Cancer Chemotherapy and Biological Response Modifiers Annual 17

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CHAPTER 1

Antimetabolites

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1. Introduction

In the past year, significant research has been directed towards determining the mechanism of cytotoxicity for each of the antimetabolites, and the design and development of new analogs continues to be an exciting area. Considerable efforts have also been made to include pharmacodynamic endpoints into clinical trials to provide a more complete understanding of the intricacies of combination chemotherapy. The various studies reviewed in this year's chapter provide further insight into the mechanisms of action of the antimetabolites and offer a more detailed understanding of both the biochemical and molecular determinants of sensitivity and resistance to these agents that is required for the design of future treatment strategies.

2. Methotrexate

Methotrexate (MTX) is a tight-binding inhibitor of the enzyme dihydrofolate reductase (DHFR), an important enzyme involved in maintaining intracellular folate homeostasis. This enzyme catalyzes the conversion of dihydrofolate (H2PteGlu) to tetrahydrofolate (H4PteGlu) with the reduced folate being a key intermediate in one-carbon transfer reactions. An intact enzyme function is therefore critical for the maintenance of de novo purine and thymidylate biosynthesis as well as for protein synthesis and various methylation pathways. As a result, DHFR represents an important target enzyme in cancer chemotherapy.

2.1. Mechanism of action

The precise mechanism(s) by which MTX exerts its cytotoxic effects remains the focus of considerable research efforts. The long-held view was that treatment with MTX resulted in depletion of the intracellular reduced folate cofactor pool via inhibition of DHFR and that depletion of these critical one-carbon donor substrates was then associated with inhibition of de novo purine and thymidylate biosynthesis. However, as has been reviewed in previous issues (Annuals 10–15), several investigators have provided evidence that the level of intracellular reduced folates is reduced by only 50–60% in response to MTX treatment, a level that would appear to be insufficient to completely account for the marked cytotoxic effects of MTX. Moreover, several groups have demonstrated that both MTX polyglutamates and dihydrofolate polyglutamates are able to directly inhibit folate-dependent enzymes other than DHFR including thymidylate synthase (TS),

glycinamide ribonucleotide (GAR) transformylase, and aminoimidazolecarboxamide ribonucleotide (AICAR) transformylase (Annuals 10–15). Taken together, these studies suggest that metabolic inhibition by MTX is a complex process mediated by both reduced folate depletion and direct inhibition of key folate-dependent enzyme pathways.

There remains considerable debate as to the relative contribution of purine and thymidylate inhibitory effects in determining the ultimate cytotoxicity of MTX and other antifolate analogs. Several studies have demonstrated that the cytotoxic activity of MTX is prevented to a varying degree by exogenous thymidine. In contrast, exogenous administration of purines has been shown to markedly potentiate MTX (Annuals 10–12, 16). Given these observations, it was postulated that inhibitors of DHFR that alter both de novo purine and thymidylate synthesis may be less cytotoxic than pure TS inhibitors since the resulting imbalance in deoxyribonucleotide triphosphate (dNTP) levels may be less severe. The development of new antifolate analogs with selective inhibitory effects on different folate-dependent enzymes involved in purine and pyrimidine synthesis provide important new tools for studying the effects of MTX treatment on folate and nucleic acid metabolism. In this regard, Chong and Tattersall [1] investigated the effects of the de novo purine synthesis inhibitor 5,10-dideazatetrahydrofolate (DDATHF) on the cytotoxic activity of the folate-based inhibitors of TS, ICI D1694 and CB3717. Using the murine leukemic L1210 cell line as their model system, they observed that DDATHF, in a dose-dependent manner, reduced the cytotoxicity of both ICI D1694 and CB3717. Following treatment with either ICI D1694 or CB3717 a significant reduction in dTTP pools occurred with a concomitant time-dependent increase in dATP levels that reached a maximum at 12 h following drug treatment. Addition of DDATHF, however, prevented the rise in dATP levels seen after ICI D1694 or CB3717 treatment. In fact, dATP levels were reduced to approximately 30% of that observed with either drug alone. Thus, the findings from this study provide further evidence that the imbalance in intracellular dTTP and dATP pools represents an important determinant of cytotoxicity in cells treated with antifolate compounds and provide support for the continued development of novel antifolate analogs that directly target thymidylate biosynthesis. Further studies are now required to determine the potential mechanisms by which dNTP pool imbalance results in cell death.

2.2. *Folate transport*

Folate transport in mammalian cells is characterized by two distinct transport systems. One system involves the folate receptor (FR), a membrane-associated, high affinity folate binding protein with affinity constants for folic acid and reduced folates in the range of 1 to 50 nM. This system is a less efficient transporter of methotrexate (MTX) with affinity constants ranging from 0.1 to 2 μM; however, it has high affinity for certain newer synthetic antifols, such as ZD1694 (tomudex), DDATHF (lometrexol), LY231514, and BW1843U89. Variable expression of the FR is found in a wide variety of normal tissues, but some malignant tumors, such as ovarian and cervical carcinomas express very high levels of the FR (Annual 16). Structurally, the FR is a membrane-associated, 38 kDa glycoprotein and it is anchored to the cellular membrane by a carboxyl-terminal glycosyl-phosphatidylinositol (GPI) tail. Proteolytic cleavage of this GPI linkage generates a hydrophilic soluble form of the FR which is present in serum and breast milk. The second folate transport system involves the reduced

folate carrier (RFC) which transports reduced folates, e.g. 5-methyltetrahydrofolate, and antifols, e.g. methotrexate, with an affinity constant ranging from 1 to 10 μM. The RFC is a much less efficient transporter of folic acid with affinity constants in the range of 200 to 400 μM.

Substantial progress continues to be made in the molecular characterization of the human RFC system. Over the past year, four separate laboratories independently isolated human cDNAs with high homology to one another, and their transfection into mutant cell lines completely restored reduced folate carrier activity [2–5]. These cDNAs encoded proteins with predicted molecular sizes of 58 to 68 kDa, and all contained consensus sequences for N-glycosylation. Using in situ hybridization, it was determined that one of the RFC cDNAs mapped to a locus on the long arm of human chromosome 21 [4]. The functional characteristics of these cDNAs strongly suggest that they code for the human RFC transporter; however, additional studies to confirm their biological function are in progress. Nonetheless, the development of these new molecular probes which are associated with human reduced folate and MTX transport activity should facilitate our understanding of the mechanism of reduced folate transport. Moreover, the characterization of the expression and distribution of this transporter in normal and malignant tissues should help define the role of this gene in clinical MTX transport resistance.

Recent molecular studies of the folate receptor transport system have identified at least three distinct FR isoforms in mammalian cells, and these have been referred to as FR-α (FR-1), FR-β (FR-2), and FR-γ [6]. The FR-α isoform was initially characterized in human nasopharyngeal KB carcinoma cells and it has often been detected in various epithelial neoplasms, with very high levels being present in most ovarian carcinomas (Annual 16). In contrast, the FR-β isoform was originally isolated from human placenta, and it is typically expressed in low to moderate amounts in most normal tissues with slightly higher levels occurring in many non-epithelial malignant tumors. More recently, a FR-γ isoform was characterized in human hematopoietic cells. Because FR-γ lacked an efficient signal for GPI modification, transfection and expression of FR-γ cDNA in Chinese hamster ovary (CHO) cells resulted in poor surface expression of the FR-γ protein [7]. However, because high concentrations of FR-γ were secreted into the tissue culture media, the FR-γ isoform may represent a secreted form of the folate receptor which is normally found at low concentrations in serum but in dramatically increased levels in folate deficient states. Like the other isoforms, FR-γ has a high affinity for folic acid; however, it does not display a stereospecific preference for binding to reduced folates as do the FR-α and FR-β isoforms [7].

The regulation of FR-α gene expression was studied in five different ovarian cancer cell lines following growth in tissue culture media containing high and low folate concentrations [8]. In four of five cell lines, stable changes in the expression of FR-α were not observed when the extracellular folate concentration was lowered to physiologic levels (2 nM). Only in one cell line (SKOV3) was there a persistent stable 2-fold increase in FR-α protein expression and this corresponded to a 1.5-fold increase in FR-α mRNA levels. These authors concluded that in vitro over expression of the FR-α isoform in ovarian cancer cell lines was generally not sensitive to extracellular folate concentrations. In another series of experiments, Orr and colleagues examined the regulation and expression of FR-α isoform in the human squamous cell carcinoma cell line, UMSCC38, which expresses four to six copies of the FR-α gene [9]. Despite the amplification of the gene, these

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