polyglutamated forms of Tomudex are 100-fold stronger inhibitors of TS than the monoglutamates, polyglutamation is thought to be essential for its antitumor activity [72]. Interestingly, Tomudex and its polyglutamates do not appear to inhibit DHFR, GAR or AICAR transformylase, suggesting that this drug is a pure TS inhibitor.

Tomudex was originally developed as a water soluble analog of CB3717, which was the first quinazoline-derived antifolate TS inhibitor to enter clinical trials. Despite showing antitumor activity in phase I studies, the development of CB3717 was halted because of unpredictable renal toxicity [9]. The poor solubility of CB3717 in low pH probably caused the drug to precipitate in the acidic environment of the renal tubules. In contrast to CB3717, Tomudex is more water soluble in acid pH, and it is a better substrate for polyglutamation by FPGS. Preclinical studies of Tomudex confirm its greater cytotoxic potency than CB3717 in most tumor cell lines [9].

**Dosage and Pharmacokinetics**—The most commonly used dose schedule of Tomudex has been 3 to 4 mg/m² infused intravenously over 15 minutes every 3 weeks. Linear increases in the area under the curve have been observed with increasing dose levels, and the drug is eliminated primarily by hepatic metabolism and biliary excretion. The elimination profile of Tomudex has been characterized by a triexponential model with a beta half-life of 1.3 to 2.6 hours and a gamma half-life of 50 to 100 hours [73].

**Clinical Toxicity**—Dose escalation was limited in two phase I studies by a profound fatigue and malaise syndrome, [73,74] with neutropenia being the only other dose-limiting toxicity observed. Additional side effects included diarrhea, reversible elevation of liver transaminases, nausea and vomiting, stomatitis, rash, fever, and mild alopecia. Overall, the drug was felt to be well tolerated, and there has been no evidence of renal toxicity.

**Antitumor Activity**—Preliminary reports of clinical trials using Tomudex have described response rates of about 25% in colorectal cancer and breast cancer ([Table 4] [75-80]). In non-small-cell lung cancer and refractory ovarian cancer, the response rates appear to be less than 10%. Further phase II testing of Tomudex is in progress. Although combinations have yet to be studied clinically, it may be particularly interesting to combine this drug with other inhibitors of TS, such as fluorouracil.

**Lometrexol**

Lometrexol (5,10-dideazatetrahydrofolate [DDATHF]) is a tetrahydrofolate analog that contains carbon atoms substituted for nitrogen atoms at the N5 and N10 positions. Lometrexol does not inhibit DHFR or TS; instead, it is a relatively specific inhibitor of GAR transformylase. The resulting
inhibition of the de novo purine synthetic pathway leads ultimately to a depletion of the adenosine triphosphate and guanosine triphosphate nucleotide pools. Lometrexol has a different pattern of activity against various tumor cell lines than methotrexate or other inhibitors of DHFR [9]. Lometrexol enters cells by active transport and must be polyglutamated by FPGS in order to become active [81]. Lometrexol polyglutamates are 100-fold more potent enzyme inhibitors of GAR transformylase than the monoglutamates, and cellular resistance to lometrexol has been observed in vitro as a consequence of decreased FPGS activity [82]. Lometrexol has been administered weekly or daily as an intravenous infusion, both with and without leucovorin rescue. In phase I testing, the drug caused severe cumulative myelosuppression, predominantly thrombocytopenia, which was much worse than predicted from animal models [83,84]. Coadministration of either folic acid or leucovorin appears to alleviate this toxicity. Other side effects included stomatitis, neutropenia, and anemia. Further clinical testing of lometrexol in combination with folic acid is in progress.

Conclusions

The study of the cellular pharmacology of methotrexate has led to the development of new antifolates with great therapeutic potential. In addition to the agents described here, several other antifolates, such as LY231514,[85] are just entering clinical trials. Of particular interest are the new synthetic antifolate inhibitors of TS, AG331 [86] and AG337, [87] which were rationally designed based on crystallographic modeling of the TS folate-binding pocket. The next few years will see a large increase in the clinical testing of new antifolates, which hopefully will lead to more effective and better treatments for human diseases such as cancer.

References:


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