New Dose-Time Relationships of Folate Antagonists to Sustain Inhibition of Human Lymphoblasts and Leukemic Cells

in Vitro

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ABSTRACT

In this study, three methods are utilized to analyze toxicity produced by methotrexate and the lipid-soluble antifolate, 2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine, in human lymphoblasts (WIL-2) and leukemic cells. These methods detect increasingly severe metabolic damage: inhibition of deoxyuridine incorporation into DNA, the reversibility of inhibition of deoxyuridine incorporation by supplementation with formyltetrahydrofolate as Ca2+ leucovorin, and the ability of cells to form clones in soft agarose. The critical dose and exposure time for establishing and maintaining the metabolic toxicity of methotrexate is examined in detail. It is shown that, if an initial loading dose of methotrexate is of high enough concentration or is maintained for a sufficient period to achieve >98% inhibition of deoxyuridine incorporation, this inhibition can be sustained by low concentrations of 2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine or methotrexate. Concentrations of methotrexate or 2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine that equal or exceed 0.1 μM are sufficient for maintenance of inhibition by an initial loading dose of methotrexate but escape from inhibition that occurs if lower levels of drug are used. The possible implications of these observations for in vivo protocols are discussed.

INTRODUCTION

This paper describes a rationale for establishing and maintaining a stringent metabolic blockade in leukemic cells by means of a brief exposure to a large priming dose of methotrexate followed by low doses either of methotrexate or of a lipid-soluble folate antagonist, DDMP3 (4, 23). Unlike the usual concept of using combinations of drugs which have different but complementary sites of action, this approach seeks to maximize drug effectiveness against a (presumed) single enzymatic target. The metabolic impact of methotrexate and other antifolates has been the subject of intensive study (3, 8, 9, 23). Experiments with acute nonlymphocytic leukemia (2, 11) suggest that methotrexate might be better utilized if infused in sufficiently high doses to maintain blood levels of methotrexate at 2 μM for as extended a period as possible. This long-term high-dose infusion may then be followed by "rescue" with citrovorum factor. Such protocols, although effective for reduction of tumor mass, involve obvious difficulty with managing drug toxicity and with precise scheduling for individual patients.

The working hypothesis of this study is based on the knowledge that methotrexate establishes an essentially irreversible inhibitory complex with its primary target enzyme, dihydrofolate reductase, and that inhibition of this enzyme leads to reduction in the intracellular tetrahydrofolate pools. Under such conditions, only sufficient concentration of free intracellular methotrexate or DDMP to inhibit newly synthesized dihydrofolate reductase should be required to maintain inhibition of tetrahydrofolate-dependent cellular functions. The in vitro work to test this concept was carried out in the human lymphoblastoid cell line (WIL-2) and in human peripheral blood leukemic cells. In either cell type, a low dose of methotrexate or DDMP can sustain the metabolic inhibition and subsequent cytotoxicity induced by a brief exposure to a high dose of methotrexate. Possible applications of shutdown drug regimens for future design of therapeutic drug programs are also discussed.

MATERIALS AND METHODS

The WIL-2 cell line, originally isolated by Levy et al. (20), was obtained from M. Hershfield, Duke University Medical School, Department of Medicine; [6-3H]deoxyuridine (24.2 Ci/mm) was obtained from New England Nuclear (Boston, Mass.); MRC-5 cells came from the American Type Tissue Culture Collection (Rockville, Md.). Unlabeled methotrexate, DDMP, and [14C]DDMP were kindly supplied by Dr. C. Nichol of Burroughs Wellcome Corp. (Research Triangle Park, N. C.); [3',5',9(n)-3H]methotrexate was provided by the National Cancer Institute, Contract N01-CM-67121, and repurified (final specific activity, 2.0 × 108 cpm/μmol) before use as described by Goldman (8). Earle’s minimal essential medium for suspension cultures utilized to support the growth of the WIL-2 cells and minimal essential medium for surface-attached cultures were the autoclavable formulations sold by Grand Island Biological Co. (Grand Island, N. Y.). Fetal calf serum (Grand Island Biological Co.) was dialyzed for 3 days against 3 changes of 10 volumes of 0.15 M sodium chloride before being used for incorporation and drug uptake experiments.

Cell Maintenance. WIL-2 cells were maintained in suspen-
sion cultures by continuous agitation on a rotary shaker at 37°
at a density of 1.5 to 8 x 10^6 cells/ml. Suspension minimal
essential medium for cell growth was supplemented with 10%
fetal calf serum (growth medium). MRC-5 cells were grown in
plastic T75 flasks (Corning Glass Co., Corning, N. Y.) and
passaged once weekly at a 1:2 ratio. Leukemic cells were
obtained from 40 ml of heparinized blood samples from se-
lected patients. RBC were sedimented in the syringe for 1 hr;
the leukocytes in plasma were removed, counted in a hemo-
cytometer, and diluted in tissue culture medium or the patient's
autologous plasma. Cells were found to be free of Mycoplasma
by the culture method of Hayflick (10) and by the thymidine
incorporation assay of Todaro et al. (30).

Cloning Assay of WIL-2 cells. Tissue culture dishes (Tissue
Culture Cluster 24; Costar, Cambridge, Mass.) were prepared
by inoculation of 8 x 10^4 MRC-5 cells into each well of the
multiwell tissue culture dish. Twenty-four hr prior to confluent
growth of the monolayer, these cells were overlaid with 1 ml of
growth medium containing 0.3% melted agarose. After another
24 hr, the plates were removed from the incubator, and sam-
ples of WIL-2 cells were diluted to 100 to 500 cells/ml and
then plated over the first agarose layer in 0.5 ml of minimal
essential medium containing 0.3% melted agarose. Clones
were counted microscopically at 7 to 10 days after seeding.
Cell counts in each well were made on 5 random microscope
fields and then normalized to the surface area of the entire
well. Since it is difficult to quantitate clones on the perimeter of
the culture wells, this counting method gave a more reliable
estimate than counting the total number of clones in each well.

Pulse-labeling Protocol. Cells used in pulse-labeling exper-
iments were first incubated for 30 min in fresh growth medium.
Cells were pulse labeled by transferring 0.2 to 0.3 ml of cells
to a tube containing 10 μl of triitated deoxyuridine (5 μCi/ml).
The reaction was stopped by the addition of 3 ml of ice-cold
phosphate-buffered saline (1 mM CaCl_2; 0.5 mM MgCl_2; 2.7 mM
KCl; 1.7 mM KH_2PO_4; 0.14 mM NaCl; 8 mM Na_2HPO_4) by cen-
trifugation and removal of the supernatant. The cells were then
resuspended and transferred to Whatman No. 3M filter paper
discs (Whatman, Inc., Clifton, N. J.). The discs were further
processed for scintillation counting as previously described
(18).

Assay of Methotrexate Uptake. The procedure for transport
studies was modified from that of Marz et al. (21). The uptake
of methotrexate by WIL-2 cells was measured by rapidly mixing
equal volumes of 5 x 10^6 cells/ml in minimal essential medium
with additional medium containing 2 times the desired final
concentration of methotrexate. The 0.3-ml samples were pe-
riodically transferred to microcentrifuge tubes containing a
mixture of silicon oil (70%, 702 diffusion pump fluid; Dow
Corning, Midland, Mich.) and light mineral oil (30%; Squibb,
Princeton, N. J.) and centrifuged for 30 sec in a microcentrifuge
(Brinkmann-Eppendorf Centrifuge 3200; Brinkmann Instru-
mements, Inc., Westburg, N. Y.). The aqueous layer on top of the
oil containing unabsorbed drug was then removed, and the tube
walls over the oil layer were washed by filling the tube
twice with deionized water. One M sucrose (0.5 ml), buffered
by phosphate-buffered saline, was then added to each tube.
Tubes were again centrifuged, and the floating oil layer was
discarded. The cells were then suspended in the remaining 0.5
ml of phosphate-buffered saline:sucrose solution and removed
to scintillation vials. The cell suspension in the microcentrifuge
tube was finally washed into the scintillation vial by 2 washes
of 1 ml Aquasol (New England Nuclear, Boston, Mass.). Three
ml of Aquasol were then added to each scintillation vial. Each
sample was shaken vigorously until clear and then counted.

RESULTS

Uptake and Efflux of Methotrexate in WIL-2 Cell Cultures.
Chart 1 shows the kinetics of uptake of labeled methotrexate
into WIL-2 cells and the efflux of drug observed following a
shift to medium containing no drug, 0.5 μM DDMP, or 10% of
the amount of labeled methotrexate at the same specific activity
as the 1 μM methotrexate loading dose. Methotrexate effluxes
from cells suspended in drug-free medium, leaving behind a
residual fraction of bound drug. This bound fraction of metho-
trexate is reduced about 50% by 0.5 μM DDMP. On the other
hand, 0.1 μM methotrexate sustains a free intracellular drug
concentration which is greater than the cell-associated fraction
observed after washing cells in drug-free medium; this incre-
ment presumably represents free drug.

Relationship of Sustained Inhibition of Deoxyuridine Incor-
poration by Low-Dose DDMP or Methotrexate to the Time
Interval of High Drug Dose. Inhibition of deoxyuridine incor-
poration into DNA caused by 30 min of preexposure of WIL-2
cells to 1 μM methotrexate can be maintained by 0.1 μM
methotrexate (Chart 2). The ability of 0.1 μM methotrexate to
sustain the inhibition of deoxyuridine incorporation caused by
1 μM methotrexate in 30 min is contrasted with the recovery of
deoxyuridine incorporation by WIL-2 cultures after centrifuging
the cells and suspending them in fresh growth medium without
drug.

Sustained inhibition of deoxyuridine incorporation of DDMP
and methotrexate is dependent upon the level of inhibition
which is achieved prior to shifting the cells to a lower concen-
tration of methotrexate. Once sufficient inhibition (around 98%) is
achieved in WIL-2 cultures by methotrexate, however, 0.1
μM methotrexate or 0.1 μM DDMP efficiently excludes de-
oxyuridine incorporation into the DNA (Chart 3). Methotrexate
(0.1 μM) maintains deoxyuridine incorporation into DNA at
approximately the level achieved at any time by the initial
exposure to a higher concentration of methotrexate. Mainte-
nance of inhibition by 0.1 μM DDMP requires a somewhat more
stringent inhibition by the initial loading dose of methotrexate
and is possible only after about 98% inhibition is achieved by
the methotrexate loading dose.

Similar experiments showed that inhibition of deoxyuridine
incorporation can also be maintained in human leukemic cells
by drug shutdown protocols. Since DNA synthesis often con-
tinues linearly for only 2 to 3 hr in such cultures, it was not
possible to evaluate long-range effects on DNA synthesis and
cell survival. However, 1 to 5 μM methotrexate characteristically
inhibited deoxyuridine incorporation into DNA at >90% within
15 min in cultures of leukemic cells suspended in growth
medium containing 60% fetal calf serum, and this inhibition
was sustained by 0.1 μM methotrexate or 0.1 μM DDMP. On
the other hand, although the usual response was similar to that
seen in WIL-2 cells (with methotrexate-induced deoxyuridine
inhibition always being maintained by 0.1 μM methotrexate),
such inhibition was not sustained by DDMP in cells from all

Antifolate Toxicity in WIL-2 and Leukemic Cells

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The Relation of Irreversible Inhibition of Deoxuryridine Incorporation to Drug Dosage or Time of Exposure. Irreversible inhibition of deoxuryridine incorporation is defined in this study as an inability of cells to recover deoxuryridine incorporation to initial control levels at 2 hr after reversal of the metabolic block induced by DDMP or methotrexate. This experiment measures a delayed effect of the drug. Chart 5 illustrates the relationship between irreversible inhibition and the dose and interval of DDMP or methotrexate drug exposure. The toxicity of each dose-time regimen was tested by assaying the ability of drug...

Chart 1. Kinetics of uptake of 1.0 μM methotrexate (MTX) and its efflux from cells. Tritiated methotrexate (2.0 × 10⁶ cpm/μmol) was added to a culture of WIL-2 cells, and its rate of influx (△) into the cells was determined as described in "Materials and Methods." Thirty min after addition of 1.0 μM methotrexate to the culture, it was divided into 4 parts, and 3 of these aliquots were washed 2 times with cold medium and suspended in medium containing no drug (C), 0.1 μM tritiated methotrexate (△), or 0.5 μM DDMP (A). The quantity of methotrexate associated with cells was determined as described in "Materials and Methods."...

Chart 2. Maintenance and recovery from inhibition of deoxuryridine incorporation induced by 1 μM methotrexate. Methotrexate (1 μM) was added to the culture of WIL-2 cells at Time 0, and the culture was subsequently pulse labeled with 2 μCi/ml of tritiated deoxyuridine for 10 min at the time indicated (△). At 30 min after drug addition, methotrexate was removed by twice washing the cells with cold medium and resuspending them in minimal essential medium without drug (C), 0.1 μM methotrexate (△), or 1.0 μM methotrexate (△). Results were plotted as percentage of Time 0 control values without drug where aliquots of 6 × 10⁶ cells incorporated 16,900 cpm.

patients (29). Up to 50% recovery of initial deoxuryridine incorporation rates was observed in 2.5 hr after shifting the culture from one chronic granulocytic leukemia patient to medium containing 0.1 μM DDMP.

Drug Levels Needed for Maintenance of Inhibition of Deoxuryridine Incorporation. A critical concentration of DDMP and methotrexate is required for maintenance of inhibition by a methotrexate loading dose. The level of drug needed to sustain inhibition achieved by 1 μM methotrexate in 30 min is shown in Chart 4. Cells begin to recover their ability to incorporate deoxuryidine when exposed to maintenance doses below 0.1 μM methotrexate and 0.1 μM DDMP. This recovery is not complete in 2 hr, however, and cells initially inhibited by 1 μM methotrexate recover much more slowly in a medium containing 0.01 μM methotrexate or DDMP than they do in drug-free medium.

Chart 3. Dependence of sustained inhibition of deoxuryridine (UdR) incorporation by low concentrations of DDMP and methotrexate on the duration of initial exposure to methotrexate. A, 1 μM methotrexate added to the cultures at 2.5 × 10⁶ cells/ml. At the times indicated by the symbols on the abscissa (C, 1 min; △, 10 min; ●, 30 min), aliquots of the cells were washed free of 1 μM methotrexate and shifted to medium containing 0.1 μM DDMP. Tritiated deoxuryridine uptake throughout the experiment was monitored in 0.2-ml samples by 10-min pulse labeling. Untreated cultures incorporated 1.2 × 10⁶ cpm/sample at Time 0. △, 1 μM methotrexate throughout the experiment. B, cell culture (2.5 × 10⁶ cells/ml) treated essentially as described in A except 0.1 μM methotrexate was utilized to maintain the inhibition achieved with 1 μM methotrexate. Intervals were C, 2 min; △, 15 min; and ●, 25 min. Pulse labeling with deoxuryridine was carried out for 6 min. ○, control with no drug; △, 0.1 μM methotrexate added at Time 0; △, 1 μM methotrexate throughout.

Chart 4. Methotrexate and DDMP concentrations required for maintenance of inhibition induced by 1 μM methotrexate. A, 1 μM methotrexate added to a culture at 2.5 × 10⁶ cells/ml, and inhibition of deoxuryridine incorporation was monitored by 6 min of labeling of 0.3-ml samples with deoxuryridine, 5 μCi/ml, after the treatments indicated. Under these conditions, untreated control cultures at Time 0 incorporated 2500 cpm. An aliquot of the cell suspension was washed 2 times with cold minimal essential medium at 30 min after addition of 1 μM methotrexate and was subsequently resuspended in medium containing 0.01 (C), 0.05 (△), 0.1 (●), and 0.5 (□) μM DDMP. B, 1 μM methotrexate added to a culture of WIL-2 with 3 × 10⁶ cells/ml, and inhibition of deoxuryridine incorporation monitored by 5 min of exposure to deoxuryridine, 5 μCi/ml, after treatment with 0.01 (C), 0.05 (△), 0.1 (●), and 0.5 (□) μM methotrexate. Under these conditions, the control culture incorporated 740 cpm at Time 0. △, methotrexate at 1.0 μM throughout the experiment.

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The amount of deoxyuridine incorporated into DNA was determined. Untreated 0.2 ml were then pulse labeled for 3 hr with tritiated deoxyuridine (5 μCi/mL) and deoxyuridine incorporated into DNA. Results were plotted in terms of percentage samples for 3 hr. after which samples were processed to determine the level of samples from control cultures incorporated 4.0 × 10^3, 5.0 × 10^3, and 5.3 × 10^3 cpm/sample at 30, 60, and 120 min, respectively. B, methotrexate (MTX) in concentrations ranging from 0.1 to 20 μM added to cultures of WIL-2 cells, and the cultures were subsequently incubated for 30, 60, or 120 min. At the end of these incubations, the cultures were washed twice with drug-free cold medium. Tritiated deoxyuridine, 5 μCi/mL, was subsequently added to 1 ml cell samples for 3 hr, after which samples were processed to determine the level of deoxyuridine incorporated into DNA. Results were plotted in terms of percentage control deoxyuridine incorporation at Time 0 in the culture containing no drug. Untreated cultures incorporated 7.0 × 10^3 cpm/sample.

Chart 5. Dependence of irreversible inhibition of deoxyuridine incorporation on drug concentration and time. A, DDMP in concentrations ranging from 0.1 to 20 μM added to cultures of WIL-2 cells at 5 × 10^4 cells/mL, and cultures subsequently incubated for 30, 60, or 120 min. At the end of the incubation, the cultures were washed free of drug with 2 washes of cold medium. Samples of 0.2 ml were then pulse labeled for 3 hr with tritiated deoxyuridine (5 μCi/mL), and the amount of deoxyuridine incorporated into DNA was determined. Untreated samples from control cultures incorporated 4.0 × 10^3, 5.0 × 10^3, and 5.3 × 10^3 cpm/sample at 30, 60, and 120 min, respectively. B, methotrexate (MTX) in concentrations ranging from 0.1 to 20 μM was also added to cultures of WIL-2 cells, and the cultures were subsequently incubated for 30, 60, or 120 min. At the end of these incubations, the cultures were washed twice with drug-free cold medium. Tritiated deoxyuridine, 5 μCi/mL, was subsequently added to 1 ml cell samples for 3 hr, after which samples were processed to determine the level of deoxyuridine incorporated into DNA. Results were plotted in terms of percentage control deoxyuridine incorporation at Time 0 in the culture containing no drug. Untreated cultures incorporated 7.0 × 10^3 cpm/sample.

DISCUSSION

The metabolic toxicity of methotrexate and combined methotrexate:DDMP regimens for cell cultures was studied by examining effects on deoxyuridine incorporation, Ca²⁺ leucovorin-induced recovery of deoxyuridine-incorporating ability, and cloning efficiency.

The first of these methods indirectly measures the inhibition of dUMP conversion to TMP as the step most sensitive metabolically to antifolate activity that occurs as an indirect consequence of the rapid depletion of tetrahydrofolate cofactors when dihydrofolate reductase activity is blocked by an antifolate. The rapid manifestation of this effect in WIL-2 cells was measured in another of our studies (28) where we showed that DDMP can induce a steady state of inhibition of deoxyuridine incorporation into DNA within 5 sec of its addition to cells. This inhibition was a direct effect on dUMP conversion to TMP and not a consequence of a blockade of deoxyuridine uptake into cells. Moreover, the kinetics of DNA inhibition of deoxyuridine by methotrexate as defined by pulse exposures to tritiated deoxyuridine closely follows the rates of drug transport into the cells as measured by labeled drug, further documenting the rapid inhibition of deoxyuridine incorporation into DNA as a consequence of antifolate action.

The second experiment measures the loss of cellular ability to incorporate deoxyuridine into DNA in the presence of citrovorum factor. Longer intervals of drug exposure or higher concentrations of drug are required to achieve inhibition levels approximating those observed when inhibition of deoxyuridine incorporation into cells in the presence of drug is examined.

The final assay, determination of cloning efficiency, demonstrates the remarkable ability of cells to survive severe tetrahydrofolate cofactor depletion. Higher drug concentrations or longer intervals of drug exposure than are needed to inhibit deoxyuridine incorporation in either of the experiments described above are required to reduce cloning efficiency.

In these studies, we have also used the above assays to define conditions for establishing and maintaining metabolic
Metabolic toxicity and cell killing by methotrexate after maintenance by low-concentration methotrexate and DDMP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1 hr</th>
<th>2 hr</th>
<th>8 hr</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
</tr>
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<tr>
<td><strong>A. Control (no drugs)</strong></td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μM methotrexate</td>
<td>20.5</td>
<td>6.8</td>
<td>1.7</td>
<td>0.9</td>
<td>1.5</td>
<td></td>
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<tr>
<td>0.5 μM DDMP</td>
<td>15.9</td>
<td>5.5</td>
<td>8.3</td>
<td>5.2</td>
<td>2.0</td>
<td></td>
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<tr>
<td>5.0 μM methotrexate; 5.0 μM methotrexate</td>
<td>0.7</td>
<td>0.5</td>
<td>0.6</td>
<td>0.1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
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<td>0.5</td>
<td>0.5</td>
<td>0.9</td>
<td>0.7</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
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<td>0.6</td>
<td>0.5</td>
<td>0.8</td>
<td>1.1</td>
<td>1.0</td>
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</tr>
<tr>
<td><strong>B. 0.1 μM methotrexate</strong></td>
<td>52.0</td>
<td>109.0</td>
<td>63.1</td>
<td>8.7</td>
<td></td>
<td></td>
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<tr>
<td>0.5 μM DDMP</td>
<td>84.0</td>
<td>112.1</td>
<td>63.0</td>
<td>16.8</td>
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<tr>
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<td>8.9</td>
<td>23.4</td>
<td>26.0</td>
<td>17.5</td>
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<tr>
<td>5.0 μM methotrexate; 0.1 μM methotrexate</td>
<td>18.8</td>
<td>28.9</td>
<td>32.4</td>
<td>13.1</td>
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<tr>
<td>5.0 μM methotrexate; 0.5 μM DDMP</td>
<td>14.0</td>
<td>27.0</td>
<td>31.4</td>
<td>9.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C. Control (no drugs)</strong></td>
<td>100</td>
<td>117</td>
<td>128 (141)</td>
<td>177 (218)</td>
<td>230 (225)</td>
<td></td>
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<tr>
<td>0.1 μM methotrexate</td>
<td>87</td>
<td>89</td>
<td>85 (138)</td>
<td>43 (63)</td>
<td>24 (64)</td>
<td></td>
</tr>
<tr>
<td>0.5 μM DDMP</td>
<td>99</td>
<td>123</td>
<td>72 (148)</td>
<td>32 (111)</td>
<td>16 (96)</td>
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<tr>
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<td>56</td>
<td>32 (186)</td>
<td>17 (69)</td>
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<td>5.0 μM methotrexate; 0.1 μM methotrexate</td>
<td>105</td>
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<td>53 (166)</td>
<td>19 (107)</td>
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<tr>
<td>5.0 μM methotrexate; 0.5 μM DDMP</td>
<td>82</td>
<td>70</td>
<td>57 (121)</td>
<td>27 (79)</td>
<td>8 (57)</td>
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</table>

* a 12,460 cpm.
* b Numbers in parentheses, total cell count.

The experiments shown above were designed to mimic in vivo conditions of administration of methotrexate where the organism is exposed to an initial high concentration of methotrexate that rapidly decreases with time. In patients receiving a rapid i.v. infusion of 120 mg methotrexate per sq m, blood levels of >10 μM methotrexate are achieved, which typically drop to <0.1 μM in 24 hr (2). In response to such administration, human cells also can increase the absolute amount of dihydrofolate reductase 3- to 4-fold over 24 to 48 hr, while the enzyme activity remains inhibited and tetrahydrofolate-dependent, TMP synthesis remains depressed (2, 11).

Although the relationship between free intracellular methotrexate and apparent folate-related toxicity has been demonstrated by Goldman (8) in the L1210 system, the extracellular drug concentrations required for maintenance of toxic intracellular drug levels in L1210 cells have not been clearly defined. We have found that low concentrations of methotrexate or DDMP (0.1 to 0.5 μM) are sufficient to maintain the inhibition achieved by exposure of WIL-2 and leukemic cells to a high initial loading dose (1 to 5 μM) of methotrexate.

The relationship between maintenance of toxic effect and intracellular methotrexate levels, however, is not clearly established in this study. It can be calculated from Chart 1, with appropriate adjustments for cell volumes,4 that methotrexate (0.1 μM) sustained a 0.05 to 0.1 μM intracellular free-drug level above the bound fraction of 0.2 μM in log-phase cultures. However, although 0.5 μM DDMP displaced about 30% of the bound methotrexate fraction, it still maintained methotrexate-induced inhibition of deoxyuridine incorporation. Further experiments on this displacement phenomenon indicate that DDMP displaces methotrexate from dihydrofolate reductase, because higher concentrations of DDMP or high concentrations of methotrexate can deplete the cell of >90% of its bound methotrexate.5 This result is consistent with the observation that cold methotrexate or pyrimethamine can displace methotrexate from L1210 cells (5).

A possible rationale for drug protocols using a combination of methotrexate and DDMP is provided by the observation that DDMP can sustain methotrexate inhibition at drug levels which alone cause little deoxyuridine inhibition and no toxicity for cloning potential in 24 hr.

This may provide an alternative strategy for the utilization of lipid-soluble drugs like DDMP with long plasma half-lives (ap-

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4 W. D. Sedwick and O. E. Brown, unpublished observations.
5 M. J. Fyte and W. D. Sedwick, unpublished observations.
proximately 180 hr). Sustained plasma levels of DDMP (approximately 5 μM) can be achieved and maintained with a single drug dose, from which hematological recovery can also be effected with leucovorin (22). However, high levels of DDMP often cause troublesome side effects on the central nervous system, skin, and gastrointestinal tract, which appear to be unrelated, or in addition to the toxicity caused by the antagonism of folic acid metabolism (22). The in vitro shiftdown protocol described in this paper shows that maintenance of the methotrexate-induced antifolate effect in WIL-2 cells requires only a fraction of the dose (1/10 to 1/50 of the usual drug levels) needed to initiate the inhibition of deoxyuridine incorporation into DNA. The DDMP maintenance level (0.1 to 0.5 μM) is well below the concentration that would cause non-folate-dependent side effects. However, the binding capacity of plasma and serum proteins may necessitate somewhat higher sustaining doses of DDMP in vivo (23).

A protocol based on these in vitro observations is currently a focus for in vivo studies in our laboratory. Since high-dose administration of methotrexate can achieve toxic concentrations of methotrexate in the central nervous system (27), the possibility presents itself of sustaining the inhibition achieved by a single high dose of methotrexate with DDMP which, because of its ability to cross the blood-brain barrier, provides a possible means of maintaining antifolate toxicity in a region which is relatively inaccessible to most drugs.

Although these results do not provide clear evidence for selective antitumor toxicity by protocols involving a shift to lower drug concentrations, differential response to drug concentrations required to maintain inhibition might be expected among cell populations. In this regard, we found that inhibition of deoxyuridine incorporation in some leukemic cells is not as efficiently maintained by DDMP as it was in the lymphoblastoid cell cultures. Differential sensitivity will be further explored in cells of normal and leukemic origin to determine whether it may be exploited to promote selective toxicity as part of individually tailored treatment strategies. Possible explanations for such sensitivity differences could include a cellular ability to synthesize dihydrofolate reductase at widely varying rates (1, 11), mutation of the enzyme to a form with a different Kf for methotrexate (12, 13), or differences in drug transport (16, 17).

In summary, low concentrations of both DDMP and methotrexate can sustain the metabolic toxicity of high-concentration loading doses of methotrexate (as measured by deoxyuridine incorporation) and cell killing (as measured by cloning ability). Utilization of high-concentration loading doses of methotrexate followed by low-dose maintenance provides a rationale for expanding the range of protocols using these drugs, which may provide a means of achieving selective cellular toxicity with overall exposure of patients to less drug. A maintenance protocol using low levels of drug might improve the efficiency of rescue protocols dependent on purging the patient of toxic levels of these drugs. Such protocols should also reduce kidney toxicity related to deposition of methotrexate and its metabolite (14) and lower drug costs for methotrexate protocols.

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