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Metabolic Inhibition by a New Antifolate, 2,4-Diamino-6-(2,5-Dimethoxybenzyl)-5-methyl-pyrido[2,3-*d*]pyrimidine (BW301U), an Effective Inhibitor of Human Lymphoid and Dihydrofolate Reductase-Overproducing Mouse Cell Lines

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SUMMARY

The human lymphoblastoid cell line, WIL-2, and a mouse cell line, 3T6R400, that overproduces a mutant dihydrofolate reductase (DHFR) having >100-fold increased resistance to methotrexate (MTX) inhibition, were used to compare the inhibitory properties of a novel lipid-soluble antifolate, 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methyl-pyrido[2,3-*d*]pyrimidine (BW301U), with the 2,4-arylpyrimidine, 2,4-diamino-5-(3,4′-dichlorophenyl)-6-methylpyrimidine (DDMP) and with MTX. These studies demonstrated that, like DDMP, BW301U rapidly entered cells and inhibited the incorporation of dUrd into DNA. Drug association with cells was temperature-independent and apparently did not require active transport. BW301U inhibited cell growth by 50% at 0.025 μM, whereas MTX caused equivalent inhibition at 0.045 μM. Inhibition of DNA synthesis produced by 90 min of exposure to BW301U was completely reversed within 2 hr after washing cells and suspending them in drug-free medium. In contrast, inhibition of DNA synthesis in MTX-treated cells was not reversed by simply removing the antifolate, but required the addition of calcium leukovorin or thymidine to the drug-free medium in order to facilitate complete reversal of DNA synthesis. Finally, like DDMP, BW301U was approximately 1000 times more effective than MTX in inhibiting dUrd incorporation into the DNA of a DHFR gene-amplified cell line of mouse 3T6 cells.

INTRODUCTION

A new lipid-solution folate antagonist, BW301U[2] (1–3), which has a high affinity for DHFR, a short half-life *in vivo*, and promising antitumor effects in animal studies, has recently been synthesized. These characteristics and the minimal effects of this drug against histamine *N*-methyltransferase and diamine oxidase (4) have led to its selection as a promising new lipid-solution antifolate. Effects on histamine metabolism were determined because of earlier studies which showed that DDMP inhibited histamine *N*-methyltransferase and caused elevated histamine levels in brain and kidney of rats (4–6). Moreover, it was concluded that elevation of histamine levels in man would provide a reasonable explanation for many of the reported side effects of DDMP (7, 8).

This communication extends our preliminary report on the inhibitory properties of BW301U in human cells (9) and MTX-resistant mouse cell lines, and extends and supports observations previously reported by Duch *et al.* (1, 2) and Grivsky *et al.* (3), who have studied the properties of BW301U in rodent tumor lines *in vitro* and *in vivo*. In order to contrast the metabolic effects of these drugs, the present study compares BW301U, MTX, and DDMP with respect to the kinetics of inhibition of dUrd and ^{32}P incorporation into DNA and the dose-response relationships attendant to inhibition of cell growth.

MATERIALS

Carrier-free [^{32}P]phosphoric acid (^{32}P) and [6-^{3}H] deoxyuridine (24.2 mCi/mmol) were obtained from New England Nuclear Corporation (Boston, Mass). MTX was kindly provided by the Developmental Therapeutics Program, Division of Cancer Treatment of the National Cancer Institute. BW301U, DDMP, and calcium leukovorin were kindly provided by Drs. C. A. Nichol, C. Sigel, and D. Duch of Burroughs Wellcome Company (Re-

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[2] The abbreviations used are: BW301U, 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methyl-pyrido[2,3-*d*]pyrimidine; DHFR, dihydrofolate reductase; DDMP, 2,4-diamino-5-(3,4′-dichlorophenyl)-6-methylpyrimidine; MTX, methotrexate; MEM, minimal essential medium; PBS, phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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search Triangle Park, N. C.). Concentrations of calcium leukovorin are expressed as the concentrations of the naturally occurring stereoisomer. The human lymphoblastoid cell line, WIL-2, originally isolated by Levy *et al.* (10), was obtained from Dr. M. Hershfield of Duke University. The MTX-resistant mouse cell line, 3T6R400, was obtained from Dr. Vera Morhenn of the Stanford University School of Medicine. The autoclavable formulation of MEM, with Earle's salts for suspension culture, and the fetal calf serum were purchased from Grand Island Biological Company (GIBCO, Grand Island, N. Y.). Dialyzed fetal calf serum from K-C Biologicals or, alternatively, mycoplasma-screened fetal calf serum from GIBCO was dialyzed two times against 10 volumes of 0.15 M NaCl for sequential 12-hr periods.

METHODS

Cell Maintenance

WIL-2 cells were maintained in suspension cultures by continuous agitation on a rotary shaker at 37° at a density of 1.5–8 × 10^5 cells/ml. MEM for use in suspension cultures was supplemented with 10% fetal calf serum.

Cell counts were obtained from WIL-2 cultures by diluting cell suspensions with one-third of their volume of trypan blue staining solution (GIBCO) and counting the cells in a Neubauer hemocytometer. The wild-type 3T6 cell line was carried in MEM medium with 5% dialyzed fetal calf serum and routinely passaged three times weekly by subdividing the culture 1:4. The mutant cell line was carried in an identical manner except that the medium contained 400 μM MTX.

Pulse Labeling Protocol

Suspension cells. Cells were preincubated for 30 min in fresh medium (MEM) containing 10% dialyzed fetal calf serum, then pulse-labeled with tritiated deoxyuridine by transferring 0.2–0.3 ml of cells to a tube containing 2 μCi of tritiated deoxyuridine. The reaction was stopped by the addition of ice cold PBS, the cells were centrifuged, and the supernatant was removed. Cells were then resuspended and spotted on Whatman filter paper discs which were further processed for scintillation counting as previously described (11).

Surface-attached cells. Cells were subcultured in 16-mm wells of tissue culture dishes (tissue culture Cluster 24; Costar, Cambridge, Mass.) for 18–24 hr prior to use. Medium containing MTX or DDMP at the desired concentration was added for designated time intervals. For each experiment, cells were incubated in Hepes-buffered MEM containing sodium bicarbonate (0.73 g/liter) and 6 mM Hepes for 30 min before addition at time zero of the designated concentrations of antifolates. The reaction was stopped by removing the medium from the well and washing the monolayer once with PBS. A 0.5-ml volume 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 PBS; the cells were collected by centrifugation at 800 × *g* for 5 min. The cells were then resuspended in 100 μl of PBS and spotted on No. 3 filter discs (Whatman, Inc., Clifton, N. J.) and processed for scintillation counting. ID_{50} values were determined by probit analysis for the inhibition obtained after 3 hr in each cell line (12).

^{32}P Incorporation

Suspension cells. The incorporation of ^{32}P into DNA was determined by a modification of the method of Schmidt and Thannhauser (13). WIL-2 cells (2 × 10^6/ml) were preincubated at 37° in MEM containing dialyzed fetal calf serum and one-half of the normal phosphate (0.5 μM). After 30 min, ^{32}P (100 μCi/ml) and either 1 μM MTX or 5 μM DDMP were added to the cell suspension. At appropriate intervals, duplicate samples were removed and added to PBS containing 1 μM sodium azide. The samples were washed once in PBS and then twice in 5% trichloroacetic acid. Samples were then incubated in 0.6 N KOH for 2 hr at 37° to hydrolyze the RNA fraction.

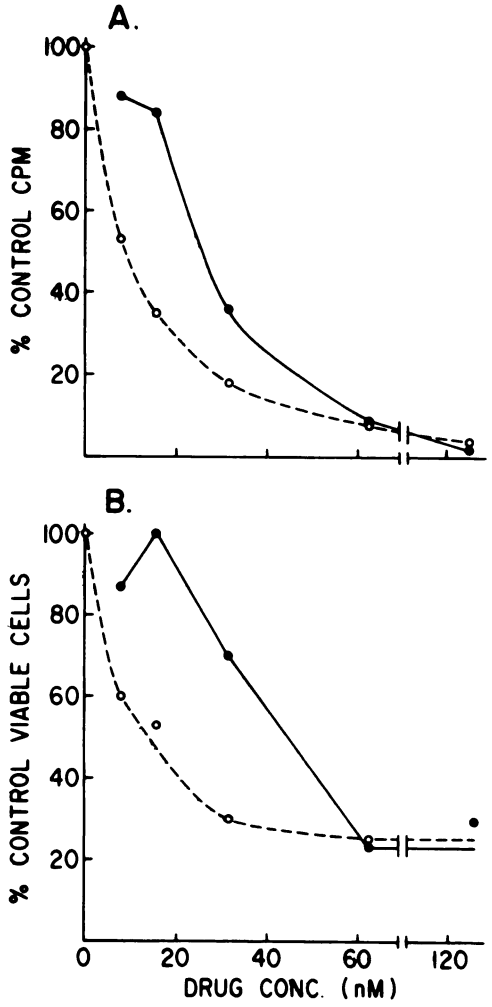

FIG. 1. *Inhibition of deoxyuridine incorporation into DNA and inhibition of cell growth by BW301U and MTX: dose response*

Triplicate cultures of WIL-2 cells were seeded at a concentration of 1.5 × 10^5 cells/ml and incubated with the indicated drug concentrations. At 24 and 48 hr, 0.5 ml of cell suspension was taken from each flask and incubated for 1 hr at 37° with [^{3}H]dUrd (8 μCi/ml). Thereafter, the cells were harvested and processed for scintillation counting as previously described (9) (A). A second sample was taken from each of the flasks at this time and incubated with trypan blue for 5 min before determination of the number of viable cells in each culture (B). ○, BW301U; ●, MTX.

The remaining DNA was precipitated by 5% trichloroacetic acid, and again centrifuged and hydrolyzed in 10% trichloroacetic acid at 90° for 1 hr. Aliquots of the DNA were counted in a scintillation counter; each point represents the average of duplicate determinations.

Monolayer cells. The incorporation of ^{32}P into monolayer cells was assayed in a manner similar to that for suspension cells. Hepes-buffered MEM containing drug was added to the 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 PBS containing 1 mM sodium azide. A 0.5-ml volume of 0.25% trypsin was added, and the cell suspension was pipetted into tubes containing 3 ml of ice-cold PBS/azide and processed for determination of isotope incorporated into RNA and DNA.

RESULTS

The relative abilities of BW301U and MTX to inhibit WIL-2 growth and dUrd incorporation are compared in Fig. 1. BW301U was somewhat more toxic than MTX for these cells. With both drugs, 2 days of exposure were required to achieve a consistent dose-response cell-killing curve.

Like DDMP (14), BW301U immediately enters cells and blocks dUrd incorporation into DNA. The kinetics of drug entry for BW301U and DDMP was not detectably affected by shifting cell cultures from 37° to 23° (Fig. 2), demonstrating that these drugs do not require an active transport mechanism for their entry into cells.

A comparison of the effects of MTX, BW301U, and DDMP on cellular DNA synthesis was carried out by labeling cells with ^{32}P and then differentially extracting the DNA and RNA to determine labeled nucleotide incorporation into these fractions. Our earlier studies (15, 16) have shown that, although inhibition of ^{32}P into DNA measures the immediate metabolic effects of antifolates less sensitively than inhibition of dUrd incorporation into DNA, it is a better indicator of the potential lethal toxicity of these drugs. When this parameter was measured, 1 μM BW301U, 1 μM MTX, and 10 μM DDMP gave equivalent levels of inhibition of DNA synthesis once a steady state was reached (Fig. 3). Reversibility of this inhibition was then evaluated after 90 min by washing the cells and resuspending them in fresh medium, medium containing 10 μM dThd, medium containing 50 μM calcium leukovorin, or medium containing 10 μM dThd and 50 μM calcium leukovorin, and continuing the incu-

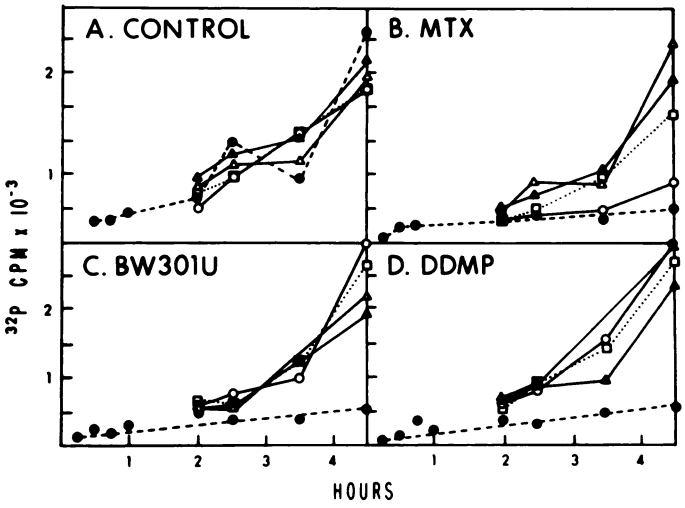

FIG. 3. *Inhibition of DNA synthesis by MTX, BW301U, and DDMP, and reversal of this inhibition in drug-free medium or medium containing calcium leukovorin or thymidine*

WIL-2 cells were suspended and incubated as described for ^{32}P incorporation (see Methods). At time zero, ^{32}P was added along with no drug (A), 1 μM MTX (B), 1 μM BW301U (C), or 10 μM DDMP (D). At 90 min after drug addition, aliquots of the cells in each culture were centrifuged and resuspended in the ^{32}P- and drug-containing medium (●), or washed once with drug-free medium and resuspended in medium containing ^{32}P but no drug (○); ^{32}P and 10 μM dThd (△); ^{32}P and 50 μM calcium leukovorin (□); or ^{32}P, 50 μM calcium leukovorin, and 10 μM dThd (▲). Samples were taken at the indicated time intervals after addition of drugs, and processed for incorporation of ^{32}P into DNA as described under Methods.

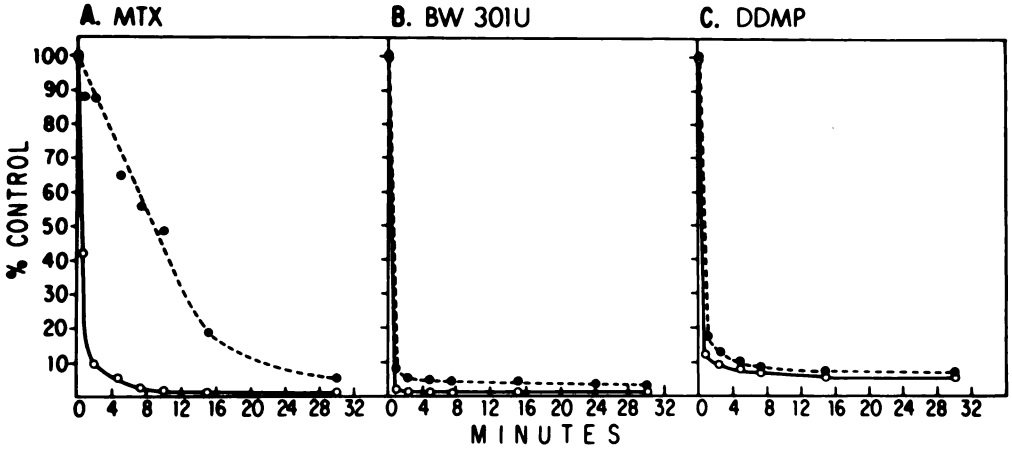

FIG. 2. *Effect of temperature on onset of inhibition of deoxyuridine incorporation MTX, BW301U, and DDMP*

WIL-2 cells (2×10^6 cells/ml) were suspended in MEM containing 10% dialyzed fetal calf serum. The cells were then incubated at 37° (○) or 23° (●) with 1 μM MTX (A), 1 μM BW301U (B), or 1 μM DDMP (C). Control incorporation values in drug-free medium for the experiments with MTX and BW301U were carried out on the same day and averaged 10,020 cpm at 37° and 2,125 cpm at 23°. Control values for the similar DDMP experiment shown in C averaged 23,558 cpm and 7,131 cpm, respectively. At the indicated time points, 200-μl aliquots from each incubation flask were pulse-labeled for 5 min with 2 μCi of dUrd and processed for counting as described under Methods.

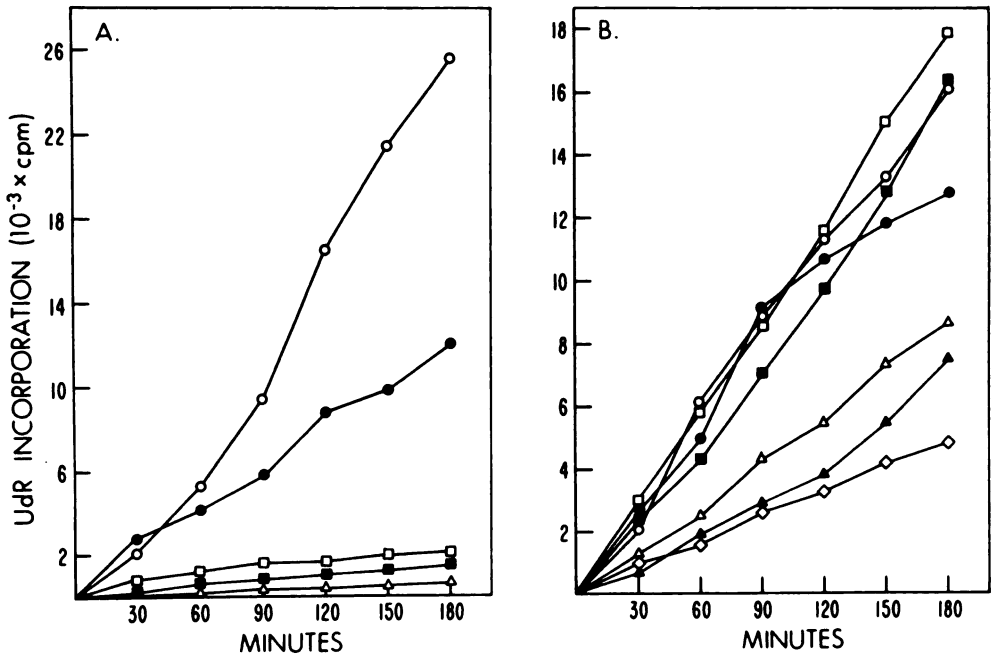

FIG. 4. *Inhibition of deoxyuridine incorporation into the DNA of 3T6R400 and 3T6 Cells by BW301U*

Cells (3×10^4/well) were subcultured in medium without drug for 24 hr prior to use in experiments with 3T6R400 cells. Drug response in wild-type 3T6 cells is shown in A contrasted with response to drug in 3T6R400 cells in B. BW301U was added at time zero, and replicate samples were pulse-labeled with [^{3}H]dUrd (*UdR*) (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. dUrd incorporation into DNA was determined as described under Methods. ○, Control, no drug; ●, 0.1 μM BW301U; □, 0.5 μM BW301U; ■, 1.0 μM BW301U; △, 5.0 μM BW301U; ▲, 10.0 μM BW301U; ◇, 25 μM BW301U.

bation for 3 hr. The DNA synthesis of cells inhibited by either BW301U or DDMP was fully reversible under all of the above conditions. The inhibitory effects of MTX on DNA synthesis, although fully reversible by dThd and calcium leukovorin, was not completely reversed by washing the cells and resuspending them in fresh medium. RNA synthesis was not significantly affected by any of the drugs during the time course of this experiment (data not shown).

BW301U was also effective in inhibiting the incorporation of dUrd into the DNA of a mouse cell line, 3T6R400, which we and others (17–19) have found to have a complex phenotype, resistant to MTX because of the overproduction of a DHFR which is 100 times more resistant to MTX than the DHFR from the parent 3T6 cell line. Probit analysis of the data in Fig. 4B showed that dUrd incorporation into DNA of 3T6R400 can be 50% inhibited by concentrations of BW301U of 6 μM, whereas an equal level of inhibition requires 10 mM MTX (19). The ^{32}P incorporation into DNA of the 3T6R400 was also more sensitive to BW301U and DDMP than to MTX (Fig. 5).

DISCUSSION

Lipid-soluble antifolates have the distinct advantage of entering cells without dependence on a cellular active transport mechanism. This consideration has prompted our focus on lipid-soluble drugs over the past several years (9, 11, 14–17). The time-course for producing metabolic inhibition by BW301U *in vitro* was similar to that of DDMP (14), which was selected in our earlier studies as a prototypic lipid-soluble antifolate. Cells approached a steady state of inhibition of deoxyuridine incorporation into DNA within minutes following addition of this drug to the culture medium. Uptake of this drug when measured indirectly by inhibition of dUrd incorporation into DNA was independent of temperature between 23° and 37°. This indirect method was chosen to study the kinetics of BW301U entry into WIL-2 cells because of our earlier studies of DDMP (14, 20). These studies suggested that DDMP is concentrated in hydrophobic spaces of the cell so that measurement of labeled drug uptake does not denote access of lipid-soluble antifolate to DHFR. However, as shown with DDMP, inhibition of dUrd incorporation occurs immediately after the addition of 1 μM BW301U to cells and all other concentrations of this drug above 0.1 μM (data not shown).

Although our *in vitro* studies showed that the continuous presence of BW301U was both more toxic and more effective in inhibiting deoxyuridine incorporation into DNA of WIL-2 cells than was MTX, inhibition by this drug was more easily reversed in the absence of continuing drug exposure than was inhibition by MTX. Inhibition of DNA synthesis by BW301U in WIL-2 cells was reversed by simply removing the drugs from the medium after 90 min. This contrasted to the result obtained with MTX in WIL-2 cells, where reversal of inhibition of DNA synthesis over a comparable time period required the further addition of dThd or calcium leukovorin to the medium. This difference could be related to the ability of cells to form polyglutamates of MTX (21), a reaction that is not possible with BW301U. Clearly, this difference in the metabolism of the two drugs could affect significantly the drug scheduling requirements for effective use

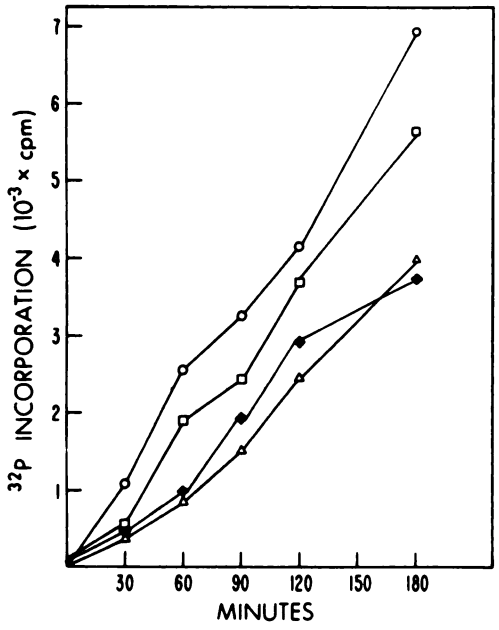

FIG. 5. *Effect of BW301U, MTX, and DDMP on ^{32}P incorporation into the DNA of 3T6R400 cells*

Cells (3×10^4/well) were subcultured in medium without drug for 24 hr prior to use. ^{32}P (100 μCi/ml) and 50 μM BW301U (◆), 50 μM MTX (□), 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 under Methods. Each point represents the average of duplicate determinations. The sample ○ indicates ^{32}P incorporation into DNA in a control culture containing no drug.

of BW301U, and, coupled with its short half-life in blood, may necessitate longer, continuous, or more frequent intermittent exposure periods to achieve optimal chemotherapeutic effects.

BW301U was also an effective inhibitor of deoxyuridine incorporation and DNA synthesis in 3T6R400 cells. This cell line has a complex resistance phenotype, since it overproduces a DHFR enzyme which also has reduced affinity for MTX (18, 19). Our studies with BW301U in 3T6R400 cells confirmed earlier studies which demonstrated the important role of the cellular active transport mechanism in the observed resistance to MTX (17). Despite a complex resistance phenotype, 3T6R400 cells were sensitive to levels of both DDMP (17) and BW301U (9) that could be achieved *in vivo*.

In conclusion, BW301U is a new lipid-soluble folate antagonist with potency against WIL-2 human lymphoblastoid cells that is somewhat greater than that of MTX, as measured by inhibition of cell growth and deoxyuridine and ^{32}P incorporation into DNA. This folate-dependent toxicity was readily reversible in cultures of WIL-2 cells after drug exposure periods of 90 min. BW301U, like DDMP, was also an effective inhibitor in 3T6R400 cells wherein the inhibition produced by this drug was consistent with their expected resistance attributable to high DHFR levels. Inhibition of this cell line by 6 μM BW301U contrasted with that obtainable with MTX, where 10 mM MTX was required to produce equivalent inhibition of dUrd incorporation. All of these biochemical and biological properties suggest that BW301U may be a promising inhibitor of cell growth, particularly in tumor cells that may be resistant to MTX.

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