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CHAPTER 1

Antimetabolites

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1. Introduction

Ongoing basic research continues to focus on the mechanisms of cytotoxicity for each of the antimetabolites. Major emphasis has also been placed on including pharmacokinetic and pharmacodynamic endpoints in clinical trials to help elucidate the optimal method of administration of single agents as well as the combination of two or more drugs. The various studies reviewed in this year's chapter provide further insight into the mechanisms of action of the antimetabolites. As will be described, emerging understanding of the biochemical and molecular determinants of drug sensitivity have provided new therapeutic strategies.

2. Methotrexate

Methotrexate (MTX) is a tight-binding inhibitor of the enzyme dihydrofolate reductase (DHFR), an essential enzyme in intracellular folate metabolism. DHFR is necessary for the conversion of dihydrofolate to tetrahydrofolate, and the reduced folates are key intermediates in one-carbon transfer reactions. An intact enyzme pathway is necessary to maintain de novo synthesis of purines and thymidine monophosphate (thymidylate). For this reason, DHFR represents a critical target enzyme in cancer chemotherapy.

2.1. *Mechanism of action*

The precise mechanism(s) by which MTX exerts its cytotoxicity remains a subject of ongoing debate. The long-held belief has been that inhibition of DHFR by MTX leads to a depletion of intracellular levels of reduced folate cofactors, with subsequent impairment in de novo synthesis of purines and thymidylate. However, studies from several laboratories have demonstrated that the level of reduced folates is depleted by only 50–70% (Annuals 10–17). Such a modest decrease in the reduced folate pool would seem to be insufficient for the cytotoxic effects observed following treatment with MTX. It is now known that the polyglutamates of both MTX and dihydrofolate, which accumulates in the presence of MTX-mediated DHFR blockade, are capable of directly inhibiting the activity of several folate-dependent enzymes in addition to DHFR, including thymidylate synthase (TS), glycinamide ribonucleotide (GAR) transformylase, and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase. Thus, metabolic inhibition by MTX represents a multifactorial process that involves partial depletion of key reduced folate substrates and direct inhibition of various folate-dependent enzymes.

Fiskerstrand et al. [1] investigated the effects of MTX-mediated folate depletion on the activity of methionine synthase. This enzyme

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catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine, thus forming methionine. Cobalamine is an important cofactor in this reaction pathway. Treatment of GaMg human glioma cells with ≤ 1 μM MTX resulted in a dose- and time-dependent reduction in both the total folate and 5-methyl-tetrahydrofolate pools in this cell line; the latter pool size was reduced by 50% at 3 h, and was barely detectable at 48 h. A significant dose-dependent reduction in the activity of methionine synthase coincided with folate depletion. MTX also reduced the intracellular methyl-cobalamine content, presumably the result of an inadequate supply of 5-methyl-tetrahydrofolate cofactor required for remethylation of homocysteine. Given that methionine synthase catalyzes a reaction that involves reduced folates, cobalamine, and sulfur amino acids, impaired function of this enzyme might have a significant impact on a number of critical 'downstream' pathways, including transmethylation reactions, polyamine synthesis, and protein biosynthesis. These findings suggest another potential cytotoxic mechanism for MTX.

Schalinske and Steele [2] employed an in vivo rat model to investigate the effects of MTX on folate-dependent, one-carbon metabolism using a sensitive tracer kinetic method to quantify the carbon flux through this pathway. Following a 7-day treatment with MTX, hepatic pools of tetrahydrofolate, 5-methyl-tetrahydrofolate, and 5-formyl-tetrahydrofolate were decreased by 63, 83 and 58%, respectively. Compared to control animals, carbon flux through the one-carbon pool from histidine to methionine was significantly reduced by nearly 60% in MTX-treated rats. These kinetic experiments demonstrate MTX treatment markedly alters the actual carbon flow through the hepatic folate-dependent, one-carbon pool, and a major effect is a reduction of carbon flow needed for the formation of both 5-methyl-tetrahydrofolate and methionine.

Nesher et al. [3] investigated the in vitro effects of MTX on polyamine levels in lymphocytes obtained from patients with rheumatoid arthritis (RA). Dose-dependent reductions in intracellular spermidine and spermine were noted, while putrescine was unaffected. Addition of either folinic acid or *S*-adenosyl-methionine prevented MTX-induced inhibition of polyamine synthesis. In contrast, incubation with hydrocortisone or D-penicillamine, two other immunosuppressive agent used in the treatment of rheumatoid arthritis, had no effect on polyamine levels. These findings suggest that inhibition of the *S*-adenosyl-methionine-dependent methyltransferase pathway by MTX interferes with spermidine and spermine synthesis in RA lymphocytes. This effect may account for the immunosuppressive action of MTX, but also may represent an alternative cytotoxic mechanism of MTX in malignant cells that are especially dependent upon polyamine biosynthesis for growth and proliferation.

In several different model systems, pretreatment of malignant cells with MTX results in the rapid intracellular accumulation of 5-phosphoribosyl pyrophosphate. This effect has been exploited as a means to biochemically modulate the antitumor activity of fluorouracil by enhancing its anabolism to the ribonucleotide level. However, studies using a chick fibroblast system suggest that PRPP may regulate the intracellular synthesis of glucose transporters. With this in mind, Fung and colleagues [4] examined the potential relationship between MTX treatment, PRPP synthesis, and glucose transport as it relates to cytotoxicity in cultured Ehrlich ascites tumor cells. Treatment with up to 20 μM MTX resulted in a 2- to 3.5-fold increase in PRPP levels, accompanied by a significant suppression in the rate of glucose transport. Co-administration of 20 μM hypoxanthine with MTX completely protected against growth inhibition, and reversed the effect of MTX on PRPP production and the rate of glucose transport. Since glycolysis serves as a major energy supply for malignant cells, these findings suggest MTX-mediated inhibition of critical glucose transport mechanisms might starve cells of essential nutrients required to maintain cellular metabolism and growth.

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2.2. *Folate transport*

Two major folate transport systems in human tissues have been well characterized at the molecular level. One is the classic reduced folate carrier (RFC) system, that has a relatively low affinity for reduced folates (affinity constants in the micromolar range). However, the RFC system has a large capacity, and is primarily responsible for MTX transport into cells at pharmacological drug concentrations. The human reduced folate carrier gene has been mapped to the long arm of chromosome 21, and it encodes a protein with a predicted molecular size of 59–68 kDa (Annual 17). A second folate transport system involves a high-affinity membrane-bound, folate receptor binding protein (affinity constants for folic acid in the nanomolar range); this system has a much reduced capacity for transport of reduced folates and MTX relative to the RFC system. The human folate receptor (FR) is a 38–40 kDa glycoprotein bound to cellular membranes by a carboxyl-terminal, glycosyl-phosphatidylinositol tail. This receptor is highly expressed on the surface of some epithelial tumors, such as ovarian cancer, making it a potentially useful target for antigen-directed anticancer therapies [5]. The human FR appears to be the major transport mechanism for the uptake of non-classical antifols such as N^{10}-propargyl-5,8-dideazafolic acid (CB3717) and (6*R*)-5,10-dideaza-5,6,7,8-tetrahydrofolic acid (lometrexol, DDATHF).

Because of the important role of the RFC in providing MTX transport, there is great interest in how the activity of the RFC gene is regulated. When cultured human leukemia CCRF-CEM cells were grown in folate-depleted media and then exposed to high concentrations of the reduced folate, leucovorin, down-regulation of the RFC protein on the cell surface was observed [6]. In contrast, addition of trimetrexate, a lipophilic DHFR inhibitor, decreased intracellular reduced folate pools and blocked RFC down-regulation, suggesting that the relative size of the intracellular reduced folate pool may be an important determinant of RFC expression. The regulation of RFC activity was also affected by other biochemical pathways dependent upon intracellular folates, such as de novo purine synthesis and DNA methylation reactions. Incubation of CCRF-CEM cells with either adenosine or *S*-adenosyl methionine also caused RFC down-regulation, but the underlying mechanism appeared to be independent of reduced folate pools. Thus, several diverse folate-dependent biochemical pathways may contribute to the complex regulation of RFC expression.

Relationships between MTX resistance and relative RFC expression were explored further in studies by Moscow et al. [7]. Transfection of RFC cDNA into a transport-deficient, MTX-resistant human breast cancer line rendered them 250-fold more sensitive to MTX. The transfected cells were 300-fold more resistant to trimetrexate, which enters cells through passive diffusion. The basis for trimetrexate resistance was attributed to enhanced uptake of reduced folates by the RFC system, which rescued cells from DHFR inhibition. Thus, increased expression of the RFC had disparate effects on sensitivity to antifolates that utilize different mechanisms for cellular entry.

Gorlick et al. [8] employed a flow cytometry method to assess MTX transport in human leukemic blast cells in which the competitive displacement of PT430, a fluorescent MTX analog, reflects reduced folate transport. Impaired MTX transport was observed in only 13% of untreated patients, compared with over 70% in patients who had relapsed following prior MTX-containing chemotherapy. Quantitation of RFC mRNA expression indicated that impaired transport activity was associated with decreased RFC mRNA expression, which supports reduced transport capacity as an important mechanism of clinical MTX resistance.

The molecular changes responsible for MTX-resistance was examined in a transport-deficient human T-cell lymphoblastic cell line [9]. No difference in RFC mRNA levels by reverse transcription–polymerase chain reaction (RT-PCR) methodology was noted compared with parental wild-type, MTX-sensitive cells. However, nucleotide se-

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