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Review paper

Characterization by flow cytometry and fluorescein–methotrexate labeling of hydrophilic and lipophilic antifolate resistance in cultured mammalian cells

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The aim of this review is to summarize currently available information on the rapid screening and initial characterization of the different mechanisms of resistance to hydrophilic [e.g. methotrexate (MTX)] and lipophilic antifolates [e.g. trimetrexate (TMTX)] in cultured mammalian cells using fluorescein–methotrexate (F-MTX) and flow cytometry. Toward this end an integrative F-MTX labeling and flow cytometry-based protocol is proposed here to facilitate the rapid identification of modes of antifolate resistance in a heterogenous drug-resistant cell population or in clonal derivatives. Following antifolate selection, drug-resistant cells are first labeled with F-MTX in order to saturate intracellular dihydrofolate reductase (DHFR). F-MTX-labeled cells are then subjected to flow cytometric analysis and mean fluorescence/cell is determined. Thus, increased F-MTX staining is an indication of overproduction of the target enzyme for antifolates, DHFR, as a result of DHFR gene amplification. In contrast, significantly reduced cellular F-MTX labeling could be an indication of the existence of a structurally altered DHFR displaying a decreased affinity for antifolates. Alternatively, antifolate-resistant cells frequently display wild-type F-MTX labeling; these cells are subjected to competition with hydrophilic and lipophilic antifolates in order to examine whether the process of antifolate accumulation is deficient. Cells that lose F-MTX labeling upon competition with lipophilic antifolates yet still retain it with hydrophilic antifolates, are likely to possess transport alteration(s) that impair or abolish the accumulation of hydrophilic but not of lipophilic antifolates. In contrast, cells that lose their F-MTX labeling after competition with hydrophilic antifolates but retain it with lipophilic antifolates, possess a deficient accumulation of lipophilic antifolates. The importance of the antifolate concentration yielding 50% displacement of cellular F-MTX labeling in the quantitative assessment of the degree of DHFR overexpression and/or antifolate transport alteration is discussed. Thus, flow cytometric analysis of F-MTX-labeled cells following competition with hydrophilic and lipophilic antifolates provides a useful tool for the rapid screening and assessment of the major modes of antifolate resistance that may appear as exclusive mechanisms or co-emerge in mammalian cells following antifolate selection.

Key words: Antifolate resistance, flow cytometry, methotrexate.

Introduction

The folic acid antagonist methotrexate (MTX) has gained wide-spread clinical use as an integral component of combination chemotherapy employed in the treatment of various human malignancies.[1] MTX is a high affinity inhibitor of the target enzyme dihydrofolate reductase (DHFR; EC 1.5.1.3). MTX, as well as other folic acid analogs, exert their cytotoxic effect on mammalian cells by blocking tetrahydrofolate biosynthesis, a key cofactor in a variety of metabolic pathways involving one-carbon transfer reactions. This results in interference with the biosynthesis of purines and thymidylate thus leading to inhibition of DNA synthesis and cell death. However, like many chemotherapeutic agents, the clinical use of the hydrophilic antifolate MTX has been limited by inherent and acquired anticancer drug resistance. Several mechanisms whereby mammalian cells can acquire resistance to MTX have been documented: (i) DHFR gene amplification and subsequent overexpression of this target enzyme,[2] (ii) alterations in the carrier-mediated transport of MTX that reduce[3–9] or abolish[10] its entry into mammalian cells, (iii) reduced affinity of DHFR for MTX due to point mutations in active site residues,[11–18] and (iv) reduced cellular retention of MTX polyglutamates due to quantitative or qualitative alterations in the enzyme folylpoly-γ-glutamyl synthetase (FPGS; EC 6.3.2.17) activity.[19–27]

During the past decade many studies have focused on the elucidation of the various

mechanisms that underlie resistance to hydrophilic and lipophilic antifolates. In this respect there is a growing need for the development of new methodologies for the rapid and reliable assessment of the different modalities of anti-cancer drug resistance in a heterogenous population of drug-resistant (e.g. antifolate-resistant) cells, such as in tumor-derived cells obtained from patients after chemotherapy. In one approach, in order to rapidly screen for one of the major mechanisms of antifolate resistance in cultured mammalian cells, i.e. DHFR gene amplification, a fluorescein-conjugate of MTX (F-MTX) was first introduced by Gapski *et al.*[28] Although initially used only to quantify cellular DHFR content in flow cytometric studies[10,12,29], F-MTX has proved to be an important intracellular probe in the identification of the various mechanisms of antifolate resistance.[10,12,30–32] It is the intent of this review to summarize published information on flow cytometric studies with F-MTX which assess mechanisms of resistance to hydrophilic and lipophilic antifolates in cultured mammalian cells. In this respect, a F-MTX-based flow cytometric protocol is presented here to facilitate the rapid identification of the various modalities of antifolate resistance in cultured mammalian cells.

Identification of mechanisms of antifolate resistance using fluorescent MTX analogs and flow cytometry

F-MTX

The high-affinity binding of MTX to DHFR is derived primarily from its diamino-substituted pyrimidine core.[33] Thus, Gapski *et al.*[28] have introduced an FITC moiety to the distal α- and γ-carboxyl groups of MTX via a diaminopentyl spacer using a carbodiimide coupling reaction. Gaudray *et al.*[32] have significantly improved this carbodiimide reaction, thus yielding a chromatographically pure F-MTX, free of uncoupled MTX and FITC. Relatively high yields of 20% are obtained in this improved synthesis as compared with the roughly 1% yield in the original method.[28] Consequently, F-MTX became commercially available (Molecular Probes, Eugene, OR). Similarly, the distal γ-carboxyl group of the glutamate residue of MTX has been used by Rosowsky *et al.*[34] as an acceptor group to which an FITC moiety was attached via a lysine linking group. Kumar *et al.*[35,36] have coupled the fluorochrome dansyl chloride to lysine or ornithine analogs of MTX.

Accumulation of F-MTX in sensitive and antifolate resistant cells

Hydrophilic folic acid antagonists such as MTX accumulate within mammalian cells via a high-affinity carrier-mediated transport system.[3–10] MTX is rapidly taken up by mammalian cells and attains steady-state levels within 20–30 min. In contrast, the uptake of F-MTX in mammalian cells is slow and saturation is achieved only after 8 h or longer.[29,37] The introduction of FITC to MTX renders F-MTX a bulky and lipid-soluble chromophore that fails to enter mammalian cells via the carrier-mediated transport of MTX. The lipophilicity of F-MTX allows for its accumulation in mammalian cells via diffusion. Indeed, flow cytometric characterization of F-MTX transport in mammalian cells has revealed that F-MTX enters mammalian cells via a mechanism distinct from the energy-dependent transport system of MTX;[37] thus, it was concluded that F-MTX accumulates in mammalian cells by a passive and/or facilitated diffusion process.[37] This was supported by the following evidence. (i) F-MTX accumulated equally well in wild-type cells as well as in mutant cells defective in the inward transport of MTX. (ii) Folic acid and/or reduced folates that block the transport of MTX inward, did not affect F-MTX accumulation. (iii) The influx of MTX exhibits a strong temperature dependence[38] (Q_{10} = 6-8), whereas the inward uptake of F-MTX displayed a low temperature coefficient[37] (Q_{10} = 1.6). (iv) Sulfhydryl reactive mercurials which block MTX transport did not reduce F-MTX transport but rather stimulated it.

Binding and specificity of F-MTX to mammalian DHFR

The apparent dissociation constant (K_d) for MTX in a MTX–DHFR–NADPH ternary complex using a purified enzyme from animal or human origin was in the picomolar range.[11,12,18,39–41] The high-affinity binding of the 4-amino folate analog MTX to DHFR is stoichiometric and derives primarily from the diamino-pyrimidine core contained in the pteridine structure.[33] Thus, an FITC moiety was conjugated to the remote α- and γ-carboxyl groups of the glutamate residue in MTX, resulting in a fluorescein conjugate of MTX that was found to inhibit mammalian DHFR only 5- to 10-fold less efficiently than MTX.[28,32,42] Although the glutamate residue of MTX poorly contributes to its binding

to DHFR, it is likely that the presence of the large FITC group renders F-MTX a bulky molecule that does not fit the hydrophobic pocket formed in the active site of DHFR so well.

The specificity of mammalian cells' labeling with F-MTX has been approached experimentally by the evaluation of three parameters: the first involves the routine recording of the basal fluorescence (i.e. autofluorescence) emitted from cells that were not labeled with F-MTX. For example, the autofluorescence in cultured CHO cells consisted of approximately 15% F-MTX labeling.[10,29–32,37] The second parameter has been the determination of F-MTX staining of animal cells that are devoid of DHFR as a result of a deletion of the diploid DHFR locus.[43,44] The fluorescence of DHFR-deficient cells was comparable with that of wild-type cells.[10,29,44] The third and key parameter used to distinguish specific from non-specific F-MTX labeling has been the use of MTX competition. As detailed above and discussed hereafter, the interaction of F-MTX with the target enzyme DHFR is of a high-affinity type; this permits the assessment of the specificity of F-MTX labeling by exposing cells to increased concentrations of MTX. Based on the preferred affinity of DHFR for MTX than for F-MTX,[32] increased MTX concentrations yield a consistent loss of fluorescence from F-MTX labeled cells.[10] Thus, any F-MTX staining in wild-type cells that is not sensitive to competition with MTX should be regarded as a non-specific component of the labeling.[32]

The binding of fluorescein analogs of MTX to purified DHFR results in a 4- to 5-fold increase in their emitted fluorescence.[36,42,45] There are at least two factors that are known to increase the emitted fluorescence of a chromophore. (i) The lipophilicity of the solvent and/or the surrounding milieu of the fluorophore; the greater the hydrophobicity of the solvent is, the larger the increase in emitted fluorescence. (ii) The extent of the immobility of the chromophore; the greater the immobilization of the chromophore is, the larger the fluorescence enhancement. Thus, it is likely that the enhancement in the fluorescence emitted from F-MTX following its binding to DHFR is a result of hydrophobic interactions of this chromophore with active site residues of DHFR. In addition, the tight binding of F-MTX to DHFR confers a large degree of immobilization to the former. These processes could explain the increased quantum yield that is followed by the enhancement of fluorescence of F-MTX upon binding to DHFR[36,42,45] (YG Assaraf and M Nakache, unpublished data).

Competition of F-MTX labeling with antifolates

Although the primary target of F-MTX is DHFR, one cannot rule out the possibility that F-MTX may also interact with folate/reduced folate/MTX active transporters,[38] with soluble and membrane-bound folate receptors,[46,47] as well as with enzymes involved in folate/reduced folate metabolism including FPGS.[19–27] However, the folate/reduced folate/MTX transporter, as well as the folate receptor, display an affinity for MTX in the micromolar range, whereas mammalian DHFR has a 1000- and 100-fold higher affinity for MTX and F-MTX, respectively; this suggests that F-MTX will interact predominantly with the high affinity target enzyme, DHFR.

The finding that mammalian DHFR displays a 10-fold higher affinity for MTX than for F-MTX suggested that when bound to DHFR, F-MTX could be readily displaced after exposure to competition with MTX. Indeed, various experiments have shown that F-MTX-labeled cells rapidly lose their fluorescence following competition with relatively low concentrations of MTX[10,29,48] as well as other high-affinity lipophilic analogues of MTX including trimetrexate (TMTX) and piritrexim (PTX). The antifolate competition of F-MTX labeling in mammalian cells proved a useful tool in the establishment of a number of flow cytometric assays aimed to determine MTX and TMTX levels in sera of cancer patients,[48] as well as to rapidly screen for, and provide an initial characterization of the various mechanisms underlying resistance to hydrophilic and lipophilic antifolates (Figure 1).

Overproduction of DHFR

As early as three decades ago, Bertino *et al.*[49] ascribed MTX resistance to elevated levels of DHFR in leukocytes and erythrocytes of patients treated with MTX for several weeks. The mechanism underlying overexpression of DHFR was discovered by Schimke and co-workers,[2,50] who demonstrated that normal and malignant cultured mammalian cells may acquire resistance to MTX due to DHFR gene amplification. Thus, various fluorescent analogs of MTX[28,34–36] were initially introduced in an attempt to determine the intracellular content of DHFR in cultured mammalian cells. In pioneering studies, Kaufman *et al.*[42] devised a F-MTX-labeling flow cytometric technique for the rapid measurement of DHFR levels in

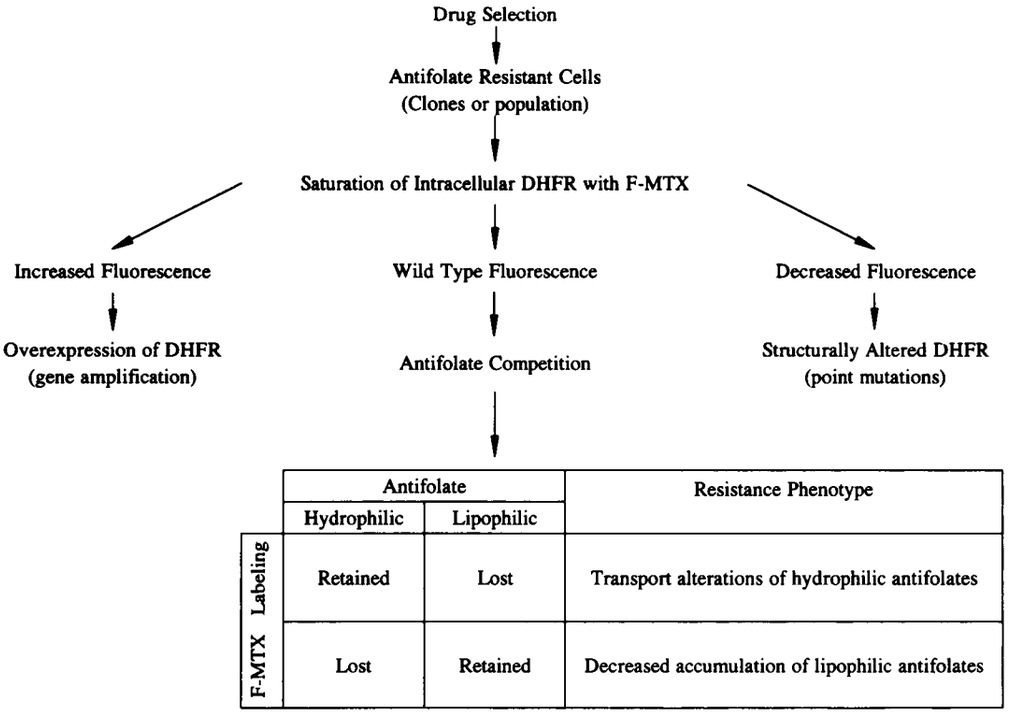

Figure 1. An integrative protocol for the rapid assessment of the various mechanisms of antifolate resistance in cultured mammalian cells using F-MTX labeling and flow cytometry.

individual cells. It was demonstrated in this work that both DHFR-specific activity and the rate of synthesis of this enzyme are proportional to the mean F-MTX fluorescence/cell. However, subsequent to the commercial availability of a pure F-MTX preparation that was free of any contaminating non-fluoresceinated MTX, several studies have shown that mean F-MTX fluorescence/cell does not reflect the DHFR gene copy number and the specific activity in a linear manner. For example, CHO B11 0.5 cells that display a 104-fold increase in the specific activity of DHFR exhibited only a 10-fold increase in F-MTX fluorescence/cell as compared with their drug-sensitive parental cells.[29,30,52] Thus, Assaraf *et al.*[52] have used MTX competition of F-MTX labeling with MTX; as discussed below, the antifolate concentration that yields 50% displacement (DC_{50}) of initial F-MTX labeling proved to be a valuable parameter. The ratio of the DC_{50} values obtained with the DHFR overexpressing cells and their wild-type counterparts tightly correlated with the levels of enzyme, e.g. when using MTX as a competing antifolate, this analysis revealed a DC_{50} ratio of 106 as compared with the 104-fold overexpression of DHFR enzyme molecules per B11 0.5 cell.[52] Thus, it appears that saturating F-MTX labeling followed by antifolate competition and DC_{50} ratio analysis is a sensitive and reliable measure for DHFR overexpression. This should be taken with some caution as drug resistant cells should be verified for an intact accumulation of the competing antifolate: in the case of an altered MTX transport, a high-affinity lipophilic antifolate competition (e.g. TMTX) should be used. Additionally, this approach of analyzing individual cells was also aimed at determining the heterogeneity in DHFR levels within various populations of parental as well as MTX-resistant cells.[42] This methodology proved useful as various studies reported the successful use of fluorescent analogs of MTX and flow cytometry for the assessment of DHFR content in individual cells selected for antifolate resistance[23,30,34,45,51–53] or in mammalian cells with no prior drug treatment yet showing spontaneous DHFR gene amplification.[54] F-MTX labeling and flow cytometry also proved as an efficient tool in the isolation of cells that are devoid of DHFR[43–44] as well as in mammalian cell transfectants expressing variable yet poor levels of DHFR.[32]

Overexpression of DHFR and P-glycoprotein in lipophilic antifolate resistant cells

Selection of cultured mammalian cells with the lipophilic antifolate TMTX, which maintains the high-affinity binding properties of MTX to DHFR, resulted in complex resistance phenotypes;[51,55,56] TMTX-resistant cells displayed overproduction of DHFR as well as of the multidrug resistance (MDR) gene product, P-glycoprotein (P-170), as a result of a consistent amplification of both the DHFR and MDR genes in individual cells.[30,51] This conclusion that individual drug-resistant cells simultaneously overexpress DHFR and P-170 was made possible by a flow cytometric analysis of individual cells concomitantly stained with F-MTX and daunorubicin; in this assay TMTX-resistant cells appeared with increased green fluorescence due to increased expression of DHFR and thus increased F-MTX labeling; at the same time these cells contained a poor red fluorescence as they overexpressed P-170 that actively extruded daunorubicin out of these MDR cells.

Reduced affinity of DHFR for antifolates

The importance of DHFR to the biochemistry of the cell and to the antifolate treatment of a variety of malignant and non-malignant diseases has made this enzyme the focus of numerous studies on its structure and function. Thus, crystal structures of DHFR from bacterial,[57] avian,[58] animal[59] as well as human origin[60] have been determined. Despite the importance of DHFR as a target for chemotherapy, relatively little work has been done to systematically investigate the role of active site residues of this enzyme. A better understanding of the structure –function relationships of mammalian DHFR started to emerge through analysis of mutants with altered catalytic activity and antifolate inhibition properties; although much less frequent than MTX transport alterations and DHFR gene amplification, antifolate-resistant mutants with altered (i.e. reduced) DHFR affinity for folate analogs were isolated and characterized. In parallel to this, site-directed mutagenesis studies of the DHFR gene were undertaken in an attempt to determine the importance of amino acid residues at the putative active site. These time-consuming studies could have been accelerated if mammalian cell cultures bearing resistance to MTX could have been rapidly screened on a single-cell basis and initially characterized for altered DHFR. Brown and Schimke[12] observed that MTX-resistant mouse 3T6-R400 cells highly overproducing DHFR stained poorly much to their surprise (i.e. at wild-type drug-sensitive cell level), even when using saturating concentrations of F-MTX. This observation was the basis for a thorough characterization of a structurally altered DHFR that displayed a 270-fold reduction in its affinity for MTX, a 20-fold decrease in dihydrofolate (i.e. the preferred substrate of DHFR) turnover number, as well as a 3-fold increase in the K_m for this substrate.[12] The dramatic reduction in the affinity of DHFR for MTX was subsequently shown to originate from a leucine to arginine substitution at position 22.[13] It was suggested that the arginine would protrude in the mutated active site more than the leucine residue in the wild-type active site and thus could hinder the positioning of antifolates either by its steric effect or by allowing the penetration of water molecules into the active site pocket.[61]

These studies suggest that when using appropriate controls (e.g. wild-type drug-sensitive cells), saturating F-MTX labeling and flow cytometry could serve as an invaluable tool for the rapid and facile identification of cells displaying a reproducible and significant reduction in F-MTX labeling in a heterogenous antifolate-resistant cell population. These cells that could be sorted out are likely to possess a structurally altered DHFR.[12,13]

Gaudray *et al.*[32] raised the possibility that reduced F-MTX labeling may reflect an altered (i.e. decreased) entry of F-MTX and not necessarily altered antifolate binding to DHFR. However, several lines of evidence suggest that mammalian cells do not contain a diminished accumulation of F-MTX and that, within a given species, cellular F-MTX labeling appears to be reproducible and consistent. First, as mentioned previously, unlike MTX uptake, F-MTX accumulation in mammalian cells occurs via diffusion and/or facilitated diffusion, which are clearly energy-independent processes.[37] Second, F-MTX accumulation is distinct from the carrier-mediated transport route of MTX because mammalian cells which are completely deficient in the transport of MTX inward[10] accumulate wild-type levels of F-MTX.[37] Third, a large number of MTX-resistant rodent clonal variants[10,51,62] as well as lipophilic antifolate-resistant cells[30,31,51,55] display wild-type F-MTX labeling when no qualitative or quantitative changes in DHFR could be observed; these suggest that changes in F-MTX labeling do not appear to result from potential alterations in F-MTX accumulation *per se*.

Decreased accumulation of hydrophilic and lipophilic antifolates

Membrane transport of folate analogs[3–10,38,63,64] is a key factor in the effectiveness of antifolate-containing chemotherapy.[1] Thus, a better knowledge as to the properties of this transport process is not limited only to biochemical interest but bears pharmacological significance as well. MTX and other 4-aminofolate analogs accumulate in mouse cells by an energy-dependent transport system,[3,9,63,64] the preferred substrate of which is the circulating plasma folate, 5-methyltetrahydrofolate.

Resistance to the classical hydrophilic antifolate MTX is observed as a primary phenomenon in tumor cells or it can be acquired by sensitive cells that undergo antifolate selection. Impaired membrane transport of MTX has been well established as an important and frequent mechanism of resistance.[3–10,38,63–65] Although a genetic and molecular characterization has not been achieved to date, transport defects are known to result from quantitative (reduced influx V_{max}) or qualitative (increased influx K_m) alterations of the reduced folate/MTX transport system, or changes in both parameters.[3–10,38,63–65] However, regardless of the exact nature of the qualitative and/or quantitative MTX transport alteration, transport defects have been characterized by reduced levels of free antifolate within the intracellular compartment.[3–10,38,63–65] Thus, given the various MTX transport alterations that appear in clonal variants following antifolate selection as well as the subsequent reduction in the intracellular concentra-

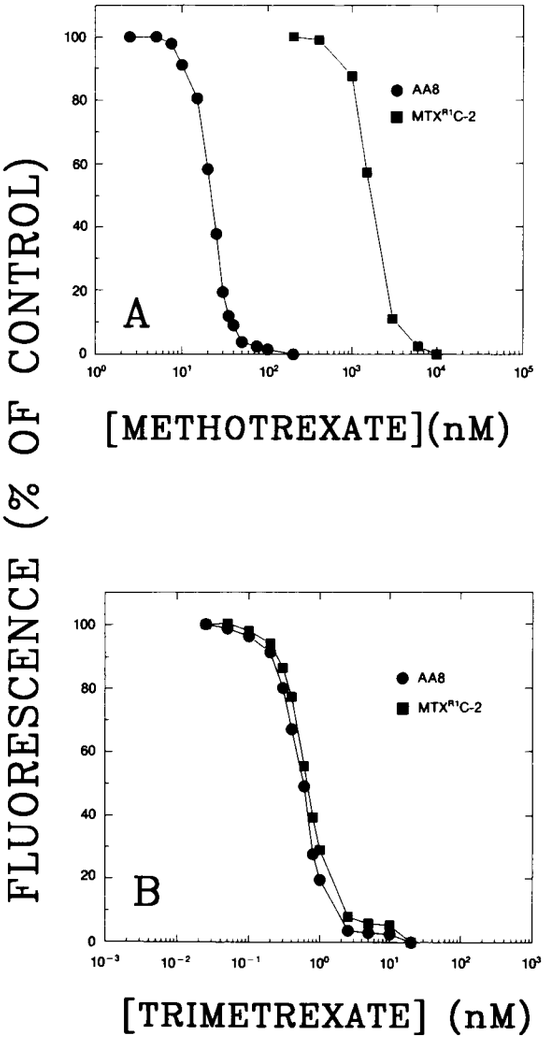

Figure 2. Displacement by hydrophilic and lipophilic antifolates of F-MTX labeling in parental CHO cells and their MTX transport deficient clonal variant $MTX^{R1}C$-2. Following growth for several generations in antifolate-free medium (α-minimal essential medium; Biological Industries, Beth Haemek, Israel), exponentially growing parental AA8 cells and their MTX-resistant $MTX^{R1}C$-2 clonal derivative (YG Assaraf, unpublished data) were stained for 8 h in medium containing 2 μM F-MTX (Molecular Probes, Eugene, OR). F-MTX staining medium was also supplemented with 5% dialyzed fetal calf serum (dFCS; Biological Industries) in order to prevent any competition with folates or their reduced forms (i.e. tetrahydrofolates) present in whole serum. The medium also contained 30 μM each of glycine, hypoxanthine and thymidine in order to protect labeled cells from the cytotoxic effect of F-MTX. Following saturation of intracellular DHFR with F-MTX some parental AA8 and $MTX^{R1}C$-2 cultures were exposed to competition with various concentrations of MTX (A) or TMTX (B) to initiate F-MTX displacement. Control cultures of parental AA8 and $MTX^{R1}C$-2 cells received an antifolate-free medium. Cells were then incubated for 2–3 h after which monolayer cells were washed twice with phosphate-buffered saline (PBS), briefly trypsinized, and resuspended to a density of 3×10^5–10^6/ml in ice-cold PBS containing 1% dFCS. Cells were then analyzed for mean linear fluorescence per cell by a flow cytometer. Flow cytometric analysis was carried out on a Becton-Dickinson FACStar plus flow cytometer (Becton-Dickinson, San Jose, CA). F-MTX was excited with a 488 nm line from an argon ion laser (Coherent, Palo Alto, CA). Emitted cellular fluorescence was collected through a 525 nm bandpass filter. A minimum of 10^4 cells were analyzed per each determination. Mean linear fluorescence values shown were corrected for the individual autofluorescence (about 15% of F-MTX labeling) emitted from unlabeled cells. Based on the fluorescence of control cells that were not exposed to competition with antifolates, the percentages of F-MTX displaced versus antifolate concentration were plotted and the 50% displacement concentration was derived for each antifolate used in each cell line.

tion of MTX, a single cell-based flow cytometric protocol that utilizes F-MTX labeling is presented here (Figure 1) in an attempt to identify the various modalities of MTX resistance in a heterogenous antifolate-resistant cell population.[10,29–32,42,44,45,51,52,54,62] In this assay antifolate-resistant cells are first labeled with F-MTX for 8–9 h in order to saturate intracellular DHFR. Cells that display wild-type F-MTX labeling are then exposed to competition with various concentrations of hydrophilic and lipophilic folate analogs (Figures 2 and 3). This competition of cellular F-MTX labeling is designed to probe for the ability of drug-resistant cells to accumulate antifolates. The larger the intracellular concentration of the antifolate is, the greater the competition of DHFR-bound F-MTX; subsequent to competition is the dose-dependent loss (i.e. displacement) of F-MTX labeling[10] (Figure 2A). Thus, cells that retain their initial F-MTX staining upon challenge with hydrophilic antifolates (Figure 2A), still lose it with a lipophilic antifol (Figure 2B), and possess a reduced (or abolished) hydrophilic antifolate (e.g. MTX) accumulation[10,52,62] (Figure 1). In contrast, cells that lose their F-MTX labeling with hydrophilic antifolates (Figure 3A), but maintain it in the presence of lipophilic antifolates (Figure 3B), are cells that display a decreased accumulation of lipid-soluble antifolates[31] (Figure 1). Moreover, during the course of the development of the F-MTX labeling and the antifolate competition flow cytometric assay presented here, it became evident that the antifolate concentration yielding 50% displacement of initial F-MTX labeling (i.e. DC_{50}) bears a major quantitative value. When the DC_{50} value obtained for an antifol in drug-resistant cells is divided by that found in wild-type cells, a reliable measure of the severity of the antifolate transport defect could be obtained[10,31,52] (Figure 3B). The higher this ratio is, the greater the severity of the transport alteration.[10,31,52]

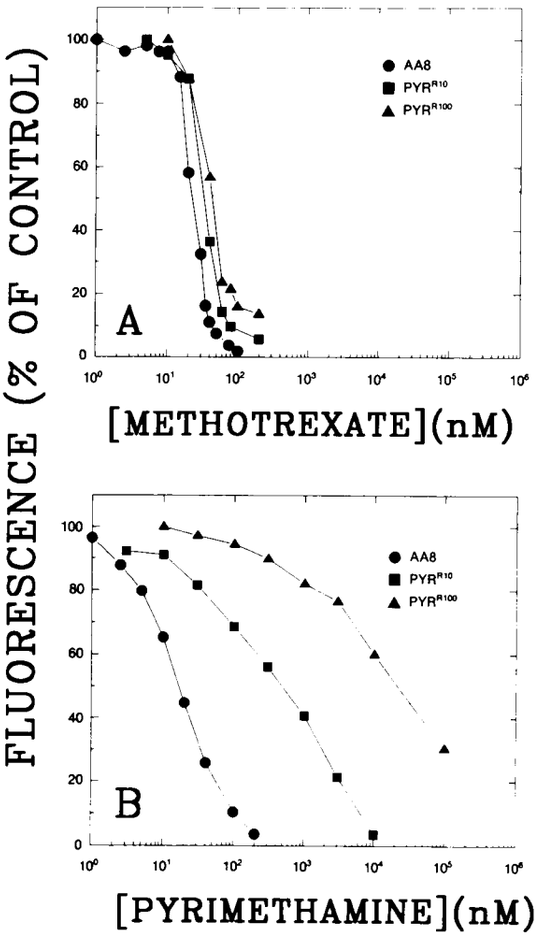

Figure 3. Displacement by hydrophilic and lipophilic antifolates of F-MTX labeling in wild-type CHO AA8 cells and their lipophilic antifolate transport deficient Pyr^{R10} and Pyr^{R100} variants.[31] Drug-sensitive AA8 cells and their pyrimethamine-resistant Pyr^{R10} and Pyr^{R100} sublines were labeled with F-MTX after which a displacement analysis was performed with MTX (A) and the lipophilic antifolate antibiotic pyrimethamine (B). This analysis was performed as detailed in the legend to Figure 2.

A successful F-MTX displacement analysis is based on two criteria of which one at least must be met: (i) the affinity of DHFR for the competing antifolate should be higher than for F-MTX and/or (ii) the higher the intracellular concentration of the competing antifolate is, the more efficient is the displacement of F-MTX. This was exploited, as mentioned, to establish a sensitive F-MTX-based bioassay capable of detecting nanomolar concentrations of MTX and other antifolates in sera of cancer patients.[48] Surprisingly, however, the 2,4-diaminopyrimidine (DAP) lipophilic antifolate pyrimethamine that is currently used as an antiparasitic agent, that bears a 32-fold less affinity for mammalian DHFR as compared with MTX, exhibited a 3-fold more efficient displacement of intracellular DHFR-bound F-MTX.[31] Therefore, based on these factors that control the efficiency of antifolate competition of F-MTX labeling, it was suggested[31] that DAP lipophilic antifolate antibiotics, including metoprine, pyrimethamine and trimethoprim, accumulate to high concentrations within mammalian cells. This increased intracellular antifolate concentration compensates well for the low affinity of DHFR for DAP, thus enabling an efficient competition of F-MTX. Therefore, antifolate competition of

cellular F-MTX labeling can also report on the intracellular accumulation of lipophilic antifolates that bear a poor affinity for mammalian DHFR.

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References

1. Frei E III. Curative cancer chemotherapy. *Cancer Res* 1985; **45**: 6523–37.
2. Schimke RT. Gene amplification in cultured cells. *J Biol Chem* 1988; **263**: 5989–92.
3. Goldman ID, Lichtenstein NS, Oliverio VT. Carrier-mediated transport of the folic acid analogue, methotrexate, in the L1210 leukemia cell. *J Biol Chem* 1968; **243**: 5007–17.
4. Niethammer D, Jackson RC. Changes in the molecular properties associated with the development of resistance against methotrexate in human lymphoblastoid cells. *Eur J Cancer* 1975; **11**: 845–54.
5. Galivan J. Transport and metabolism of methotrexate in normal and resistant rat hepatoma cells. *Cancer Res* 1979; **39**: 735–43.
6. Hill BT, Bailey BD, White JC, *et al.* Characteristics of transport of 4-amino antifolate compounds by two lines of L5178Y lymphoblasts, one with impaired transport of methotrexate. *Cancer Res* 1979; **39**: 2440–6.
7. McCormick JI, Susten SS, Freisheim JH. Characterization of the methotrexate transport defect in a resistant L1210 lymphoma cell line. *Arch Biochem Biophys* 1981; **212**: 311–8.
8. Sirotnak FM, Moccio DM, Keller LE, *et al.* Relative frequency and kinetic properties of transport-defective phenotypes among methotrexate-resistant L1210 clonal cell lines derived *in vivo*. *Cancer Res* 1981; **41**: 4447–52.
9. Sirotnak FM. Obligate genetic expression in tumor cells of a fetal membrane property mediating 'folate' transport: biological significance and implications for improved therapy of human cancer. *Cancer Res* 1985; **45**: 3992–4000.
10. Assaraf YG, Schimke RT. Identification of methotrexate transport deficiency in mammalian cells using fluoresceinated methotrexate and flow cytometry. *Proc Natl Acad Sci USA* 1987; **84**: 7154–8.
11. Flintoff WF, Essani K. Methotrexate-resistant Chinese hamster ovary cells contain a dihydrofolate reductase with an altered affinity for methotrexate. *Biochemistry* 1980; **19**: 4321–7.
12. Haber DA, Beverley SM, Kiely M, *et al.* Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured mouse fibroblasts. *J Biol Chem* 1981; **256**: 9501–10.
13. Simonsen CC, Levinson A. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. *Proc Natl Acad Sci USA* 1984; **80**: 2495–9.
14. Melera PW, Hession CA, Davide JP, *et al.* Antifolate-resistant Chinese hamster cells: mRNA directed overproduction of multiple dihydrofolate reductases from a series of independently derived sublines containing amplified dihydrofolate reductase genes. *J Biol Chem* 1982; **257**: 12939–49.
15. Melera PW, Davide JP, Hession CA, *et al.* Phenotypic expression in *Escherichia coli* and nucleotide sequence of two Chinese hamster lung cell cDNAs encoding different dihydrofolate reductases. *Mol Cell Biol* 1984; **4**: 38–48.
16. Lewis JA, Davide JP, Melera PW. Selective amplification of polymorphic dihydrofolate reductase gene loci in Chinese hamster lung cells. *Proc Natl Acad Sci USA* 1982; **79**: 6961–5.
17. Melera PW, Davide JP, Oen H. Antifolate-resistant Chinese hamster cells: molecular basis for the biochemical and structural heterogeneity among dihydrofolate reductases produced by drug-sensitive and drug-resistant cell lines. *J Biol Chem* 1988; **263**: 1978–90.
18. Srimatkandada S, Schweitzer BI, Moroson B, *et al.* Amplification of a polymorphic dihydrofolate reductase gene expressing an enzyme with decreased binding to methotrexate in a human colon carcinoma cell line, HCT-8R4, resistant to this drug. *J Biol Chem* 1989; **264**: 3524–8.
19. McBurney MW, Whitmore GF. Isolation and biochemical characterization of folate-deficient mutants of Chinese hamster cells. *Cell* 1974; **2**: 173–82.
20. Cowan KH, Jolivet JA. A methotrexate resistant human breast cancer cell line with multiple defects including diminished formation of methotrexate polyglutamates. *J Biol Chem* 1984; **259**: 1073–80.
21. Frei E III, Rosowsky A, Wright JE, *et al.* Development of methotrexate resistance in a human squamous cell carcinoma of the head and neck in culture. *Proc Natl Acad Sci USA* 1984; **81**: 2873–7.
22. Chabner BA, Allegra CJ, Curt GA, *et al.* Polyglutamation of methotrexate. Is methotrexate a prodrug? *J Clin Invest* 1985; **76**: 907–12.
23. Wright JE, Rosowsky A, Waxman DJ, *et al.* Metabolism of methotrexate and γ-tert-butyl methotrexate by human leukemic cells in culture and by hepatic aldehyde oxidase *in vitro*. *Biochem Pharmacol* 1987; **36**: 2209–14.
24. Pizzorno G, Mini E, Coronelio M, *et al.* Impaired polyglutamylation of methotrexate as a cause of resistance in CCRF-CEM cells after short-term high dose treatment with this drug. *Cancer Res* 1988; **48**: 2147–55.
25. Shane B. Folylpolyglutamate synthetase in the regulation of folate and antifolate metabolism. *Vitam Horm (NY)* 1989; **45**: 263–335.
26. Shane B, Lowe K, Osborne C, *et al.* Role of folylpolyglutamate synthetase in the regulation of folate and antifolate metabolism. In: Curtius HC, Ghisla S, Blau N, eds. *Chemistry and biology of Pteridines 1989*. Berlin: de Gruyter 1990: 891–5.
27. McCloskey DE, McGuire JJ, Russell CA, *et al.* Decreased folylpolyglutamate synthetase activity as a mechanism of

methotrexate resistance in CCRF-CEM human leukemia sublines. *J Biol Chem* 1991; **266**: 6181–7.
28. Gapski GR, Whiteley JM, Rader JI, *et al.* Synthesis of a fluorescent derivative of amethopterin. *J Med Chem* 1975; **18**: 526–8.
29. Sherwood SW, Schimke RT. Flow cytometric analysis of gene amplification in cultured mammalian cells. In: Yeh A, ed. *Flow cytometry; advanced applications.* Boca Raton FL: CRC 1989; 85–98.
30. Sherwood SW, Assaraf YG, Molina A, *et al.* Flow cytometric characterization of antifolate resistance in cultured mammalian cells using fluoresceinated methotrexate and daunorubicin. *Cancer Res* 1990; **50**: 4946–50.
31. Assaraf YG, Slotky JI. Characterization of a lipophilic antifolate resistance provoked by treatment of mammalian cells with the antiparasitic agent pyrimethamine. *J Biol Chem* 1993; **268**: 4556–66.
32. Gaudray P, Trotter J, Wahl GM. Fluorescent methotrexate labeling and flow cytometric analysis of cells containing low levels of dihydrofolate reductase. *J Biol Chem* 1986; **261**: 6285–92.
33. Matthews DA, Alden RA, Bolin JT. *et al.* Dihydrofolate reductase: X-ray structure of the binary complex with methotrexate. *Science* 1977; **197**: 452–5.
34. Rosowsky A, Wright JE, Shapiro H, *et al.* A new fluorescent dihydrofolate reductase probe for studies of methotrexate resistance. *J Biol Chem* 1982; **257**: 14162–7.
35. Kumar AA, Freisheim JH, Kempton RJ, *et al.* Synthesis and characterization of a fluorescent analogue of methotrexate. *J Med Chem* 1983; **26**: 111–3.
36. Kumar AA, Kempton RJ, Anstead GM, *et al.* Fluorescent analogues of methotrexate: characterization and interaction with dihydrofolate reductase. *Biochemistry* 1983; **22**: 390–95.
37. Assaraf YG, Seamer LC, Schimke RT. Characterization by flow cytometry of fluorescein-methotrexate transport in Chinese hamster ovary cells. *Cytometry* 1989; **10**: 50–5.
38. Sirotnak FM, Chello PL, Brockman RW. Potential for exploitation of transport systems in anticancer drug design. *Methods Cancer Res* 1979; **16**: 382–442.
39. Hakala MT, Zakrzewski SF, Nichol CA. Relation of folic acid reductase to amethopterin resistance in cultured mammalian cells. *J Biol Chem* 1961; **236**: 952–8.
40. Werkheiser WC. Specific binding of 4-amino folic acid analogs by folic acid reductase. *J Biol Chem* 1961; **236**: 888–93.
41. Schweitzer BI, Srimatkandada S, Gritsman H, *et al.* Probing the role of two hydrophobic active site residues in the human dihydrofolate reductase by site-directed mutagenesis. *J Biol Chem* 1989; **264**: 20786–95.
42. Kaufman RJ, Bertino JR, Schimke RT. Quantitation of dihydrofolate reductase in individual parental and methotrexate-resistant murine cells. *J Biol Chem* 1978; **253**: 5852–60.
43. Urlaub G, Chasin LA. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc Natl Acad Sci USA* 1980; **77**: 4216–20.
44. Urlaub G, McDowell J, Chasin LA. Use of fluorescence-activated cell sorter to isolate mutant mammalian cells deficient in an internal protein, dihydrofolate reductase. *Somat Cell Mol Gen* 1985; **11**: 71–7.
45. Henderson GB, Russell A, Whiteley JM. A fluorescent derivative of methotrexate as an intracellular marker for dihydrofolate reductase in L1210 cells. *Arch Biochem Biophys* 1980; **202**: 29–34.
46. Elwood PC, Kane MA, Portillo RM, *et al.* The isolation, characterization and comparison of the membrane-associated and soluble folate-binding proteins from human KB cells. *J Biol Chem* 1986; **261**: 15416–23.
47. Saikawa Y, Knight CB, Saikawa T, *et al.* Decreased expression of the human folate receptor mediates transport-defective methotrexate resistance in KB cells. *J Biol Chem* 1993; **268**: 5293–301.
48. Assaraf YG, Molina A, Schimke RT. A fluorescein–methotrexate-based flow cytometric bioassay for measurement of plasma methotrexate and trimetrexate levels. *Anal Biochem* 1989; **178**: 287–93.
49. Bertino JR, Donohue DM, Simmons B, *et al.* Induction of dihydrofolate reductase activity in leukocytes and erythrocytes of patients treated with amethopterin. *J Clin Invest* 1963; **42**: 466–75.
50. Alt FW, Kellems RE, Bertino JR, *et al.* Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J Biol Chem* 1978; **253**: 1357–70.
51. Assaraf YG, Molina A, Schimke RT. Sequential amplification of dihydrofolate reductase and multidrug resistance genes in Chinese hamster ovary cells selected for stepwise resistance to the lipid-soluble antifolate trimetrexate. *J Biol Chem* 1989; **264**: 18326–34.
52. Assaraf YG, Feder JN, Sharma RC, *et al.* Characterization of the coexisting multiple mechanisms of methotrexate resistance in mouse 3T6 R50 fibroblasts. *J Biol Chem* 1992; **267**: 5776–84.
53. Serrano EE, Schimke RT. Flow cytometric analysis of mammalian glial cultures treated with methotrexate. *Glia* 1990; **3**: 539–49.
54. Johnston RN, Beverley SM, Schimke RT. Rapid spontaneous dihydrofolate reductase gene amplification shown by fluorescence-activated cell sorting. *Proc Natl Acad Sci USA* 1983; **80**: 3711–5.
55. Sharma RC, Assaraf YG, Schimke RT. A phenotype conferring selective resistance to lipophilic antifolates in Chinese hamster ovary cells. *Cancer Res* 1991; **51**: 2949–59.
56. Assaraf YG, Borgnia MJ. Differential reversal of lipophilic antifolate resistance in mammalian cells with modulators of the multidrug resistance phenotype. *Anti-Cancer Drugs* 1993; **4**: 395–406.
57. Bolin JT, Filna DJ, Matthews DA, *et al.* Crystal structures of *Escherichia coli* and *Lactobacillus casei* dihydrofolate reductase refined at 1.7 Å resolution. I. General features and binding of methotrexate. *J Biol Chem* 1982; **257**: 13650–62.
58. Volz KW, Matthews DA, Alden RA, *et al.* Crystal structure of avian dihydrofolate reductase containing phenyltriazine and NADPH. *J Biol Chem* 1982; **257**: 2528–36.
59. Stammers DK, Champness JN, Beddell CR, *et al.* The structure of mouse L1210 dihydrofolate reductase-drug complexes and the construction of a model of the human enzyme. *FEBS Lett* 1988; **218**: 178–84.
60. Oefner C, D'Arcy A, Winkler FK, *et al.* Crystal structure of human dihydrofolate reductase complexed with folate. *Eur J Biochem* 1988; **174**: 377–85.
61. Thillet J, Absil J, Stone SR, *et al.* Site-directed mutagenesis of mouse dihydrofolate reductase. *J Biol Chem* 1988; **263**: 12500–8.
62. Sharma RC, Schimke RT. Enhancement of the frequency of methotrexate resistance by γ-radiation in Chinese hamster ovary and mouse 3T6 cells. *Cancer Res* 1989; **49**: 3861–6.

63. Sirotnak FM. Membrane transport of antineoplastic agents. In: *International encyclopedia of pharmacology and therapeutics: section 118*. Oxford: Pergamon Press 1986: 241–75.
64. Goldman ID, Matherly LH. Membrane transport of antineoplastic agents. In: *International encyclopedia of pharmacology and therapeutics: section 118*. Oxford: Pergamon Press 1986: 283–302.
65. Schuetz JD, Matherly LH, Westin EH, *et al.* Evidence for a functional defect in the translocation of the methotrexate transport carrier in a methotrexate-resistant murine L1210 leukemia cell line. *J Biol Chem* 1988; **263**: 9840–7.

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