MOLECULAR PHARMACOLOGY, 20:637–643

Toxicity of Methotrexate and Metoprine in a Dihydrofolate Reductase Gene-Amplified Mouse Cell Line

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Received April 23, 1981; Accepted July 15, 1981

SUMMARY

The comparative effects of methotrexate (MTX) and the lipid-soluble antifolate metoprine [DDMP, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine] on DNA synthesis and cell viability were studied in an MTX-resistant mouse fibroblast 3T6R400 cell line. MTX levels as high as 1000 μM inhibited deoxyuridine incorporation into cellular DNA by only 28%, whereas a relatively low concentration (3 μM) of DDMP produced 50% inhibition. Inhibition of DNA synthesis and of cytotoxicity (measured by cloning efficiency) were approximately proportional with both drugs. Partially purified dihydrofolate reductase (DHFR) from the mutant cell was about 100 times more resistant to both MTX and DDMP than was enzyme from the wild-type cell. Furthermore, the resistant cell line took up MTX as efficiently as did the sensitive cell line. However, since the mutant cell contained very high levels of drug-resistant DHFR, a normal rate of drug entry apparently did not permit sufficient MTX to saturate the dihydrofolate reductase binding sites; consequently, cells escaped toxicity. Low concentrations of DDMP (relative to MTX) inhibit deoxyuridine incorporation by resistant cells. This inhibition occurs presumably because DDMP is not dependent on active transport. DDMP also was slightly more inhibitory (relative to MTX) for the DHFR of the mutant cell line than for this enzyme from the wild-type cells. Because toxicity to antifolates is mediated by a variety of factors in this mutant cell line, its observed sensitivity to lipid-soluble antifolates lends added importance for further developmental work on these compounds. We suggest that such drugs may be useful against tumors where resistance is a result of the complex interplay of transport limitations, altered target enzyme affinity, and changes in the amount of target enzyme.

INTRODUCTION

Development of resistance to MTX[2] is an important drawback to its therapeutic use in patients with cancer. There are a number of known mechanisms for development of acquired resistance to MTX *in vitro*. Resistance can be due to (a) decrease in the amount of drug entering the cell (1–3); (b) increase in the amount of target enzyme (4–8); (c) structural alteration in the active site of DHFR which leads to reduced efficiency of the MTX-enzyme interaction (9–12); or (d) a variety of other complex metabolic events not directly related to DHFR sensitivity (13–15).

This work was supported by Grants CA 08800 and CA 11265 from the National Cancer Institute and by Grant DRG-236-F from the Damon Runyon-Walter Winchell Cancer Fund (to M. H.).

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[2] The abbreviations used are: MTX, methotrexate; DHFR, dihydrofolate reductase; DDMP, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Mutant cells which have developed resistance to MTX by means of gene amplification have recently been characterized (16–18). Certain of these cell lines have increased copies of the gene which codes for DHFR. These genes are contained on small bits of chromosomal material called "double minute" chromosomes (19) or are integrated into the chromosome as a region of tandemly repeated genetic material (20).

3T6R400 is a cell line that contains "minute chromosomes" described by Kellems *et al.* (17), in which an overproduction of enzyme results in a greater than 10,000-fold increase in resistance to MTX (21). In this study, we have characterized and compared the resistance of this cell line to MTX and to a lipid-soluble antifolate (22), DDMP. We studied deoxyuridine incorporation into DNA as a measure of drug effectiveness, since the conversion of deoxyuridine to thymidine can be used as a very sensitive indicator of antifolate effectiveness (23–25). In order to monitor toxic cellular responses, this means of analysis was complemented by investigation of additional effects of these antifolates on thymidine and ^{32}P incorporation into DNA, as well as on cell-cloning efficiency. Uptake of labeled MTX was also measured in

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order to characterize the active transport capacity of the mutant and wild-type 3T6 cells. Finally, the inhibition of isolated DHFR by MTX and DDMP was compared in partially purified extracts from both 3T6R400 and 3T6 wild-type cells.

Normal drug uptake apparently restricts intracellular MTX to a concentration below that required for DHFR inhibition. This work supports the general conclusion that transport-independent drugs like DDMP (22) may be effective inhibitors of cells in which resistance is due to a complex interaction of drug transport and target enzyme changes.

EXPERIMENTAL PROCEDURE

Materials

[6-^{3}H]deoxyuridine (24.2 Ci/mmole) and [*methyl*-^{3}H] thymidine (50.5 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). Unlabeled MTX and [3′,5′,9(n)-^{3}H]MTX were generously provided by the Developmental Therapeutics Program, Division of Cancer Treatment of the National Cancer Institute (Bethesda, Md.). [^{3}H]MTX was repurified before use (final specific activity, 1.9×10^9 cpm/μmole) as described by Goldman *et al.* (26). DDMP was kindly provided by Drs. C. Nichol and C. Sigel of Burroughs-Wellcome Laboratories (Research Triangle Park, N. C.). The autoclavable formulation of Earle's minimal essential medium for monolayers was obtained from Grand Island Biological Company (Grand Island, N. Y.) and dialyzed fetal calf serum from K-C Biologicals, Inc. (Lenexa, Kan.). ^{32}P, as orthophosphoric acid (carrier-free), was purchased from New England Nuclear Corporation, and [*G*-^{3}H]folic acid (1–5 Ci/mmole) from Amersham Corporation (Arlington Heights, Ill.). The MTX-resistant 3T6 line and the parent cell line, isolated and characterized by Kellems *et al.* (17), were kindly provided by Dr. V. Morhenn, Stanford University (Stanford, Calif.).

Methods

Cell maintenance. The MTX-resistant and parent cell lines were cultured in Earle's minimal essential medium containing 5% dialyzed fetal calf serum in a humidified atmosphere containing 5% CO_2. The resistant line, referred to as 3T6R400, was maintained in the presence of 400 μM MTX. The cells were found to be free of mycoplasma by the culture method of Hayflick (27).

Pulse-labeling protocol. Cells were subcultured for 24 hr prior to use in 16-mm wells in tissue-culture dishes (tissue-culture cluster 24) in medium without drug. For each experiment, Hepes-buffered medium (with 6 mM Hepes), containing MTX or DDMP at the desired concentration, was added at time zero and the cells were pulse-labeled for 30 min with [^{3}H]deoxyuridine or [^{3}H] thymidine (2 μCi/ml) at the times indicated. This level of labeled nucleoside gives linear incorporation for up to 2 hr (not shown). The reaction was stopped by removing the medium from the well and washing the monolayer once with phosphate-buffered saline (25). One-half milliliter of 0.25% trypsin was then added, and after 10 min the cell suspension was removed and pipetted into tubes containing 3 ml of ice-cold phosphate-buffered saline and collected by centrifugation at $800 \times g$ for 5 min. Cells were then resuspended in 100 μl of phosphate-buffered saline and spotted on No. 3M filter discs (Whatman Inc., Clifton, N. J.) and processed for scintillation counting as previously described (23). Successive pulses were added together and plotted as total accumulated counts for the course of the experiment. ID_{50} values were determined by probit analysis for the inhibition obtained after 3 hr in each cell line (28).

^{32}P incorporation. The incorporation of ^{32}P into DNA was determined by a modification of the method of Schmidt and Thannhauser (29). Hepes-buffered medium containing a 50 μM concentration of drug was added to cells in 16-mm tissue-culture plates, and ^{32}P (100 μCi/ml) was added to each well. At the times indicated, the medium was removed and the reaction was stopped by washing the monolayer in phosphate-buffered saline containing 1 mM sodium azide. One-half milliliter of 0.25% trypsin was added and the cells were scraped off and transferred to tubes containing 3 ml of ice-cold phosphate-buffered saline-azide and processed for incorporation into DNA as described previously (30).

Assay of MTX uptake. The uptake of [^{3}H]MTX was measured in cells which were plated in medium in 100-mm tissue-culture plates without drug for 24 hr prior to use. Hepes-buffered medium (0.3 ml) containing varying concentrations of [^{3}H]MTX (1.66×10^7 cpm/mmole) was added and the plates were incubated at 37°. At the times indicated, working as rapidly as possible, the medium was aspirated and the plates were placed on ice and washed twice with ice-cold phosphate-buffered saline. After that, 1 ml of cold phosphate-buffered saline was added, and the cells were scraped off the plates and transferred to a tube with an additional 0.5-ml wash. The cell suspension was centrifuged, the supernatant was removed, and the cell pellet was washed once with ice-cold phosphate-buffered saline and processed for scintillation counting. Each point is the average of duplicate determinations. Cell number was determined by counting a duplicate set of plates. Cellular drug levels are expressed in molarity, using the intracellular volumes determined as described. Linear regression analysis was used to determine the best fit to the data and to calculate the initial rates and kinetic constants for MTX uptake.

Cell volume. The intra- and extracellular space of both the parent and resistant cells were estimated using a modification of the method of Wohlhueter *et al.* (31). A solution containing 2.5 μCi of [^{3}H]inulin or 12 μCi of ^3H_2O per milliliter in Hepes-buffered medium was added to cells in a 100-mm plate containing cells. Quadruplicate samples were incubated for 2 min, after which the cells were scraped off the plates, transferred to a tube, and centrifuged; the supernatant was removed and the pellets were processed for scintillation counting. The intracellular volume was calculated as the difference between the total water space (^3H_2O) and the extracellular space ([^{3}H]inulin). The number of cells per plate was determined by counting a quadruplicate set of plates.

Clonal assay of 3T6R400 cells. Mutant 3T6R400 cells were plated in 100-mm tissue-culture plates in medium without drug for 24 hr prior to use. Five milliliters of medium containing the indicated concentration of MTX or DDMP were added to each set of duplicate plates, and

the plates were incubated in a CO_2 incubator. After 24 hr the medium was aspirated and the cells were washed gently with 2 ml of medium without drug. Samples were then trypsinized to remove cells from the plates, and the number of cells was determined by counting in a hemocytometer. Each plate was replated in duplicate (approximately 2000 cells/plate) into fresh 100-mm plates in 5 ml of medium containing 50% conditioned medium and 20% dialyzed fetal calf serum. After 8–10 days, when colonies were easily visible, the plates were fixed in 10% formalin and stained with methylene blue, and the colonies per plate were counted. Cloning efficiency was measured as the average of duplicate determinations on replicate samples and was expressed as a percentage of the number of cells plated.

DHFR activity of cells. DHFR activity of the cells was assayed according to the method of Rothenbert (32) as modified by Nakamura and Littlefield (33), using [^{3}H] folic acid as a substrate. The cells, with added Hepes-buffered medium containing varying drug levels, were incubated at 37° in a CO_2 incubator. After 3 hr, the medium was removed, and the cells were washed once with fresh medium and scraped with a rubber policeman into 1 ml of phosphate-buffered saline. The cells were then centrifuged and washed twice by recentrifugation after suspension in 2 ml of ice-cold phosphate-buffered saline. The pellet was resuspended in 0.2 ml of 0.02 M Tris (pH 7.2), and the plasma membranes were ruptured by expressing the cell suspension 10 times through a 25-gauge needle. The homogenate was centrifuged at 15,000 × *g* for 30 min, and the DHFR activity of the supernatant was determined. Protein was measured by the method of Lowry *et al.* (34). The specific activity of the enzyme is expressed as micromoles of folate reduced per hour per milligram of supernatant protein. Each value is the average of duplicate determinations on replicate plates.

DHFR activity was also assayed by the method of Rothenberg *et al.* (35). This method utilizes ^{3}H-labeled dihydrofolate as a substrate at pH 7.2, and thus is a more sensitive and physiological assay of DHFR activity than is the original assay method of Rothenberg (32).

RESULTS

Metabolic inhibition of 3T6R400 and 3T6 wild-type cells. As shown in Fig. 1A, MTX inhibited deoxyuridine incorporation into the parent cell line in a time- and concentration-dependent manner. This was also true for DDMP (Fig. 1B). In the MTX-resistant line, however, MTX levels as high as 1000 μM inhibited deoxyuridine incorporation by only 28% after 3 hr (Fig. 2A). On the other hand DDMP, 5 μM (Fig. 2B), inhibited deoxyuridine incorporation by 55% at 3 hr. In the parent line, DDMP was about one-seventh as potent as MTX. Therefore, the 3T6R400 mutant showed a greater than 10,000-fold increase in resistance to MTX with only a 5-fold change in sensitivity to DDMP.

The inhibition of deoxyuridine incorporation into DNA by DDMP was not attributable to inhibition of deoxyuridine uptake by the cell. Concentrations of DDMP as high as 400 μM increased the over-all amount of deoxyuridine taken up by the cells (not shown). These results generally agreed with those shown previously in

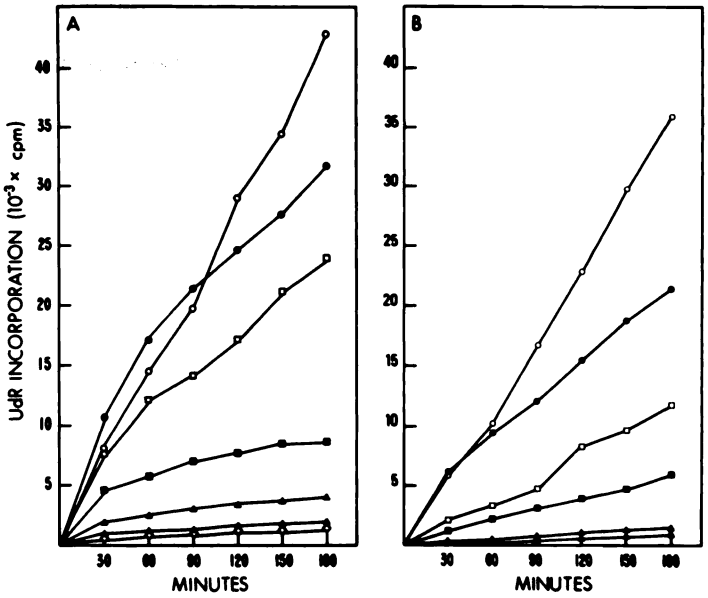

FIG. 1. *Inhibition of deoxyuridine incorporation into wild-type 3T6 cells by MTX and DDMP*

Cells (3 × 10^4/well) were subcultured in medium without drug for 24 hr prior to use.

A. MTX was added at time zero at the concentrations indicated below, and replicate samples were pulse-labeled with [^{3}H]deoxyuridine (2 μCi/ml) for 30-min intervals at the times specified. Successive pulses were then added together and plotted as total accumulated counts for the course of the experiment. Deoxyuridine incorporation into DNA was determined as described under Methods. ○, Control, no drug; ●, 0.05 μM MTX; □, 0.1 μM MTX; ■, 0.5 μM MTX; △, 1.0 μM MTX; ▲, 5.0 μM MTX; ◇, 10.0 μM MTX.

B. Samples were treated as described in A, except that DDMP was added at the indicated concentrations at time zero. ○, Control, no drug; ●, 0.1 μM DDMP; □, 0.5 μM DDMP; ■, 1.0 μM DDMP; △, 5.0 μM DDMP; ▲, 10.0 μM DDMP.

WIL-2 cells (24, 30) wherein DDMP also increased the initial rate of deoxyuridine uptake into acid-soluble products.

In addition to inhibiting the incorporation of deoxyuridine into DNA, DDMP also blocked the incorporation of thymidine into DNA of the 3T6R400 cell. Whereas the inhibition of deoxyuridine incorporation by DDMP was instantaneous and at a steady-state level within seconds, the inhibition of thymidine incorporation into the 3T6R400 line was both time- and concentration-dependent (Fig. 3). However, thymidine incorporation into the parent line was not significantly reduced after 2 hr by concentrations of DDMP that were inhibitory for deoxyuridine incorporation (Fig. 4A).

The final series of determinations of DNA synthesis employed measurements of ^{32}P incorporation, and again DDMP was more inhibitory than MTX against the 3T6R400 line. Figure 5 shows that MTX at a concentration of 50 μM had no significant effect on DNA synthesis but that 50 μM DDMP decreased DNA synthesis by 42% after 3 hr.

The toxicity of MTX and DDMP against the 3T6R400 line was assessed by measuring the ability of cells to form clones following a 24-hr exposure to various concentrations of each drug. As shown in Table 1, the cells showed

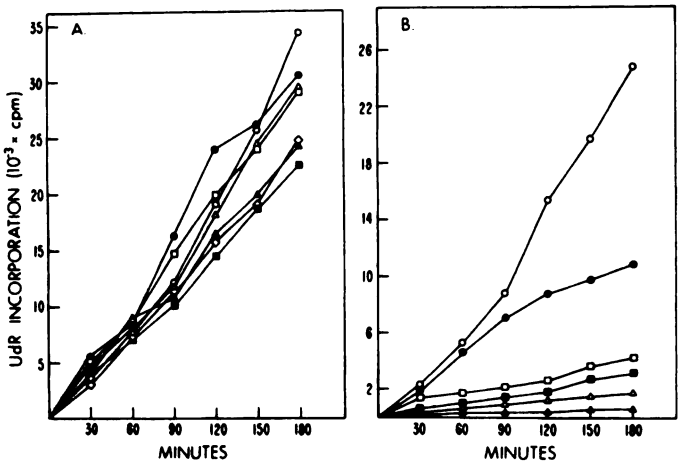

FIG. 2. *Inhibition of deoxyuridine incorporation into 3T6R400 cells by MTX and DDMP*

Cells (3 × 10^4/well) were subcultured in medium without drug for 24 hr prior to use.

A. MTX was added at time zero, and replicate samples were pulse-labeled with [^{3}H]deoxyuridine (2 μCi/ml) for 30-min intervals at the times indicated. Successive pulses were then added together and plotted as total accumulated counts for the course of the experiment. Deoxyuridine incorporation into DNA was determined as described under Methods. ○, Control, no drug; ●, 5 μM MTX; □, 50 μM MTX; ■, 100 μM MTX; △, 200 μM MTX; ▲, 400 μM MTX; ◇, 1000 μM MTX.

B. Samples were treated as described in A, except that DDMP was added at the indicated concentrations at time zero. ○, Control, no drug; ●, 5 μM DDMP; □, 50 μM DDMP; ■, 100 μM DDMP; △, 400 μM DDMP; ▲, 1000 μM DDMP.

no decrease in cloning ability after exposure to MTX concentrations of up to 1000 μM. By contrast, DDMP inhibited growth by more than 90% at 400 μM.

Uptake of MTX by 3T6R400 and 3T6 cells. The ability of the sensitive and resistant cell lines to take up MTX was examined. Both 3T6 and 3T6R400 cells treated with 1 μM MTX reached a steady state in 2 hr (data not shown). The V_{max} for MTX transport in the 3T6R400 cell line was 0.66, and in the wild-type 3T6 cells it was 0.61 pmole/min/10^6 cells in these studies. The initial velocity values for MTX accumulation in both cell lines over a range of MTX concentrations were also identical. A plot

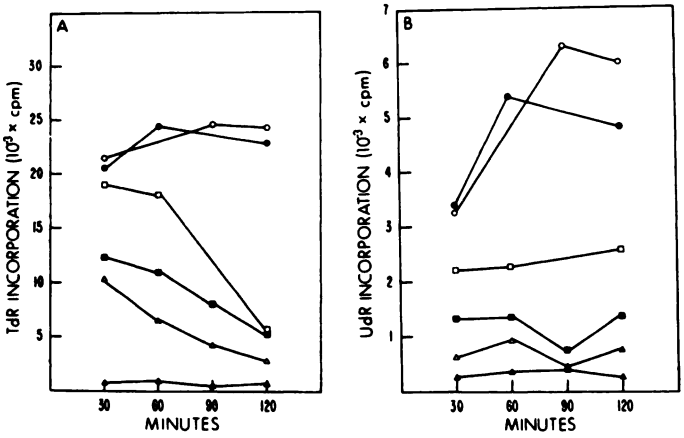

FIG. 3. *Effects of DDMP on thymidine and deoxyuridine incorporation into 3T6R400 cells*

Cells (3 × 10^4/well) were subcultured in medium without drug for 24 hr prior to use. At time zero, DDMP at the concentrations indicated below was added.

A. Replicate samples were pulse-labeled with [^{3}H]thymidine (2 μCi/ml) for 30 min at the times indicated. Incorporation into DNA was determined as described under Methods. ○, Control, no drug; ●, 1 μM DDMP; □, 10 μM DDMP; ■, 50 μM DDMP; △, 100 μM DDMP; ▲, 400 μM DDMP.

B. Samples were treated as described in A, except that they were pulse-labeled with [^{3}H]deoxyuridine (2 μCi/ml) for 30 min at the times indicated.

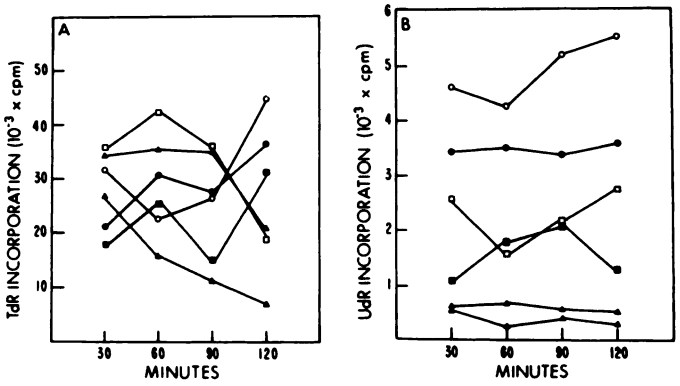

FIG. 4. *Effect of DDMP on thymidine and deoxyuridine incorporation into wild-type 3T6 cells*

Cells (3 × 10^4/well) were subcultured in medium without drug for 24 hr prior to use.

A. DDMP was added at the concentrations indicated below at time zero. Replicate samples were pulse-labeled with [^{3}H]thymidine (2 μCi/ml) for 30 min at the times indicated. Incorporation into DNA was determined as described under Methods. ○, Control, no drug; ●, 0.1 μM DDMP; □, 0.5 μM DDMP; ■, 1.0 μM DDMP; △, 5.0 μM DDMP; ▲, 10.0 μM DDMP.

B. Samples were treated as described in A, except that they were pulse-labeled with [^{3}H]deoxyuridine (2 μCi/ml) for 30 min at times indicated.

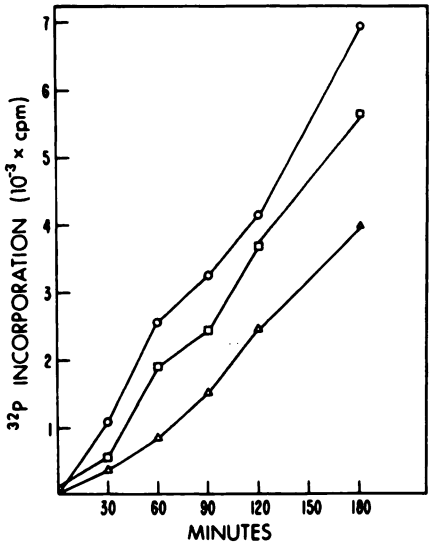

FIG. 5. *Effect of MTX and DDMP on ^{32}P incorporation into DNA of 3T6R400 cells*

Cells (3 × 10^4/well) were subcultured in medium without drug for 24 hr prior to use. ^{32}P (100 mCi/ml) and either 50 μM methotrexate (□) or 50 μM DDMP (△) were added at time zero. Duplicate samples were taken at each time point, and ^{32}P incorporation into DNA was determined as described. Each point is the average of duplicate determinations. The sample (○) indicates ^{32}P incorporation into DNA in a control culture containing no drug.

TABLE 1

Cell toxicity of MTX and DDMP in 3T6R400 cells

Resistant cells were subcultured in medium without drug for 24 hr prior to use. To each set of duplicate plates was added medium containing the indicated concentrations of MTX or DDMP. After 24 hr, the cells were plated for measurement of colony-forming ability, as described under Methods. Cloning efficiency was measured as the average of duplicate determinations of replicate samples and expressed as a percentage of the number of cells plated.

Drug concentration	Cloning efficiency	% Control
	% cells plated	
MTX (μM)		
0	14.1 ± 1.4[a]	
1	12.9 ± 3.3	91
50	13.9 ± 8.1	98
400	15.0 ± 3.7	106
1000	10.4 ± 5.6	74
DDMP (μM)		
1	16.6 ± 1.1	117
50	8.7 ± 0.4	55
400	0.5 ± 0.1	3
1000	0.0	0

[a] Mean ± standard deviation.

of the log of these initial velocities versus the log of the MTX concentrations gave the two parallel lines shown in Fig. 6 ($r = 0.98$). The K_m values for the accumulation of [^{3}H]MTX by the sensitive and resistant cells were 18.8 and 20.5 μM, respectively.

Cell volume determinations in the parent and resistant cell lines showed no differences between the two cell lines, with values of 1.23 and 1.20 μl/10^6 cells, respectively.

Finally, Table 2 demonstrates that 3T6R400 cells concentrated (1 μM) extracellular MTX 4-fold over a 3-hr exposure period, whereas at high MTX concentrations (>1000 μM), intracellular MTX levels were only ‰ of extracellular drug concentrations.

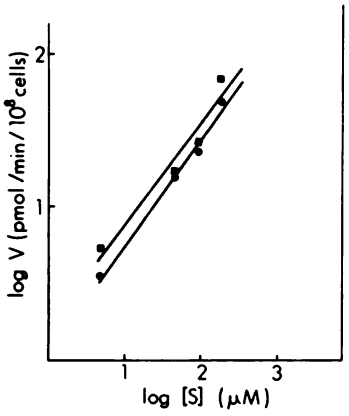

FIG. 6. *Relationship of initial velocity to external drug concentration*

The initial velocity of MTX accumulation in both the sensitive and resistant cell lines was determined over a range of MTX concentrations, as described under Methods. The initial velocity was calculated from the rate of uptake of [^{3}H]MTX in the first 10 min. The log of the initial velocities (picomoles per minute per 10^6 cells) was plotted versus the log of the corresponding external MTX concentration (micromolar) for the sensitive (●) and resistant (■) cell lines. Linear regression analysis was used to determine the best line through the points for each cell line.

TABLE 2

Exchangeable MTX and DHFR inhibition in the 3T6R400 cell line

Resistant cells were subcultured in medium without drug for 24 hr prior to use. MTX (2×10^7 cpm/μmole) was added to the cells, and the steady-state uptake level after 3 hr was determined in duplicate, as described under Methods. At 3 hr after drug addition, the drug containing medium was removed, the cells were washed three times with cold medium, and drug-free medium (at 37°) was added. After an additional 3 hr, the amount of drug still bound to cells was determined. The steady-state exchangeable drug pool was calculated as the difference between the total drug uptake and the bound level after washout. Each point is the average of duplicate determinations adjusted for cell volumes. The residual DHFR activity of the supernatant fraction after a 3-hr exposure to MTX was determined as described under Methods, using the [^{3}H]folate assay. Each point is the average of duplicate determinations of replicate plates.

External MTX	Exchangeable MTX	DHFR activity	% Inhibition DHFR
μM	*μM*	*μmoles/hr/mg*	
0	—	8.59 ± 0.31[a]	—
1	4	4.61 ± 0.26	45
50	10	5.52 ± 0.90	36
400	17	5.91 ± 0.73	31
1,000	25	4.74 ± 0.37	45
5,000	106	2.60 ± 0.28	70
10,000	222	0.52 ± 0.39	94

[a] Mean ± standard deviation.

An altered DHFR in 3T6R400 cells. Table 2 shows the DHFR activity present in the supernatant fraction of 3T6R400 cell extracts following 3 hr of exposure to MTX, compared with the concentration of exchangeable MTX. The specific activity of partially purified extracts of DHFR at pH 5.9 with folic acid as substrate was 3.68×10^6 cpm/mg/hr in 3T6R400 versus 4×10^5 cpm/mg/hr in 3T6 cells. Dialysis at pH 8.5 against 0.05 M KPO, or extract preparation through the 25% $(NH_4)_2SO_4$ precipitation step as described by Alt *et al.* (4) resulted in approximately 2-fold increases in DHFR activity in extracts from 3T6R400 grown in 400 μM MTX.

DHFR of intact mutant cells exposed to drug for 3 hr was inhibited only 55% by 1.0 mM MTX, whereas DHFR of wild-type cells was inhibited by >98%. Significant levels of DHFR activity remained in extracts from mu-

TABLE 3

ID_{50} values for inhibition of DHFR from 3T6 and 3T6R400 cells by MTX and DDMP

Quantities of 0.2 g (wet weight) of 3T6 and 0.5 g of 3T6R400 cells were extracted as described by Alt *et al.* (4). DHFR was partially purified from a postmitochondrial supernatant fraction after adjusting its pH to 5.0, centrifuging it to remove the acid-precipitable fraction, and further ammonium sulfate precipitation as described (4). The assayed fraction was a 60–100% ammonium sulfate cut which was dialyzed overnight at pH 7.2 against 0.01 M KPO_4 buffer. DHFR was assayed at pH 5 and pH 7.2 using the substrates as described under Methods, with increasing concentrations of MTX or DDMP. The values are the amount of drug required to inhibit the control activity by 50%.

Cell line	MTX, pH 5	MTX, pH 7.2	DDMP, pH 5	DDMP, pH 7.2
	M	*M*	*M*	*M*
3T6	2×10^{-9}	6×10^{-10}	4×10^{-9}	2×10^{-9}
3T6R400	2×10^{-7}	2×10^{-7}	1.5×10^{-7}	1.2×10^{-7}

tant cells (Table 2) even after 3 hr of treatment with 1.0 mM MTX. Moreover, deoxyuridine incorporation into DNA (Fig. 2A) was inhibited only by 28% under these conditions.

Relative sensitivity to MTX and DDMP of DHFR from 3T6R400 and 3T6 cells. DHFR from 3T6R400 was 100 and 267 times less sensitive, respectively, to MTX and DDMP inhibition at pH 5.0 than was enzyme from wild-type cells (Table 3). 3T6 and 3T6R400 activities were 2.0 and 0.75 times as sensitive to MTX as to DDMP when assayed at pH 5.0 with folic acid as substrate. A much larger difference in relative sensitivity to DDMP and MTX in 3T6R400 and 3T6 DHFR was observed when the extracts were assayed at pH 7.2 with dihydrofolate as substrate. In this case, resistance to MTX increased 3000-fold, whereas resistance to DDMP increased only 167-fold.

DISCUSSION

The 3T6 cell line and its MTX-resistant mutant are exceedingly useful for evaluating the sensitivity *in vitro* of cells to antifolate drugs. DHFR, the target enzyme for antifolates, can be amplified selectively so that it becomes the major soluble protein being synthesized in log-phase cells. The rate of DHFR synthesis in the MTX-resistant line is 10- to 20-fold greater than that of the parent line, so that 3.3% of the total soluble protein in log-phase cells is DHFR (17). As shown here, DHFR isolated from this cell line is also 100 times less sensitive to inhibition by MTX than that from the parent cell line. The cloned 3T6 line used was isolated by selection over many generations with progressively increasing concentrations of MTX (17). Since resistance in such cell lines is a stepwise process, a high-level resistance phenotype, such as in the 3T6R400 mutant, might not be expected to develop *in vivo* during a normal course of therapy. However, increased DHFR production might be expected from a lower order of gene amplification (5). Consequently, enzyme overproduction, although imparting significant resistance to MTX, might not lead to biologic resistance to therapeutically achievable levels of a transport-independent drug such as DDMP.

Uptake studies indicate that both the sensitive and resistant cells are at a steady state after 3 hr, with respect to total cell-associated MTX and DDMP, and to inhibition of deoxyuridine incorporation into DNA (21). We therefore further studied the inhibition of deoxythymidine incorporation into DNA, the inhibition of DHFR activity, and DNA synthesis over this time period. Whereas DDMP inhibition of thymidine incorporation into the resistant line was time-dependent and still increasing even after 2 hr, the inhibition of deoxyuridine incorporation was immediate and constant (Fig. 3). ^{32}P incorporation into DNA was similarly inhibited by DDMP (Fig. 5). Since reduction in cloning efficiency requires longer drug exposure times than measurements of deoxyuridine incorporation into DNA (24, 25, 30), cloning was examined after 24 hr of drug exposure. MTX at all concentrations tested (up to 1000 μM) had no significant effect on cloning efficiency of resistant cells, whereas DDMP was able to reduce cloning efficiency at 400 μM by >90%. Although these several methods measure different levels of toxicity, cellular responsiveness to DDMP and MTX was proportional with each.

This study examined several properties of the drug-cell interactions which contribute to high levels of resistance to MTX. Identical slopes for the plot of initial velocity of uptake versus MTX concentration showed that the resistant cell line accumulated MTX as well as the parent cell line (Fig. 6). The values obtained for K_m and V_{max} for the accumulation of MTX are the same in both the sensitive and resistant cell lines. These values were obtained using medium containing serum, in contrast to the media employed by others (3, 13, 26), of balanced salt solutions without serum. Studies in this laboratory have shown that the choice of medium and its components is an important parameter in determining a drug's physiological effect (36). Nonetheless, the values obtained, although somewhat higher, are in the same range as those reported by others. Earlier studies in other cells (37, 38) indicated that resistant cells were not able to accumulate intracellular MTX sufficient to saturate the binding sites for dihydrofolate on DHFR. Toxicity depends upon the balance between achievable intracellular drug concentrations and DHFR levels (3, 13, 39). The similar uptake rates observed in wild-type and 3T6R400 cells and the residual DHFR activity in the mutant cell line following 3 hr of drug exposure suggest that a similar mechanism of resistance may pertain in part in our studies.

The 3T6R400 cells were significantly more sensitive to DDMP than to MTX. It is clear from our work (24) and earlier studies of others (40, 41) that DDMP and MTX have different pharmacological properties in that the activity of DDMP is not affected by transport limitations (22, 42) at the concentrations used. Therefore, the restrictions on intracellular MTX accumulation are not relevant to DDMP wherein intracellular drug levels are directly proportional to extracellular drug concentrations (36). However, other mechanisms also contribute to the resistance of the 3T6R400 cell line. The resistant cell line has a DHFR with altered properties which makes it 100-fold more resistant than wild-type enzyme to both DDMP and MTX. This change in enzyme sensitivity is large relative to changes observed in studies of others (9, 10, 12). Bruckner *et al.* (43) observed a similar disproportionate increase in the amount of MTX required to overcome resistance in cells with increased DHFR. The contribution of any changes in the characteristics of the enzyme was not determined in this study. Our study leaves unresolved the question of whether this change is caused by a mutation in the DHFR structural gene or to the emergence of a different DHFR isozyme during the process of amplification. There is also some suggestion that the enzyme of the resistant cell may be somewhat more sensitive to DDMP than to MTX, according to assays with dihydrofolate as substrate at pH 7.2. Finally, these studies do not exclude the possibility that DDMP may have a site of action other than DHFR (44, 45), although we believe that the role of the mechanisms documented in this study are sufficient to explain the changes in sensitivity observed. The relative contribution of each mechanism to the resistance phenotype, however, has not yet been elucidated.

The results of this study suggest that lipid-soluble antifolates may be effective in treating cells with a pleiotropically resistant phenotype that includes transport limitations, target enzyme overproduction, and changes in target enzyme-drug affinity. The relatively greater sensitivity of such cells to lipid-soluble antifolates further supports the need for development programs for the design and testing of these transport-independent drugs.

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