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Uptake and efficacy of trimetrexate (TMQ, 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl] quinazoline), a non-classical antifolate in methotrexate-resistant leukemia cells *in vitro**

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Methotrexate (MTX) has been used for 30 years in the treatment of malignant and some non-malignant disorders. It is a key drug for the treatment of childhood leukemia, a disease in which 50% of patients may be cured. Despite this extensive use, clinical resistance is a problem and is not yet adequately explained or overcome. Efforts to circumvent resistant have included use of high-dose MTX regimens and the development of other anti-folates, both "classical", such as dichloromethotrexate, and "non-classicial", such as Baker's antifol, triazinate, and quinazolines [1, 2]. This report documents the uptake and efficacy of trimetrexate, a quinazoline compound, against a highly MTX-resistant murine leukemia, L1210RR.

Materials and methods

The cell line studied, L1210RR, has a 30- to 40-fold elevation of dihydrofolate reductase (DHFR) and a decreased transport for MTX as compared to the parent line [3]. Both lines are maintained in Fischer's medium containing 10% horse serum; in addition, the resistant line is also grown in the presence of 10^{-4} M MTX. Growth for 1 year in the absence of MTX has not changed the characteristics or quantity of DHFR or transport properties of the L1210RR line. Details of cell growth and MTX transport of both the parent and resistant line have been presented elsewhere [4].

Radiolabeled [^{14}C]trimetrexate monoacetate, sp. act. 9.4 mCi/mmole, was supplied by Dr. Robert Engel of the National Cancer Institute. Purity was checked by high performance liquid chromatography (HPLC) [5]. The drug was more than 95% pure for all experiments presented. Tritiated MTX (13 Ci/mmole, Amersham-Searle) was purified by HPLC or DEAE cellulose and was 99% pure as detailed [4]. Trimethoxyaniline and 2,4-diamino-5-methyl-6-quinazoline-carbonitrile were obtained from Warner-Lambert.

Drug uptake experiments were done according to procedures detailed earlier [4]. Specifically, cells ($1–10 \times 10^7$/ml) were incubated in Fischer's medium (without serum or folic acid) with drug as indicated. At selected times 0.05 to 0.10 ml aliquots of cell suspension were diluted 10- to 20-fold in ice-cold saline and centrifuged for 30 sec in a microfuge. The cell pellet was washed with 100 vol. of cold saline once, pelleted, lysed in 0.5 ml distilled water, and transferred quantitatively to 5.0 ml of scintillation fluid (Triton/toluene, 1/2, v:v) containing 9 g preblend 2a70 (RPI). Radioactivity was determined in a Beckman scintillation spectrophotometer. Counting efficiency was approximately 30% for ^{3}H and 65% for ^{14}C. The washing procedure resulted in a 0.1% background in "O" time cells with [^{3}H]MTX uptake [4]. In experiments where unlabeled trimetrexate was quantitated, the cells were harvested in a similar manner, suspended in 0.5 ml potassium phosphate buffer (0.05 M, pH 7.5), boiled for 5 min, centrifuged for 10 min at 10,000 *g* and assayed for drug by a sequential radioligand binding assay using [^{3}H]MTX [6]. The assay sensitivity for MTX was 0.05 pmole and for trimetrexate, 1 pmole.

Efflux experiments were done according to methods detailed elsewhere [4] and are described in the legend of Fig. 4.

Results

Representative time courses of [^{14}C] trimetrexate uptake by both L1210 and L1210RR are shown in Fig. 1. Table 1 shows that drug uptake at 37° was proportional to extracellular concentration. Measurement of cellular [^{14}C] trimetrexate during the initial velocity phase of uptake (first minute) confirmed that drug accumulation was linear with respect to extracellular drug concentration from 10^{-8} to 10^{-4} M. Experiments done at 10^{-9} M extracellular trimetrexate used unlabeled drug and were analyzed by radiobinder assay because the radiolabeled drug was not of high enough specific activity to yield significant amounts of detectable radioactivity. The intracellular concentration of MTX in the sensitive cell line (L1210) has been detailed previously [4]. For comparison, the steady-state intracellular concentration of MTX at an extracellular concentration of 10^{-5} M is shown in Fig. 1. At 10^{-4} M extracellular MTX, L1210RR cells accumulate less than 5% of the MTX accumulated by L1210 cells in 10^{-6} M MTX, and no significant MTX accumulates in L1210RR cells at 10^{-5} M extracellular MTX [4].

In an attempt to further define the nature of trimetrexate accumulation by L1210 cells, temperature, analogs and metabolic inhibitors known to affect MTX transport were studied for their effects on cellular accumulation of [^{14}C] trimetrexate. Figure 2 shows that the trimetrexate analogs, 2,4,diamino-quinazoline and trimethoxyaniline and MTX, did not affect trimetrexate uptake. Further, 4×10^{-4} M folic acid or 5-methyltetrahydrofolic acid also did not alter the time course or total amount of [^{14}C]trimetrexate taken up. Preincubating cells for 10–20 min with these same compounds prior to addition of the [^{14}C]trimetrexate also did not reduce ^{14}C accumulation.

The effect of temperature on trimetrexate uptake was examined at five temperatures between 4° and 37° (Fig. 3). The calculated Q_{10} was 2.71. Although this is suggestive of an enzymatic or carrier mediated transport process, the metabolic inhibitors *p*-chloromecuribenzoate (PCMB), $5–20 \times 10^{-6}$ M, and sodium azide, up to 10^{-2} M, had no effect (data not shown). These inhibitors have been shown to alter MTX transport [7].

Since it is also known that MTX effluxes by an energy-mediated process (low concentrations of azide can even increase net uptake of MTX [8]), the efflux of trimetrexate was also studied. The results are shown in Fig. 4. In both the L1210RR and L1210 cells the efflux of the drug was rapid, and a steady state approximately equal to three to five times their respective DHFR concentrations occurred. These results were not affected by the presence of PCMB or sodium azide at the concentrations noted above. In several experiments (both uptake and efflux), the radioactivity in both medium and cells was found to be >90% trimetrexate, indicating little intracellular metabolism. Analysis was by HPLC as recently described [5].

Although the K_i of trimetrexate for DHFR is fifty times greater than that of MTX (4×10^{-9} M and 2×10^{-11} M), respectively [9], the increased uptake of trimetrexate still

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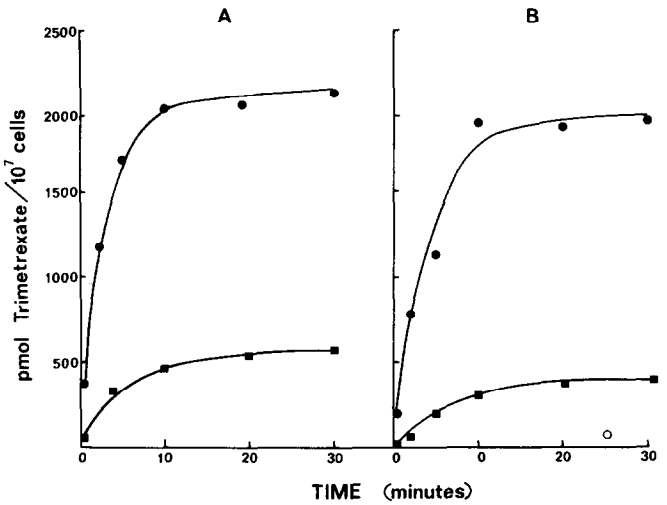

Fig. 1. Uptake of trimetrexate by L1210RR (A) and L1210 (B) cells. Transport experiments were carried out as described in the text. Key: (●) 10^{-5} M extracellular drug; (■) 2×10^{-6} M extracellular drug; (○) MTX accumulation in L 1210 at 10^{-5} M extracellular MTX.

Table 1. Trimetrexate accumulation by L1210RR cells*

Extracellular drug (M)	Trimetrexate (pmoles/10^7 cells)
10^{-9}	0.2
10^{-8}	1.8
10^{-7}	16
10^{-6}	200
10^{-5}	1,850
10^{-4}	17,500

* Uptake experiments were done as described. Amount of drug associated with cells was determined after a 20-min incubation. Results are means of triplicate determinations and are ±10%.

made it highly effective against the L1210RR despite the elevated level of DHFR in this line. The concentration of trimetrexate needed to decrease growth of L1210RR 50% (at 48 hr of culture) was 1.5×10^{-7} M. This compares to 5×10^{-4} M for MTX. Trimetrexate is approximately 5000 times more potent than MTX against L1210RR.

Discussion

Trimetrexate is a new, lipophilic antifol in which animal toxicity and pharmacologic studies have been initiated [5], interaction with DHFR has been partially studied [2, 9], and tumor specificity has been examined [2]. Uptake of trimetrexate by both MTX-sensitive and -resistant L1210 cells is presented in this study. Accumulation was equal in the two lines and proportional to the extracellular drug concentration. This finding is similar to results obtained

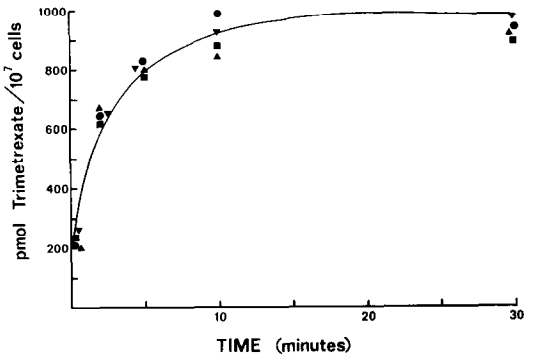

Fig. 2. Effects of trimetrexate analogs on ^{14}C drug uptake in L1210 cells. The experimental conditions are described in the text. The experiment was initiated by adding an aliquot of cells ($5–10 \times 10^7$ cells/ml) to a flask containing [^{14}C]trimetrexate and competing compound. Key: (●) control [^{14}C]trimetrexate, 4×10^{-6} M; (▼) 2×10^{-4} M 2,4-diamino-5-methyl-6-quinazoline-carbonitrile; (▲) 2×10^{-4}M trimethoxyaniline; and (■) 4×10^{-4} M MTX.

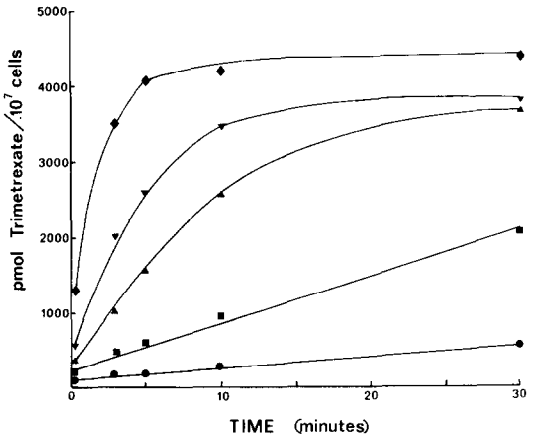

Fig. 3. Effect of temperature on [^{14}C]trimetrexate accumulation in L1210 cells. The cells were incubated for 10 min at the indicated temperature at $5–10 \times 10^7$ cells/ml and then [^{14}C]trimetrexate (2×10^{-5} M) was added. The reaction was stopped at times indicated by aliquoting 10^7 cells into ice-cold saline and washing as described. Key: (●) 4°; (■) 11°; (▲) 21°; (▼) 30°; and (◆) 37°.

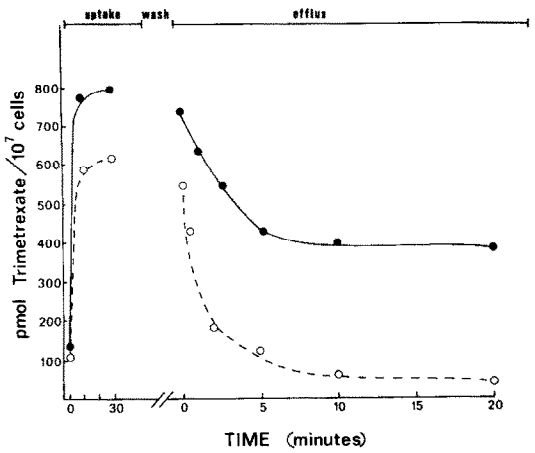

Fig. 4. Efflux of trimetrexate from L1210 and L1210RR cells. [^{14}C]Trimetrexate (4×10^{-6} M final concentration) was incubated with cells in Fischer's medium (minus folic acid) for 30 min. The cells were then collected by low speed centrifugation (5 min × 1000 *g*), washed in medium once, and resuspended to starting concentration (5×10^7 cells/min). At the time indicated, 10^7 cells were removed, washed, and processed for counting, as before. The DHFR in the L1210 cells is approximately 4 pmoles/10^7 cells and in the L1210RR, 160 pmoles/10^7 cells. Key: (●) L1210RR; and (○) L1210.

for another non-classical anti-folate, triazinate,* and may reflect an enormous nonspecific cell binding capacity. In fact, experiments done with trimetrexate to determine saturability of the uptake process here were limited by the solubility of the acetate salt (4 mg/ml). Preliminary experiments with an unlabeled isethionate salt (eight times more soluble) have yielded results similar to the ^{14}C data, namely uptake proportional to extracellular drug concentration. Other important aspects of trimetrexate uptake by L1210 cells shown here include: (a) lack of competition for uptake with folate, reduced folate compounds, or MTX, (b) lack of effect of certain metabolic inhibitors, and (c) little to no intracellular metabolism (as measured by HPLC). In this regard, the absence of a glutamate residue precludes metabolism to polygammaglutamyl derivatives.

The observation that trimetrexate uptake is thirty times greater than MTX (at 10^{-5} M extracellular drug) and is not affected by the presence of MTX or folate suggests that this class of drug could be useful in situations where drug penetration of tumor or cell transport is in doubt, e.g. trimetrexate could possibly be used instead of high-dose MTX. This rapid transport and tissue accumulation (*in vivo* and *in vitro*) are similar to other non-classical lipophilic antifols such as 2,4-diamino-5-(3′,4′-dichlorophenyl)-methylpyrimidine (DDMP) as previously documented by others [10–12]. *In vitro* the efficacy of trimetrexate was demonstrated in an L1210RR cell line in which the presumed target enzyme, DHFR, was elevated 30- to 40-fold and in which the cells could not effectively transport MTX.

The *in vitro* efficacy of trimetrexate in a doubly MTX-resistant (decreased transport and increased DHFR) murine leukemia cell line warrants further exploration. This compound or analogs may be important in expanding the clinical usefulness of anti-folate drugs in cancer therapy. Specifically, trimetrexate could be highly selective when used with leucovorin protection against tumors resistant to MTX due primarily to impaired uptake of MTX, if the cells were also deficient in reduced folate uptake. This has been shown for a MOLT cell line *in vitro* by Dr. Ohnuma and coworkers [13].

In summary, the uptake and efficacy of trimetrexate (2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]-quinazoline), a lipid soluble, anti-folate, in a methotrexate-sensitive and -resistant murine leukemia (L1210RR) were evaluated *in vitro*. Trimetrexate was transported rapidly by both methotrexate-sensitive and -resistant cells. Kinetic studies showed uptake to be proportional to extracellular drug between 10^{-9} and 10^{-4} M extracellular concentrations. Uptake was temperature dependent but not inhibited by *p*-chloromecuribenzoate or sodium azide at concentrations known to affect methotrexate accumulation. Folate or methotrexate did not block trimetrexate accumulation. Although trimetrexate has a 50-fold higher K_i for murine dihydrofolate reductase, it attained sufficient intracellular concentration to inhibit 50% growth of L1210RR cells at an extracellular drug concentration of 1.5×10^{-7} M; this compares to a concentration of 5×10^{-4} M for methotrexate.

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