Selective Toxicity of a New Lipophilic Antifolate, BW301U, for Methotrexate-resistant Cells with Reduced Drug Uptake

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ABSTRACT

Three methotrexate (MTX)-resistant cell lines and their MTX-sensitive counterparts have been used to examine 2,4-diaminoo-6-(2,5-dimethoxybenzyl)-5-methyl-pyrido[2,3-d]pyrimidine (BW301U), a novel lipophilic antifolate, and compare its cytotoxicity with MTX and metoprine. Collateral sensitivity for both BW301U and metoprine was observed in CCRF-CEM/MTX R cells, where MTX resistance appeared to be primarily due to a deficiency in drug uptake. This was particularly pronounced with BW301U which proved to be as effective in killing CCRF-CEM/MTX R as was MTX with the parental CCRF-CEM cell line. This effect was not seen in other cell lines, L5178Y/MTX or L1210/MTX R, where resistance to MTX was correlated with either an overproduction of 5,6,7,8-tetrahydrofolate:nicotinamide adenine dinucleotide phosphate oxidoreductase EC 1.5.1.3 (DHFR) or with combined uptake defect and increased DHFR levels, respectively. In each case, however, BW301U and metoprine, especially at high concentrations, were more effective than MTX in treating MTX-resistant cells.

INTRODUCTION

MTX has been successfully used in the treatment of a number of commonly occurring tumors in humans. However, the development of resistance to further treatment with MTX in tumors which have initially responded to the drug remains a major clinical problem. The evolution of this acquired resistance to MTX has been shown, primarily in experimental systems, to occur by a variety of mechanisms which include: (a) decreased net uptake of MTX into cells (11, 12, 15, 30, 37); (b) increased cellular production of the target molecule DHFR (1, 2, 10, 18, 25, 30, 37); and (c) reduced affinity for MTX of an altered form of DHFR (9, 13, 23, 30).

Of these mechanisms of resistance to MTX, those involving drug transport and overproduction of DHFR have been examined in most detail. At low concentrations of MTX, the drug is thought to be transported into the cell by the same active transport system used for the uptake of naturally occurring reduced folates (28, 32, 35, 36). In transport-resistant cells, both the V_m and K_m of this transport system for MTX can be altered in such a way as to reduce the net accumulation of intracellular MTX (36). Attempts to overcome this type of resistance have involved the use of high-dose MTX or lipophilic MTX analogues such as DDMP where the primary route of cellular uptake is by passive diffusion (6, 7, 9, 16, 17, 21, 29, 32, 33, 36, 41). In addition, it has been suggested that such resistant cells also exhibit changes in transport kinetics of naturally occurring reduced folates. These changes may potentiate the effects of antifolates capable of penetrating such folate-compromised cells (32, 36).

Overproduction of DHFR, on the other hand, has been shown to occur by increased enzyme synthesis due to selective amplification of DHFR genes (for review, see Refs. 2 and 20). This type of MTX resistance can also be partially overcome by the use of lipophilic antifolates such as DDMP where membrane diffusion occurs so rapidly that even high levels of DHFR can be saturated (19). More recently, a novel technique involving the use of tirtated deoxyuridine has also been reported as a means of overcoming resistance due to increased levels of DHFR, but this has yet to be clinically evaluated (40).

High-dose MTX has been used to overcome acquired MTX resistance; however, in head and neck cancer, at least this treatment can result in severe normal tissue complications and marginal clinical benefit (43). Of the lipid-soluble antifolates used in clinical practice, DDMP has a limited efficacy at tolerated doses and is associated with undesirable side effects which are thought to be related to its effects on histamine metabolism and relatively long half-life in vivo (8, 29).

A new lipid-soluble inhibitor of DHFR, BW301U (7, 9, 17, 33), has recently been reported to be effective in overcoming MTX resistance due to overproduction of DHFR (33). In addition, BW301U has a relatively short half life in vivo and only minimal influence on histamine synthesis, suggesting a possible clinical role as an alternative to DDMP (7, 8).

In this study, we have examined the cytotoxicity of BW301U on a number of MTX-resistant cell lines and their MTX-sensitive counterparts and have compared these results with the cellular response to MTX and DDMP treatment.

MATERIALS AND METHODS

Cell Lines

All cells were maintained as static suspension cultures at 37° in RPMI Medium 1640 supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (2 × 10^{-2} M), L-glutamine (2 × 10^{-2} M), penicillin/streptomycin (100 units/ml), and 10% fetal calf serum. Nondialyzed serum was used throughout this study. All cell counts and determinations of cell viability were made by phase-contrast microscopy. MTX-resistant cell lines were cultured in the absence of methotrexate for several months prior to study.

CCRF-CEM. This is a human leukemic T-cell line (14) which has been maintained in our laboratory for several years. Its MTX-resistant subline CCRF-CEM/MTX R was derived by continuous exposure to 2 × 10^{-4} M MTX for a period of 4 weeks. Our previous studies (39) have shown that at this concentration of MTX, cell death and cell division are balanced, resulting in no net increase in cell numbers. After 1 month, cells in 2 × 10^{-4} M MTX were growing at the same rate as non-drug-treated controls. The MTX concentration was then increased from 2 × 10^{-4} M to 4 × 10^{-7} M.
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m over a further period of 6 weeks. At this time, the cells could maintain control growth in 4 x 10^6 M MTX, and samples of them were stored at -70°C. Subsequent subculturing of CCRF-CEM/MTX R was made in MTX-free medium.

The cell population doubling time for both cell lines was about 22 hr. In our hands, the CCRF-CEM cell line does not clone reliably in soft agar, and so determinations of cell viability after drug treatment were made by phase-contrast microscopy.

L1210 and L5178Y. These are mouse leukemic cell lines which have been maintained in our laboratory for a number of years. Their MTX-resistant counterparts, L1210/MTX R and L5178Y/MTX R, were obtained several years ago from Dr. J. Berlino, Yale University, New Haven, CT. The cell population-doubling times for all 4 cell lines were 12 to 14 hr. All 4 cell lines were cloned in soft agar, although both the L1210 cell lines had a very much higher plating efficiency (90 to 100%) than the L5178Y cells (15 to 20%). Conditions used for soft-agar cloning have been fully described elsewhere (38).

Drug Treatment

MTX was supplied by Dr. A. Hellestrand, Cyanamid (Australia) Pty, DDMP, and BW301U were supplied by Dr. C. A. Nichol, Burroughs Wellcome Co., Research Triangle Park, NC.

All drugs were made up as concentrates at 30-fold of the final required concentration in distilled water and sterilized by Millipore filtration. Solutions of BW301U were solubilized by the addition of NaOH (1 M). The solubility of DDMP was increased by the addition of 1 to 2 molar equivalents of lactic acid.

MTX Uptake Studies

Prior to uptake experiments, [3',5',7-3H]MTX (Amersham Australia, Pty. Ltd.) was diluted to a specific activity of 1200 Ci/mol and purified by gel permeation chromatography on Fractogel TSK 40 (F) (BDH Australia) in a manner similar to that of Whitehead et al. (42).

Cells from exponentially growing cultures were adjusted to approximately 5 x 10^6 cells/ml (CCRF-CEM) or 5 x 10^5 cells/ml (L1210 and L5178Y) 24 hr before drug addition. For all cytotoxicity data shown, the drug exposure time approximates 2 cell population-doubling times, i.e., 48 hr for CCRF-CEM and 24 hr for L1210 and L5178Y, in order to facilitate comparison between cell lines.

RESULTS

Net uptake of MTX and levels of DHFR activity were examined in all 3 MTX-resistant cell lines and compared with their MTX-sensitive counterparts in order to determine the primary mode of MTX resistance prevailing in each case (Chart 1; Table 1). The

Approximately 5 x 10^6 cells were harvested and washed twice with 10-ml volumes of ice-cold phosphate-buffered saline. Cell pellets were resuspended with 5 ml of 0.1 m potassium phosphate buffer at pH 7.0 and homogenized by sonication. Homogenates were stored at -70°C until assayed. After thawing, the homogenate was centrifuged at 4°C for 1 hr (48,000 x g), and the clear supernatant was used in the assay. This preparation was stable with respect to DHFR activity for three days when stored at 4°C.

Assays were carried out at 37°C in disposable plastic cuvetts, and the decreasing absorbance at 340 nm was measured in a Cary 219 spectrophotometer. The final assay volume was 1.2 ml containing 60 μmol of potassium phosphate buffer (final pH, 7.0), 1.2 mg of bovine serum albumin, 12 μmol of 2-mercaptoethanol, 720 μmol of KCl, 20 μl of enzyme preparation, 48 nmol of NADPH, and 12 nmol of dihydrofolate. The assay was initiated by the addition of dihydrofolate, and the resulting rate corrected for a small background reaction where dihydrofolate was omitted. Inhibition of DHFR was assayed by the addition of MTX (final concentration, 10^-10 - 5 x 10^-9 M) to the assay mixture. Protein concentrations in the cell extract were estimated by the method of Lowry et al. (26).

DHFR Assay

Crude extracts from cell cultures were assayed for DHFR activity using a modification of the spectrophotometric technique described by Mathews et al. (27).

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Table 1

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>DHFR activity*</th>
<th>% of MTX inhibition</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10⁻¹⁰ M</td>
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<td>CCRM-CEM</td>
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<td>86</td>
</tr>
<tr>
<td>CCRM-CEM/MTX R</td>
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<td>86</td>
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<tr>
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<td>11</td>
<td>63</td>
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<td>L1210/MTX R</td>
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<td>44</td>
</tr>
<tr>
<td>L5178Y</td>
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<td>36</td>
</tr>
<tr>
<td>L5178Y/MTX R</td>
<td>3220</td>
<td>63</td>
</tr>
</tbody>
</table>

* Activity expressed in nmol/mg/min at 37° using 10 μM dihydrofolate and 40 μM NADPH.

DHFR activities in MTX-resistant and -sensitive cell lines

Expressed as percentage of remaining DHFR activity in the presence of MTX.

Chart 2. Response of CCRM-CEM (○) and CCRM-CEM/MTX R (●) cells to MTX, DDMP, and BW301U. CCRM-CEM/MTX R cells are MTX-resistant primarily due to a drug uptake defect. Cell counts are of viable cells estimated by phase-contrast microscopy. Points, means of 2 to 3 individual experiments. The range between experiments was not greater than 20%.

CCRM-CEM/MTX R-cells were found to have a much reduced uptake of MTX and no major change in DHFR levels. In contrast, while little variation in initial MTX uptake was seen between the L5178Y cell lines, L5178Y/MTX R-cells had a 500-fold increase in DHFR levels compared to MTX-sensitive controls. This fact would probably account for the higher intracellular levels of MTX observed in the resistant subline after approximately 30 min of drug uptake (Chart 1). These cell lines were, therefore, judged to be resistant primarily through a transport defect and through DHFR overproduction, respectively. The L1210/MTX R-cell line, on the other hand, had both a reduced MTX uptake and an increased level (24-fold) of DHFR activity and is therefore probably resistant to MTX by virtue of both mechanisms. We found no evidence to suggest that there was any substantial difference between sensitive or resistant cells in the degree to which DHFR was inhibited by MTX (Table 1).

Chart 2 shows that CCRM-CEM/MTX R-cells are approximately 100-fold resistant to MTX compared with controls, and that this resistance can be overcome by high concentrations of MTX. When, however, these MTX-transport defective cells are treated with either DDMP or BW301U (Chart 2), they appear to be more sensitive to these agents than normal CCRM-CEM cells. This contrasts with the response of the DHFR overproducing L5178Y/MTX R-cell line which is extremely resistant to MTX (~10,000-fold) and also shows comparable resistance to both DDMP and BW301U although at high concentrations, both of these agents are superior to MTX (Chart 3).

Chart 3. Response of L5178Y (○) and L5178Y/MTX R (●) cells to MTX, DDMP, and BW301U. L5178Y/MTX R cells are MTX-resistant due to the overproduction of DHFR. Surviving fraction was estimated from a colony-forming assay in soft agar. Points, means of 2 individual experiments.

The doubly resistant L1210/MTX R-cells (Chart 4) show responses to all 3 agents intermediate to those described above for CCRM-CEM/MTX R and L5178Y/MTX R. This cell line demonstrates a 5000-fold increase in resistance to MTX and a marked sensitivity to both DDMP and BW301U compared to
DISCUSSION

This study shows that both DDMP and BW301U are selectively cytotoxic to cells that are primarily resistant to MTX by virtue of reduced net drug uptake. Similar findings have been reported previously for DDMP and other lipophilic antifolates and are thought to be a consequence of the altered transport kinetics for naturally occurring reduced folates in these cells (32, 36). This collateral sensitivity assumes potential importance in the light of other studies where it was found that transport defects formed the major category of acquired resistance to MTX in both human and murine cell lines (30, 37). In this respect, BW301U appears to be more effective than DDMP with a molar drug concentration required to decrease cell viability to 10% of control values for CCRF-CEM/MTX R-cells identical to that of MTX for the MTX-sensitive parent line. Approximately 10-fold more DDMP was required under comparable circumstances. This increase in molar efficiency of BW301U compared to DDMP was also observed to a variable degree in all other cell lines examined in this study.

It must be noted, however, that the collateral sensitivity of both DDMP and BW301U to transport defect phenotypes is lost when the mode of resistance is compounded by overproduction of DHFR. Under these circumstances, although doubly resistant cells are more sensitive to DDMP and BW301U than to MTX, these cells are relatively resistant to both compounds when compared with the MTX-sensitive parent line. In the studies mentioned previously (30, 37), the amplification of DHFR activity in the transport defect phenotype was found to occur at a high frequency, particularly in human lymphoblastoid cells.

In circumstances where over production of DHFR appeared to be the reason for MTX resistance, DDMP and BW301U proved to be more cytotoxic than MTX only at high dose levels, and such cells were relatively resistant to both drugs when compared to their MTX-sensitive counterparts as reported previously (19, 33).

Our experimental data do not rule out the possibility that MTX resistance has developed, in part, through the mechanisms of altered DHFR affinity of the drug (13) or decreased polyglutamylation (31). However, any presumed changes in DHFR inhibition kinetics would probably be of minor significance given the data presented in Table 1 and the extent of the altered DHFR activity and/or MTX uptake that are apparent in resistant variants that we describe. Furthermore, assuming that MTX polyglutamates may enhance MTX cytotoxicity by virtue of retained intracellular drug after removal of the external drug supply (31) an alteration in polyglutamylation ability would be of little or no selective advantage to any of the these cell lines where resistance has been established in a system where extracellular drug is constantly available. Therefore, it would appear most unlikely that the polyglutamylation status of these MTX-R lines has any bearing on our basic observations which can be reasonably accounted for without invoking mechanisms other than defective drug uptake and/or increased DHFR activity.

To date, there have been few studies characterizing the mechanisms involved in the development of clinically relevant resistance to MTX in humans, although it appears that transport defects, amplified DHFR activity and altered DHFR inhibition kinetics, have been identified (3–5, 22, 24). The relevance of models of resistance such as we describe here, based as they
are on immense selective pressures (e.g., constant drug exposure) and resulting in predictably dramatic changes in phenotype, can be questioned. Currently, we are investigating resistance mechanisms as they arise in intermittent drug treatment protocols that are more closely related to the clinical schedules used in cancer treatment.

The data presented in this study suggest that lipophilic antifolates such as DDMP and BW301U have a potential role in the treatment of MTX-resistant tumors. These drugs may be particularly effective in MTX-resistant tumors exhibiting defective MTX uptake. The combined administration of MTX and a lipophilic antifolate such as BW301U may be advantageous as initial treatment, particularly in those tumor types where MTX transport defects are prone to occur.

REFERENCES

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