Paradoxical Effect of BW 301U, a Lipophilic Antifolate, on Methotrexate-inhibitable Deoxyuridine Incorporation by Human Hematopoietic Cells

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ABSTRACT

The ability of methotrexate and BW 301U, a lipophilic folate antagonist, to inhibit tritiated deoxyuridine incorporation into acid-precipitable material by human bone marrow cells was evaluated before and after five sequential daily infusions of BW 301U. After in vivo BW 301U therapy, bone marrow cells from five of the six patients exhibited significantly reduced inhibition by 1 μM methotrexate in vitro, whereas the response to 1 μM BW 301U remained unchanged. Megaloblastic marrow morphology and decreased myeloid progenitor cloning efficiency were also observed following five daily BW 301U infusions of 21 and 71 mg/m², respectively. A similar reduction in the ability of methotrexate to inhibit tritiated deoxyuridine incorporation was also seen in HL-60 cells, a human acute promyelocytic leukemia cell line, after incubation in vitro with cytostatic concentrations of BW 301U for 3 days. Concomitant changes in the response to BW 301U did not occur. While it is premature to infer clinical significance from this preliminary observation of BW 301U-induced asymmetry in the response to subsequent antifolates, our results augment a growing body of evidence which suggests that lipophilic folate antagonists might be effective in the treatment of methotrexate-resistant neoplasms.

INTRODUCTION

Folic acid antagonists have been used in the treatment of malignant disease for 35 years. MTX, the 4-amino-N10-methyl analogue of folic acid, possesses a broad spectrum of antitumor activity and is the most widely used antimetabolite of this type. For example, MTX plays an important part in the therapy of acute lymphoblastic leukemia of childhood, breast cancer, choriocarcinoma, and head and neck cancer.

Resistance to MTX (4, 19) has been studied in both animal and human tumor cell lines. Several mechanisms of resistance have been demonstrated, including: (a) active transport defects (27); (b) DHFR overproduction due to gene amplification (8, 21); (c) antifolate-dependent DHFR accumulation (16); (d) decreased affinity of DHFR for MTX (18); (e) decreased polyglutamation of MTX (7); and (f) decreased thymidylate synthetase activity (1). The relative significance of each of these mechanisms in the development of clinically significant MTX resistance has not been adequately determined. However, both gene amplification leading to DHFR overproduction (2, 8) and decreased sensitivity of DHFR to inhibition by MTX (5) have been observed in malignant cells obtained from patients with methotrexate-resistant neoplasms.

Two distinct approaches have been adopted in attempts to circumvent MTX resistance. The first has used high-dose MTX regimens in which normal tissues are rescued by sequential administration of folic acid (13) or thymidine ± inosine (9). Improved responses with such regimens have been claimed for patients with head and neck cancer, osteosarcoma, and non-Hodgkin’s lymphomas (19). The second approach has focused on the synthesis of lipophilic folate antagonists, agents that are capable of circumventing several of the above-mentioned mechanisms of MTX resistance. An additional advantage of these compounds is their potential for improved penetration into cerebrospinal fluid.

BW 301U, 2,4-diamino-6-{2,5-dimethoxybenzyl}-5-methylpyrido[2,3-d]pyrimidine, is a novel lipophilic folate antagonist (14) which exhibited many favorable characteristics in preclinical studies (25). Several attributes of this drug suggest that it has greater promise as a chemotherapeutic agent than do earlier lipophilic folate antagonists such as DDMP. It has a greater affinity for its target enzyme, DHFR, than does DDMP (12), and its DHFR-inhibitory capacity is comparable to that of MTX (12, 22); it has a much shorter and therefore more favorable half-life than does DDMP (6, 17); it produces significantly less inhibition of histamine N-methyltransferase (12) and should therefore cause fewer histaminergic side effects; and it produces greater cytotoxicity than DDMP in cell culture systems (12). In addition, our laboratory has previously shown that BW 301U is effective against cells with documented MTX resistance in vitro (15, 24). For these reasons, the drug is currently undergoing Phase I and II evaluation (17).

The design of the studies described here stemmed from our previous work with DDMP, which demonstrated that transient biological resistance to MTX and DDMP can develop in human lymphoblastoid (WIL-2) cells following in vitro exposure to DDMP (16). At least part of the biochemical basis of this resistance appears to be related to accumulation of DHFR which occurs during the period of DDMP exposure. This observation prompted us to explore the possibility that short-term exposure of normal cells to BW 301U could induce changes in the response of these cells to subsequent administration of either BW 301U or MTX. We therefore examined the in vitro response of normal human hematopoietic cells to MTX and BW 301U, both before and after in vivo administration of BW 301U, by quantitating the ability of these antifolates to inhibit incorporation of [3H]dUrd into acid-precipitable material (5, 23). The possibility that similar changes could occur in malignant cells was examined by modeling anal-
ogous experiments in HL-60 cells, a human acute promyelocytic leukemic cell line, before and after in vitro exposure to BW 301U.

MATERIALS AND METHODS

Materials. BW 301U for the in vitro studies was a kind gift from Drs. C. W. Sigel and C. A. Nichol of Burroughs-Wellcome Co., Research Triangle Park, NC. MTX was provided by the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. HL-60 cells were kindly provided by Dr. R. Gallo, National Cancer Institute, and were maintained in suspension culture in RPMI Medium 1640 (Grand Island Biological Co., Grand Island, NY), supplemented with 10% fetal calf serum, 0.1 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and antibiotics (RPMI 1640-FCS). Cultures were kept at 0.3 to 1.0 x 10^6 cells/ml in a humidified incubator under 5% carbon dioxide.

[methyl-3H]dThd, 80.1 Ci/mmol, was obtained from New England Nuclear, Boston, MA; and [5-3H]dUrd, 15.0 Ci/mmol, was from Amersham, Arlington Heights, IL. Unlabeled dThd came from Sigma Chemical Co., St. Louis, MO. Preservative-free heparin (Pan-Heparin), was obtained from O'Neal, Jones, and Feldman, St. Louis, MO. Scintillation counting was performed in Betasfluor (National Diagnostics, Somerville, NJ), and Ficoll-sodium diatrizoate (P = 1.077 to 1.080) was purchased from Bionetics, Kensington, MD.

Bone Marrow Samples from Patients Treated with BW 301U. Pre- and posttreatment bone marrow samples were aspirated from the posterior iliac spines of 6 consenting patients who were participating in a Phase I trial of BW 301U for refractory cancer at Duke University Medical Center. Only one patient had previously received MTX treatment, and that had concluded 10 months earlier. The treatment program consisted of 5 consecutive daily i.v. infusions of BW 301U in doses ranging from 13 to 105 mg/sq m/day. The drug was infused at a concentration of 0.144 mg/ml in 5% dextrose, so that the duration of each infusion ranged from 19 to 200 min. Pretreatment marrow aspirates were obtained no more than 72 h prior to BW 301U therapy, and posttreatment samples were obtained 30 to 45 min following completion of the final infusion.

Treatment of HL-60 Cells with BW 301U in Vitro. A model system utilizing HL-60 cells was also developed to study the effects of treatment with BW 301U on the response of leukemic cells to sequential antifolates. To simulate treatment with BW 301U, we first evaluated the antiproliferative effects of BW 301U in HL-60 cells so as to identify BW 301U concentrations which would produce moderate impairment of cell proliferation without excessive cell death. Aliquots of HL-60 cells were incubated with varying concentrations of BW 301U (0 to 10 μM) for 4 days. Growth curves were constructed by performing daily viable cell counts for each concentration of BW 301U (Chart 1). Viability was determined by trypan blue exclusion. From this experiment, we identified 3 days of exposure to 0.05 and 0.1 μM BW 301U as suitable for in vitro treatment of HL-60 cells.

Effect of dThd Pool Size on Metabolic Inhibition by Antifolates. The metabolic efficacy of MTX and BW 301U was quantitated by their ability to inhibit bone marrow and HL-60 cell incorporation of [3H]dUrd. A number of factors suggested that it was essential to control the dThd pool size for these studies: (a) under the antifolate exposure conditions utilized in these experiments, most of the [3H]dUrd is eventually incorporated into acid-precipitable material as [3H]dTTP; (b) these studies were to be performed both before and after several days of BW 301U therapy, during which significant contraction of the intracellular dThd pool size was likely to occur, thus artifically raising the specific activity of the incorporated tritiated nucleotide. We therefore examined the effect of varying dThd concentrations of the incorporation of [3H]dUrd and [3H]dTTP in the presence and absence of 10 μM MTX and BW 301U, in order to determine the optimal dThd pool size for tritiated nucleoside incorporation experiments.

HL-60 cells (10^6/ml, >95% viability), were suspended in Iscove's medium (Grand Island Biological Co.) containing 10% dialized FCS to remove dThd. Unlabeled dThd was then added (final concentration, 0 to 50 μM), and the cells were equilibrated by incubation at 37°C for 30 min. Cells (2 x 10^5, 0.2 ml) were then treated with 10 μM MTX or BW 301U for 2 h and subsequently pulse-labeled with 2 μCi of [3H]dThd or [3H]-dUrd for 1 h. Pulse-labeling was terminated by washing the cells twice in 3 ml of ice-cold phosphate-buffered NaCl solution (in g/liter: NaCl, 8.0; Na2HPO4, 7H2O, 2.16; KCl, 0.2; and KH2PO4, 0.2, centrifugation, and removal of the supernatant. The cells were then resuspended and transferred to Whatman No. 3 filter paper discs. The discs were dried, soaked in 5% trichloroacetic acid, and then washed twice in 95% ethanol prior to scintillation counting.

In Vitro Metabolic Inhibition of Bone Marrow. Marrow for in vitro studies was aspirated into preservative-free heparin, diluted with an equal volume of RPMI 1640-FCS, layered over 5 ml LSM, and centrifuged at 400 x g for 30 min. The interface cells were washed, and the cell concentration was adjusted to 10^5 nucleated cells/ml with additional RPMI 1640-FCS. On the basis of the dThd pool size experiments (see "Results"), unlabeled dThd was added to give a final concentration of 1 to 2 μM, and the cells were then incubated at 37°C for at least 30 min.

Cells (200 μl; 2 x 10^5 nucleated cells) were then incubated at 37°C for 2 h with 2 μl of MTX or BW 301U. The first 4 patients were studied at final antifolate concentrations of 0, 0.01, 0.1, 1.0, and 10.0 μM. The last 2 patients were treated at 0.05, 1.0, 5.0, and 10.0 μM MTX and BW 301U. Cells were then pulse-labeled with 2 μCi of [3H]dUrd for 1 h and processed for scintillation counting as described above. Control samples (without antifolate) and samples at each antifolate concentration were tested in duplicate; the mean of the incorporated cpm at each antifolate concentration was expressed in terms of the degree of inhibition relative to the mean number of cpm incorporated by control samples.

Bone Marrow Morphology. Wilson-stained slides of pre- and posttreatment bone marrow were prepared for morphological assessment and differential counts. In addition to plentiful hematopoietic precursors, scattered clumps of tumor cells were identified in the pre- and posttreatment marrow samples of one patient.

Quantitation of CFU-GM. Bone marrow granulocyte-monocyte colony forming units (CFU-GM) were quantitated before and after treatment in 2 patients who were treated with 71 and 105 mg BW 301U/sq m/day, respectively. Duplicate 35-mm plates, each containing 10^5 LSM-separated mononuclear interface cells in 1 ml of growth medium (1.2% methylcellulose in Iscove's medium, supplemented with FCS, antibiotics, glutamine, and 10% human placental conditioned medium as a source of colony-stimulating activity), were incubated for 9 to 10 days in a humidified incubator at 37°C under 5% carbon dioxide.

Metabolic Inhibition of HL-60 Cells. MTX- and BW 301U-induced inhibition of [3H]dUrd incorporation by HL-60 cells was quantitated before in vitro treatment with BW 301U as described above. The final concen-
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Concentrations of MTX and BW 301U studied were 0, 0.033, 0.1, 0.333, 1.0, and 5.0 μM. Pretreatment viability was >95%, and pulse-labeling was performed using 2 × 10^6 viable cells/sample. These baseline studies represented the model’s counterpart of the pretreatment bone marrow studies.

Three aliquots of cells (0.33 × 10^6 viable cells/ml) were then cultured for 3 days with 0 (control cells) or 0.05 and 0.1 μM BW 301U, respectively. The posttreatment viable cell count for control cells was 1.19 × 10^6/ml, with >95% viability. For cells treated with 0.05 μM BW 301U, the viable cell count was 0.57 × 10^6/ml (91.9% viability) and 0.38 × 10^6/ml for cells incubated with 0.1 μM BW 301U (83.4% viability). The ability of MTX and BW 301U to inhibit posttreatment cells was then determined by pulse-labeling with [3H]dUrd as described above. In each of the 3 treatment aliquots, control samples (without antifolate) were tested in quadruplicate, and samples at each antifolate concentration were tested in duplicate.

RESULTS

Effect of dThd Pool Size on Metabolic Inhibition by Antifolates. At 0 μM dThd (i.e., dialyzed FGS without added dThd), antifolate-treated cells incorporated approximately twice as much [3H]dThd as did control cells due to antifolate-induced contraction of the dThd pool (Chart 2A). At dThd concentrations above 1 μM, the discrepancy between antifolate-treated cells and control cells disappeared. When cells were labeled with [3H]-dUrd (Chart 2B), the incorporation of isotope relative to control cells was stable up to approximately 2 μM dThd; at higher dThd concentrations, the relative isotope incorporation was dramatically increased. We attribute this to inhibition of ribonucleotide reductase by high intracellular levels of dTTP which can cause depletion of dUrd levels, thus artificially increasing the specific activity of subsequently introduced [3H]dUrd. Thus antifolate effect on incorporation of both [3H]dThd and [3H]dUrd appeared to be independent of the dThd pool size only at dThd concentrations in the range of 1 to 2 μM. We therefore routinely allowed bone marrow and HL-60 cells to equilibrate with 1 μM dThd in experiments which used inhibition of [3H]dUrd incorporation as a measure of antifolate efficacy.

Metabolic Inhibition of Bone Marrow. In vitro inhibition of aspirated bone marrow cells by MTX and BW 301U was assayed before and after 5 daily infusions of BW 301U. The mean inhibition (±SE) of [3H]dUrd incorporation for all patients tested at each antifolate concentration is illustrated in Chart 3.

Prior to therapy, the inhibition of [3H]dUrd incorporation produced by equimolar concentrations of MTX and BW 301U was similar. After in vivo treatment with BW 301U, in vitro inhibition produced by BW 301U was unchanged (Chart 3A). However, inhibition by 0.5 and 1.0 μM MTX was markedly reduced (Chart 3B). Serial results of 1 μM MTX- and 1 μM BW 301U-induced inhibition for all 6 patients are presented in Chart 4. The reduction in MTX-induced inhibition was statistically significant (P < 0.02, 2-tailed Mann-Whitney U test), whereas inhibition by BW 301U was unchanged (P > 0.05).

The pretreatment marrow of one patient was relatively insensitive to inhibition by BW 301U in vitro (points marked #4 in
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Chart 4). Interestingly, this patient's posttreatment marrow was also the only one which did not exhibit a prominent decrease in inhibition produced by 1 \( \mu M \) MTX after therapy with BW 301U.

Bone Marrow Morphology. No megaloblastic changes were present in pretreatment aspirates, whereas mild to moderate megaloblastic changes were identifiable in the posttreatment marrow aspirates of all patients who received at least 20 mg/sq m/day (Fig. 1). The greatest degree of morphological abnormalities was seen in the erythroid series; as expected, the severity of the megaloblastic abnormalities appeared to be dose dependent (data not shown). Serial differential counts revealed a reduction in the proportion of erythroid precursors, together with a relative increase in myeloid cells (granulocytes and their precursors) after treatment with BW 301U (Table 1).

Effect of in Vivo BW 301U on CFU-GM. Although morphological abnormalities in the myeloid series were less pronounced, reduction in CFU-GM after BW 301U therapy was seen in each of 2 patients whose marrows were cultured serially. The number of CFU-GM were inhibited by 42% in the patient treated with 71 mg BW 301U/sq m/day and by 51% in the patient treated with 105 mg BW 301U/sq m/day.

Metabolic Inhibition of HL-60 Cells. In the model system developed in HL-60 cells, results similar to those described above for bone marrow were observed. Antifolate-induced inhibition of \([^{3}H]dUrd\) incorporation was evaluated prior to and following 3 days of incubation with 0 \( \mu M \) (control cells) or 0.05 or 0.1 \( \mu M \) BW 301U. No change in response to BW 301U was observed (Chart 5), and posttreatment control cells showed only a slight reduction in inhibition by MTX (Chart 5A). In contrast, MTX-induced inhibition was markedly impaired in cells which had been incubated with either 0.05 or 0.1 \( \mu M \) BW 301U (Chart 5B and C).

DISCUSSION

The place of BW 301U in the chemotherapeutic armamentarium remains to be defined. Additional Phase I and II studies of BW 301U are in progress. If BW 301U is to become a viable alternative to other antifolic agents.
alternative to MTX, it must be shown to be at least as potent against cells that are sensitive to MTX and more effective against some cells that are resistant to MTX. From the results presented in Charts 3 and 4, it can be seen that equimolar concentrations of MTX and BW 301U were equipotent with respect to pretreatment inhibition of $[^{3}H]$dUrd incorporation into normal bone marrow in 5 of the 6 patients. This finding is in agreement with preclinical studies conducted on leukemic and normal cells in our laboratory (22). Furthermore, Duch et al. (12) have shown that BW 301U and MTX produce equivalent inhibition of DHFR (purified from human leukemia cells), and cytotoxicity of cultured tumor cells produced by BW 301U is equal to or greater than that produced by MTX. In addition, we have confirmed the antifolate effects of BW 301U by demonstrating both the rapid appearance of megaloblastic bone marrow morphology and the direct inhibition of hematopoietic cell cloning efficiency following in vivo treatment with BW 301U.

For the purpose of these studies, we made an attempt to develop a method that would maximize the interpretability of antifolate-induced inhibition of $[^{3}H]$dUrd incorporation, particularly in the context of sequential antifolate treatment. In developing this approach, we chose to evaluate the dThd pool from a functional viewpoint in order to define a steady state condition for antifolate-induced inhibition that was independent of nucleotide pool perturbations. The results indicated that there was a fairly narrow range of dThd concentration (1 to 2 $\mu$M) which provided optimal conditions for pool-independent pulse-labeling analysis with both $[^{3}H]$dThd and $[^{3}H]$dUrd in the presence of antifolates. Therefore, the dThd concentration was maintained within this range for the $[^{3}H]$dUrd incorporation studies presented in this paper.

In the studies reported here, we have shown that normal human bone marrow can exhibit a change in its response to MTX over time. Curiously, this change was not induced by exposure to MTX, but by treatment with BW 301U, a lipophilic folate antagonist. Of particular importance was the finding that BW 301U-treated cells did not undergo simultaneous changes in the response to subsequent BW 301U. Although BW 301U therapy was associated with a change in the cellular composition of posttreatment bone marrow with respect to lineage (Table 1), the asymmetrical effects of MTX and BW 301U, together with the parallel findings that were observed in BW 301U-treated HL-60 cells, suggest that the change in MTX-inhibitable $[^{3}H]$dUrd incorporation after BW 301U therapy does not simply reflect the selection of a subpopulation of marrow cells that are intrinsically less sensitive to inhibition by folate antagonists.

Several alternatives might be considered to explain these observations in posttreatment cells, including: increased levels of DHFR, the target enzyme of MTX; accumulation of dihydrofolate, the substrate for DHFR, resulting in displacement of MTX from DHFR; appearance of a mutant form of DHFR with decreased affinity for MTX; defects in the active transport mechanism by which MTX enters and leaves the cell; inhibition of MTX polyglutamation; and inhibition of thymidylate synthetase.

The rapidity with which the change in response to MTX develops suggests that a mutant or genetically amplified DHFR is an unlikely possibility. Furthermore, Duch et al. (12) have shown that BW 301U does not inhibit thymidylate synthetase. Since the major advantage of BW 301U over MTX is its lack of dependence on active transport for entry into cells, reduced MTX inhibition of $[^{3}H]$dUrd incorporation could arise from the acquisition of a MTX transport defect (e.g., down-regulation of the membrane components involved in tetrahydrofolate/MTX transport), although this mechanism has not been shown to be a common in vivo event. Nevertheless, such an explanation is consistent with previous observations of collateral sensitivity to lipophilic antifolates in MTX-resistant cells (10, 20, 26). Thus, it is possible that cells which are exposed to cytostatic BW 301U concentrations (as in our HL-60 model) may develop alterations in their tetrahydrofolate-MTX transport system, such that they exhibit reduced responsiveness to subsequent MTX but not to subsequent BW 301U. The fact that decreased responsiveness to MTX was also seen in patients treated with a wide range of BW 301U doses in vivo further supports this notion.

Alternatively, a transient increase in DHFR synthesis (3, 11, 16) could lead to a differential response to MTX versus BW 301U in the absence of changes in the transport characteristics of the cell. Previous reports from our laboratory have considered the interplay of MTX transport and DHFR activity in detail (15, 24). In these studies, the lipid-soluble antifolates, BW 301U and DDMP, were shown to be effective in overcoming biochemical resistance of a 3T6 mouse cell line selected for resistance to MTX (3T6R400). This cell line exhibited normal MTX transport, increased DHFR levels, and a reduced DHFR affinity for these antifolates. Although the 3T6R400 cells were more than 10,000-fold resistant to MTX when compared with the parent 3T6 cell line, there was only a 5- to 10-fold resistance to lipid-soluble antifolates (DDMP and BW 301U), consistent with the increased DHFR activity and decreased DHFR affinity of the cell line. Significantly, these studies indicated that a normal transport system can compound the expression of MTX resistance (due to quantitative or kinetic DHFR abnormalities) by restricting intracellular accumulation of MTX and thus preventing stringent inhibition of DHFR, whereas significant inhibition of 3T6R400 cells by lipophilic folate antagonists was demonstrable at extracellular drug concentrations that are easily achievable in vivo. With respect to the present study, preliminary evaluation of normal human bone marrow and HL-60 cells, before and after treatment with BW 301U, showed significant heterogeneity in DHFR protein levels of bone marrow samples but did not demonstrate significant changes in the overall level of DHFR protein as identified by peroxidase-coupled assay of anti-DHFR antibody reaction.

Regardless of the mechanism responsible for the findings presented here, the development of a reduced ability of MTX to inhibit $[^{3}H]$dUrd incorporation did not appear to be dependent upon the severity of biological antifolate toxicity induced by BW 301U. Decreased MTX responsiveness was observed in patients who were treated with a wide range of BW 301U doses in vivo, and HL-60 cells incubated at 2 different concentrations of BW 301U developed a similar reduction in response to MTX. In contrast, megaloblastic bone marrow morphology and cytotoxicity in HL-60 cells were both dose dependent. These results, taken together with earlier evidence from our laboratory (15, 24) and from others, are consistent with the suggestion that this new antifolate could play an important part in the treatment of MTX-resistant tumors and may limit the development of some resistance phenotypes.

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5 Y-C. Cheng, unpublished data.
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REFERENCES

Fig. 1. Pre- and posttreatment marrow morphology in a patient who received 5 infusions of 71 mg BW 301U/sq m/day. A, normal morphology prior to treatment; B, megaloblastic megakaryocyte after treatment; C to E, megaloblastic erythroid and myeloid precursors after treatment. Wilson's stain, × 680.
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