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Current understanding of methotrexate pharmacology and efficacy in acute leukemias. Use of newer antifolates in clinical trials

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Background and Objectives. Methotrexate (MTX) is a key drug in the curative regimen of children with acute lymphocytic leukemia. This drug is widely used not only in the treatment of neoplastic diseases such as leukemia, lymphoma, choriocarcinoma, head and neck cancer and osteogenic sarcoma, but also for various autoimmune diseases, e.g., rheumatoid arthritis and psoriasis, and for the prevention of graft-versus-host disease after transplantation. The development of drug resistance is the limiting factor in the use of MTX. This review will outline the mechanisms of acquired and natural resistance to MTX that have been studied in patients affected by acute lymphocytic leukemia and acute myelocytic leukemia and the cell cycle genes involved in MTX resistance. This information may improve the use of MTX or could lead to the development of better drugs. Moreover a short description of newer antifolates with their mechanisms of action is presented.

Evidence and Information Sources. The authors of this review have a long-standing interest in the mechanism of action of and resistance to MTX and other antifolates. Information from journal articles covered by the Science Citation Index,® and Medline,® has been reviewed together with work performed by the authors.

Perspectives. Antifolates continue to be an extremely important class of drugs for the treatment of non- neoplastic as well as neoplastic diseases. New inhibitors that target dihydrofolate reductase as well as other folate-dependent enzymes are being evaluated in the clinic, and show promise.

Key words: methotrexate, drug resistance, cell cycle, newer antifolates

Since 1948, when Farber *et al.* showed that the antifolate aminopterin could produce remissions in children with acute leukemia, the folate-dependent enzymes have been a major target of chemotherapy because of the essential role they play in the synthesis of DNA precursors.[1,2]

Methotrexate (MTX) (Figure 1) is the antifolate most widely used not only in the treatment of leukemia, lymphoma, choriocarcinoma, head and neck cancer and osteogenic sarcoma, but also for the treatment of various autoimmune diseases, e.g., rheumatoid arthritis and psoriasis and for the prevention of graft-versus-host disease after transplantation.[3] MTX is a key drug in the curative regimen of children with acute lymphocytic leukemia (ALL) but is effective in only approximately 10% of patients with acute myelocytic leukemia (AML). The development of drug resistance is the limiting factor in the use of this drug. The mechanism of acquired or natural resistance to MTX has been studied both in experimental systems and in patients affected by leukemia in order to obtain information that could lead to an improvement in the use of MTX as well as to the development of better drugs.

Mechanism of action

MTX and its polyglutamates are tight-binding inhibitors of dihydrofolate reductase (DHFR) and as a consequence of this inhibition, N^5-N^{10} methylene tetrahydrofolate and N^{10} formyl tetrahydrofolate, the essential cofactors in the biosynthesis of thymidylate and purines, respectively, are depleted. The result is an inhibition of DNA replication and cell death. Moreover, glycinamide ribonucleotide (GAR) transformylase and aminoimidazole carboxamide (AICAR) transformylase, enzymes involved in the *de novo* synthesis of the purines, are also inhibited by MTX polyglutamates. MTX also competes with reduced folates for transport and poly - glutamylation.[1] The MTX inhibition of DNA biosynthesis is therefore multifactorial, including both partial depletion of reduced folates and direct inhibition of folate dependent enzymes. The effectiveness of MTX is depen-

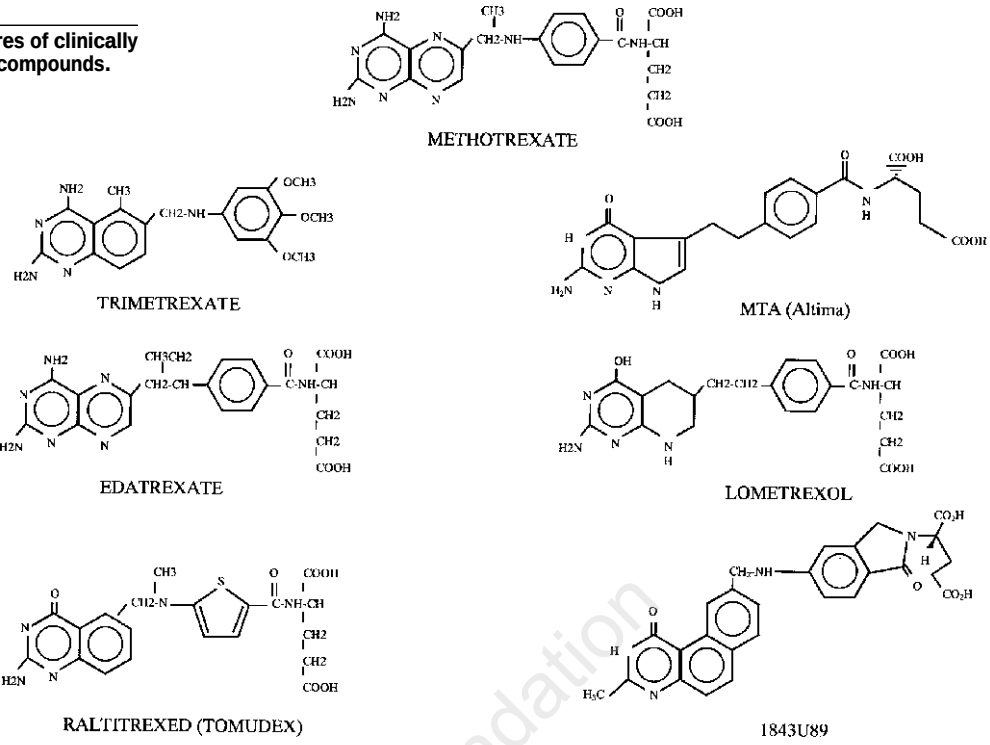

Figure 1. Structures of clinically useful antifolate compounds.

dent on its concentration and retention in cells. The rate of MTX influx into cells is governed by the reduced folate carrier protein (RFC), which efficiently transports folates and MTX into cells. A second low-capacity transport system which is mediated by a membrane-bound folate receptor is also described. However this system has not been shown to be involved in resistance to MTX.[4] Like folates, intracellular MTX is extensively metabolized to polyglutamate derivatives by the enzyme folylpolyglutamate synthetase (FPGS) which adds glutamyl groups to MTX. Formation of MTX polyglutamates is an important determinant of MTX cytotoxicity, as long chain polyglutamates $-glu_{(3-6)}-$ are retained, in the absence of extracellular drug, much longer than MTX or $MTXglu_{(1)}$ which efflux from the cells more rapidly.[1-5] The steady-state level of both folate as well as MTX polyglutamates also depends on the activity of an exopeptidase, γ-glutamyl hydrolase (γGH) (folylpolyglutamyl hydrolase), that catalyzes the hydrolysis of intracellular polyglutamates. Negatively charged polyglutamates enter lysosomes through a simple mobile carrier system with the property of exchange diffusion that appears to favor the transport of MTX with increasing γ-glutamyl chain length. The monoglutamate forms of MTX efflux rapidly from the lysosomes and the cells.[6,7] (Figure 2).

Resistance to methotrexate

Various mechanisms of resistance, both intrinsic and acquired have been described in both *in vivo* and *in vitro* studies and include: 1) defective MTX transport; 2) dihydrofolate reductase (DHFR) gene amplification resulting in high levels of the enzyme; 3) altered affinity of MTX for DHFR; 4) decreased accumulation of MTX polyglutamates which may result from reduced synthesis or increased degradation; and 5) increased MTX efflux due to elevated levels of the multidrug resistance protein (MRP).

Acquired resistance

Transport resistance is a common mechanism of acquired resistance to MTX. A useful assay, using the fluorescent lysine analog of MTX N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{6}-(4'fluorescein thiocarboyl)-L-lysine (PT430) in flow cytometric analysis and its displacement by MTX and trimetrexate (TMTX), has been employed to identify defects in MTX transport in cultured cells and in blasts from leukemia patients.[8] Defective transport can be an important factor of MTX resistance in childhood acute lymphocytic leukemia.[8-10] Gorlick *et al.*,[11] using a complementary DNA for the RFC, showed a strong correlation between low levels of expression of the RFC and impaired uptake of MTX, as measured by the PT430 displacement assay. In the same study minimal displacement of PT430 with trimetrexate was reported in 28% of cases of relapsed ALL and in 25% of cases of AML. This finding might be due to increased levels of P-glycoprotein which has been reported to be capable of effluxing trimetrexate. Cell lines exposed to increasing concentrations of MTX enhanced their DHFR activity with corresponding aug-

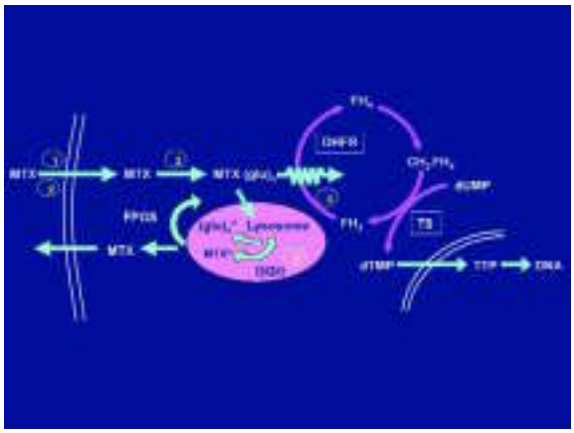

Figure 2. Mechanism of action of methotrexate. Uptake of methotrexate is mediated by the reduced folate carrier (1) and by the membrane-bound folate receptor (2); inside the cell methotrexate is extensively metabolized to polyglutamate derivatives by the enzyme FPGS (3); methotrexate and its polyglutamates inhibit DHFR and as a consequence methylene tetrahydrofolate and N10-formyl tetrahydrofolate are depleted (4); the result of this reaction is the inhibition of thymidylate, methionine, glycine and purines so that DNA replication is damaged. Lysosomal γ-glutamyl hydrolase catalyzes the hydrolysis of intracellular polyglutamates so that the monoglutamate forms of methotrexate efflux rapidly from the cells (5).

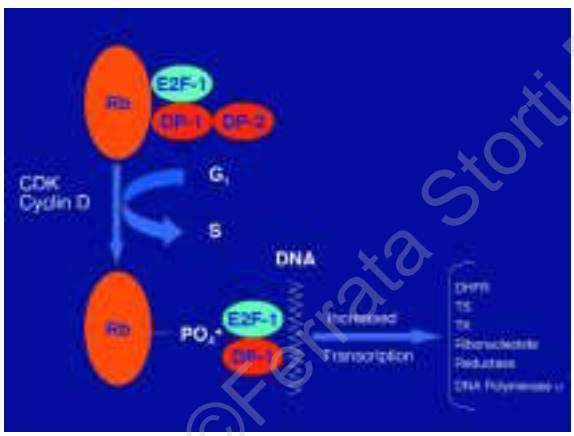

Figure 3. Deregulation of tumor suppressor genes. During the G1-S phase pRb is phosphorylated and free E2F is released. Free E2F forms a heterodimer with its binding protein DP-1 or DP-2 and promotes the transcrption of E2F target genes as DHFR, TS, TK, DNA polymerase α, etc. The consequence of the loss of pRb function, that downregulates E2F, is an increase in E2F transcriptional activity that is a critical factor in the deregulation of the cell cycle.

mentation in the number of gene copies, resulting in MTX resistance.[10] In patient samples, DHFR gene amplification has also been associated with acquired MTX resistance. Goker *et al*.,[12] using a dot blot assay, showed that 9 out of 29 blast samples from patients with relapsed ALL had low levels of DHFR gene amplification as well as increased DHFR enzyme activity. Blasts from 4 of 38 patients with newly diagnosed ALL showed gene amplification whereas only 1 of 53 with AML did so.

Decreased binding of MTX to DHFR due to mutations of the target enzyme has been observed in experimental tumor cells. Samples from leukemia patients have been tested but no decreased MTX binding at the protein level or alterations in the coding region of DHFR have been observed. Although mutations in the DHFR gene are unlikely mechanisms of resistance to MTX in acute leukemias, mutations of this gene in malaria parasites are usually associated with resistance to antifolates.[13,14]

Natural (inherent) resistance

Improved overall survival in children with ALL has been correlated with blasts that accumulate high levels of intracellular MTX long chain polyglutamates after incubation for 24 h with [^{3}H]MTX.[15] *In vitro* accumulation of long chain MTX polyglutamates in pediatric B-lineage blasts was higher than in blasts from adults and children with T-lineage ALL and from adults with B-lineage ALL.[16,17]

Patients with hyperdiploid B-lineage lymphoblastic leukemia who are considered to have a favorable prognosis accumulate higher concentrations of long chain MTX polyglutamates.[18] Decreased MTX long chain polyglutamate formation is considered an inherent mechanism of resistance to MTX.[19] Blast cells from patients with AML, a disease that is naturally resistant to MTX, show low levels of long chain polyglutamate accumulation when incubated with [^{3}H]MTX.

Short-term exposure to MTX was compared in ALL and AML blast cells and no differences in inhibition of DNA biosynthesis was detected. But when AML cells were washed and incubated in drug-free medium, DNA synthesis recovered more rapidly than in ALL cases, indicating that the rapid efflux of MTX was due to defective polyglutamylation.[20,21]

The basis for differences in long chain MTX polyglutamate accumulation between AML and ALL was investigated using leukemia cell lines and blasts from leukemic patients.[22] The major finding that emerged from this study was the different K_m values of FPGS with MTX as substrate between the AML and ALL cell lines (Figure 2). The K_m of FPGS in AML was over two-fold higher than the K_m of FPGS in ALL. This result was reproducible on patient samples from which similar data were obtained. These findings raised the possibility that isoenzymes are present in AML and ALL. The possibility that an inhibitor of FPGS enzyme was present in AML was excluded after purification of FPGS proteins in AML and ALL cells.

These findings of different FPGS activities in AML and ALL with different affinities for MTX may be explained by the identification of alternative splice variants of the FPGS gene, and provide further evidence for the tissue-specific encoding of the isoforms of FPGS.[23-25] It is also possible that these differences depend on a post-translational modification of both enzymes.[26]

As the metabolic turnover of the γ-polyglutamates is modulated by the γGH enzyme and with the evidence that enhanced γGH activity produces natural (inherent) or acquired resistance to methotrexate,[27-29] FPGS and γGH activities and accumulation of polyglutamates were measured in 15 blast samples from patients with AML and ALL. The ratio of FPGS and γGH enzyme activity at diagnosis seems to be a good predictor of the response to MTX therapy and outcome.[30-31]

Blasts from patients with M5 (acute monocytic leukemia) and patients with M7 (acute megakaryocytic leukemia) accumulate MTX-long chain polyglutamates, after *in vitro* exposure to [^{3}H]MTX, to similar levels as those found in childhood ALL blasts.[32,33] Thus, MTX may be effective in the treatment of acute monocytic and megakaryocytic leukemias, and deserves further study in these diseases.

Cell cycle genes and resistance to methotrexate

The deregulation of tumor suppressor genes has a critical effect on MTX resistance. During the G_1-S progression retinoblastoma gene product (pRb) is phosphorylated and free E2F is released from the complexes containing E2F and the hypophosphorylated form of pRb. The free E2F forms a heterodimer with its binding protein DP-1 or DP-2 and promotes the transcription of E2F target genes such as DHFR, TS, TK, DNA polymerase α, involved in entry and progression through the S phase of the cell cycle. E2F may regulate DHFR via a consensus E2F binding sequence located in the promoter region. As a consequence of loss of pRb function, there is an increase in E2F transcriptional activity that is a critical factor in the deregulation of the cell cycle and perhaps tumorigenesis and drug resistance.[34-36] (Figure 3). Lack of functional pRb was found to increase MTX and fluorodeoxyuridine resistance in an osteosarcoma cell line.[37] Osteosarcoma cells become more sensitive to MTX after introduction of a cDNA encoding pRb.[38] Although low levels of pRb were reported in about 20% of patients with ALL and AML, the correlation between lack of pRb and sensitivity of leukemia patients to chemotherapy has not yet been demonstrated.[39,40]

D type cyclins in association with cyclin-dependent kinases cdk 4 and cdk 6 participate in the phosphorylation and functional inactivation of the retinoblastoma gene product pRb. In particular, overexpression of cyclin D1, a gene usually expressed in early G_1 phase, in transfected fibrosarcoma cells resulted in increased levels of DHFR associated with decreased sensitivity to MTX.[41]

The expression of p53 can arrest cells at the G_1-S phase allowing DNA repair, thus increasing the ability of cells to survive DNA damage. On the other hand, p53 can promote apoptosis reducing cell survival.[42] The disruption of p53 enhances or reduces DNA-damage sensitivity in various tumor model systems.[43-45] In patients with ALL, p53 gene mutations were correlated with DHFR gene amplification and associated with MTX resistance. Three of 38 ALL patients who showed p53 mutations and DHFR gene amplification at diagnosis, were MTX resistant and relapsed and died within a year. Among a second group of relapsed ALL patients, those with p53 mutations had a poorer survival than those patients without p53 gene mutations.[12] As in certain other malignancies, in ALL patients, the presence of p53 mutations may be a negative prognostic factor.

Newer antifolates

Currently new antifolate drugs are in clinical development. These compounds have been designed in order to overcome the potential mechanisms of inherent or acquired MTX resistance observed in cell lines and patient samples, such as defective drug transport into cells, impaired polyglutamylation, decreased affinity of the drug for the folate-dependent enzymes, DHFR mutations and increased expression of DHFR protein. Compared to MTX, most of these newer antifolates show greater lipid-solubility, better membrane transport and/or improved ability to form long chain polyglutamates. Moreover other folate-dependent enzymes such as TS and GAR transformylase are specific targets for some of them.

Trimetrexate (TMTX) is a non-classic, quinazoline-derived lipophilic antifolate that achieves high intracellular concentrations in cells that are resistant to MTX because of defective transport. Unlike MTX, TMTX enters cells by a non energy-dependent pathway and is not polyglutamated. The high intracellular levels of TMTX achieved result in greater DHFR inhibitory activity than MTX, and depletion of the intracellular reduced folate pool and inhibition of *de novo* purine biosynthesis.[46] Resistance *in vitro* to trimetrexate has been shown to result from increased expression of p-glycoprotein, enhanced DHFR expression or reduced uptake via mechanisms not yet well understood. Although TMTX was developed to treat malaria, it is now used in combination with leucovorin against *Pneumocystis carinii* pneumonia infection and also as an anticancer drug. The effectiveness of this combination depends on the fact that TMTX enters *Pneumocystis carinii* by passive diffusion but leucovorin does not because there is no reduced folate membrane transport system in the parasite. The combination of TMTX with leucovorin was used in SCID mice bearing MTX-transport-resistant human ALL blasts, and tumor regression was obtained, with minimal toxicity.[47] This drug is currently in clinical trial to treat mycosis fungoides, and in patients with relapsed ALL and osteosarcoma (in combination with leucovorin) who are resistant to MTX.[48,49] Newer restricted analogs of TMTX are now under evaluation and show more potency and selectivity.[50]

Edatrexate (EDX) is a second generation antifolate DHFR inhibitor structurally similar to MTX. It is transported by the RFC into cells where is polyglutamated by FPGS. This mechanism, which is more efficient for EDX than for MTX, may explain the better EDX therapeutic index as compared to MTX.[51,52] Clinical trials with this agent are in progress, as well as with a third generation antifolate piritrexate, a N10-propargyl analog of EDX.

Thymidylate synthetase (TS) inhibitors

During recent years there has been a great deal of interest in developing folate inhibitors of TS, because of the activity of 5-fluorouracil (5Fura), that targets TS in epithelial cancers.

Raltitrexed (Tomudex®) is a pure TS inhibitor that enters the cells via the RFC active transport system and is a substrate for polyglutamylation by FPGS.[53] Raltitrexed is as effective in colon cancer treatment as 5Fura, and it has been shown in preclinical studies to be synergistic in combination with Fura.[54] It is now in phase II trials with 5Fura, oxaliplatin or irinotecan.[55,56] Preclinical *in vitro* studies showed its effectiveness against AML and ALL cells, and a clinical trial for AML is planned.[22]

MTA (Altima) (Multi-targeted antifolate, LY231514) inhibits TS, DHFR and GAR transformylase, and its transport appears to be more concentrative than that of MTX in leukemia cells.[57] This drug is now in phase III trials and has shown activity in phase II studies of several solid tumors. *GW1843U89* is a potent TS-inhibitor, which is converted intracellularly to the diglutamate form. Cytotoxic activity in leukemic cell lines has been shown previously,[22] and a phase I trial has been completed.

Purine synthesis inhibitors

Lometrexol [5,10-dideazatet rahydrofolate, DDATHF] is a specific inhibitor of GAR transformylase with consequent inhibition of the *de novo* synthesis of purines. Recently resistance to lometrexol in murine leukemia cells has been associated with mutations of the FPGS gene.[58] This drug is in phase II trial, with folic acid supplementation.

Conclusions

The continued understanding of the molecular mechanisms of inherent and acquired resistance to MTX have led to the development of second and third generation antifolates that have improved therapeutic indices compared to MTX, e.g., EDX, or are effective against tumors that are resistant to MTX, e.g., TMTX with leucovorin protection. Folate-requiring enzymes in addition to DHFR have also been targeted, and new folate analogs that are potent inhibitors of TS and glycine amidoformyl transferase have been developed. Finally, the generation of drug-resistant variants of DHFR and TS has been utilized for gene transfer to marrow progenitors, to protect this organ from cytotoxicity of folate antagonists.[59] These advances are in various stages of pre-clinical and clinical development, and provide the basis for improved treatment of patients with cancer.

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Both authors contributed equally to this paper.

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