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Transient Induction of Phenotypic Resistance in Human Lymphoblastoid Cells following Sequential Use of Two Antifolates

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SUMMARY

HAMRELL, M. R., J. LASZLO, AND W. D. SEDWICK. Transient induction of phenotypic resistance in human lymphoblastoid cells following sequential use of two antifolates. *Mol. Pharmacol.* **19**:491–495 (1981).

This paper demonstrates an unexpected antagonism of cytoxicity with the use of two antifolates. Cell killing depended on the order of administration, as sequential *in vitro* exposures, to a lipid-soluble antifolate, metoprine (DDMP), and methotrexate (MTX). This was manifested in greatly increased survival of cultures following a program of 4 days of exposure of human lymphoblastoid (WIL-2) cells to 5 μM DDMP, 2 days of recovery in medium without drug, then 4 days of exposure to either 5 μM DDMP or 1 μM MTX. By contrast, 1 μM MTX in the first cycle, regardless of the drug used in the second cycle, gave no survivors. Serial measurement of dihydrofolate reductase levels showed that DDMP led to increased levels of free dihydrofolate reductase during the recovery period which were not inhibited by a second exposure to drug. These results emphasize the importance of sequence and timing when drugs are used in multiagent protocols, even when the drugs have a common enzyme target.

INTRODUCTION

Many multiagent chemotherapeutic drug programs are currently being used to treat patients with cancer. Since such protocols were originally designed for theoretic reasons (different modes of action and toxicities) and tested on experimental tumors (1, 2), it is not surprising that controversy exists as to whether some of these combinations are synergistic or antagonistic (3). Recent reports on the use of MTX[2] in combination with other anticancer drugs (cytosine arabinoside and fluorodeoxyuridine) suggest that the timing as well as the sequence of administration may be very crucial (4, 5). Schedule-dependent antagonistic and synergistic interactions are not well understood for many combination chemotherapy regimens in current use. However, it seems probable that such interactions can underlie the success or failure of both single drug and combination drug regimens (6).

Many chemotherapy programs have been specifically designed to employ drugs having different biochemical targets, often at different loci in a given pathway. In this paper we have examined the efficacy of the combined use of two distinct types of antifolate drugs against a population of human lymphoblastoid cells, a prototypic lipid-soluble antifolate (DDMP), and the classical antifolate, MTX.

Our original hypothesis was that the combined use of MTX and the lipid-soluble drug DDMP might decrease the incidence of emergence of surviving cells in a population as compared with the use of each drug alone. This hypothesis was based on the ability of DDMP to enter cells without dependence on active transport, and on the higher avidity of MTX for their common target enzyme, DHFR. The protocol was set up to determine the frequency of survival of cultures after exposure to a lipid-soluble drug alone, MTX alone, and the two drugs in combination. We show that these antifolates can, depending upon the sequence of administration, create a transient metabolic state of resistance that gives a greatly reduced drug response.

MATERIALS AND METHODS

[*methyl*-^{3}H]Thymidine (50.5 mCi/mmole) and [6-^{3}H]-deoxyuridine (24.2 mCi/mmole) 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. DDMP was kindly provided by Dr. C.

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[2] The abbreviations used are MTX, methotrexate; DDMP, [2,4-diamino-5-(3′,4′-dichlorophenyl)]-6-methylpyrimidine (metoprine); DHFR, dihydrofolate reductase.

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A. Nichol of Burroughs Wellcome Company, Research Triangle Park, N. C. The human lymphoblastoid cell line, WIL-2, originally isolated by Levy *et al.* (7), was obtained from Dr. M. Hershfield at Duke University. The autoclavable formulation of minimal essential medium with Earle's salts for suspension culture and fetal calf serum were obtained from Grand Island Biological Company, Grand Island, N. Y. Dialyzed fetal calf serum was obtained from K-C Biologicals, Lenexa, Kan. Dihydrofolate was prepared from folic acid according to the method of Blakley (8).

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. The medium used for cell growth was supplemented with 10% fetal calf serum (growth medium).

Selection of surviving cells. Four groups of cells were set up in culture tubes on a rotary shaker. Each group had 21 tubes containing 3 ml of cell suspension per tube at 3.3 × 10^5 cells/ml in medium containing 10% dialyzed fetal calf serum and 6 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (experimental medium). The cells were exposed to either 1 μM MTX or 5 μM DDMP, as indicated in Table 1. After 4 days, the cells were allowed to settle at 1 *g*, the medium was aspirated, and fresh growth medium was added. Two days later, the medium was again removed, and the second drug was added in fresh experimental medium. After 4 days of exposure to the second drug, the medium was aspirated, and the cells were placed in fresh growth medium (drug-free) and observed for cell growth. Surviving cultures were determined after 10–12 days in those tubes showing growth, as indicated by a change in the pH of the medium and by microscopic examination of the cultures. Viability of control cultures was >90% throughout the course of the selection until overgrowth of the culture occurred about 4 days after the final drug treatment period. The surviving cultures were designated by group (I–IV) and the tube number in the group (1–21).

Pulse labeling protocol. Cells used in pulse labeling experiments were first incubated at 37° for 45 min in fresh experimental medium, after which either 1 μM MTX or 5 μM DDMP was added. Cell suspensions were pulse-labeled by transferring 0.3 ml (1 × 10^6 cells/ml) to a tube containing either [^{3}H]deoxyuridine or [^{3}H]thymidine, 2 μCi/ml. The samples were processed for incorporation of label into DNA as previously described (9).

Dihydrofolate reductase levels during selection. Three groups of cells were established in culture bottles containing 300 ml of cells at 3.3 × 10^5 cells/ml in experimental medium. One bottle was used as a control group, and the other two bottles were exposed to drug as described for Groups I and IV in Table 1. Every 2nd day, an aliquot of cells from each bottle was removed, stained with trypan blue, and counted on a hemocytometer to determine cell number and viability. Duplicate samples of approximately 2 × 10^6 cells were then removed, centrifuged, washed once with 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 M NaCl:8 mM Na_2HPO_4), and frozen at −70°C for dihydrofolate reductase determination. Viability as measured by trypan blue staining of the control group remained at >90% during the course of the experiment.

Cell extract. DHFR activity and protein concentrations were measured in cell extracts prepared from the frozen pellets. The cells were resuspended in 0.25 ml of 0.02 M Tris-HCl buffer, pH 7.5, disrupted by sonic oscillation and the cell debris removed by centrifugation at 15,000 × *g* for 30 minutes.

DHFR activity. DHFR activity was determined by measuring the decrease in absorbance at 340 nm of dihydrofolate and NADPH (10) at 32° in Tris buffer at pH 7.0 and pH 8.5 (11). The assay mixture contained 50 μmoles of Tris buffer, 50 μmoles of KCl, 150 nmoles of NADPH, 70.5 nmoles of dihydrofolate, and 100 μl of extract in a total volume of 0.5 ml. Activity was measured by using a Cary Model 118 recording spectrophotometer. NADPH oxidase was determined at both pH values and found to be negligible. A molar extinction coefficient of 12,300 was used to calculate the quantity of NADPH converted to NADP (12). Protein was measured by the method of Lowry *et al.* (13), and values are expressed as the average of duplicate determinations. Significance of values obtained for each curve were determined relative to the control group by using Student's *t*-test.

RESULTS

In Groups I and II to which MTX was added in the first cycle, no resistant cells were found. No survivors were found among 1000 cells examined for trypan blue dye exclusion in each of 21 tubes in each group regardless of which drug was used in the second cycle. In groups III and IV, where DDMP was added first, a number of cultures survived. These results are summarized in Table 1. Instead of the 100% killing of the cell cultures seen when MTX was given first, greatly reduced cell killing was produced in the groups receiving DDMP first. Furthermore, although the levels of DDMP chosen in the experiment inhibited deoxyuridine incorporation into DNA in a manner similar to MTX at the concentration used (9), much less cell killing was observed with DDMP during the selection.

TABLE 1

Sequence of drug exposure and frequency of survival in drug-treated cultures

WIL-2 cells were set up in four groups, each having 21 tubes containing 1 × 10^6 cells. The cells were exposed to either 1 μM MTX or 5 μM DDMP in the first cycle for 4 days followed by 2 days in drug-free medium. The cells were then exposed to the same drug levels in the second cycle for 4 days, according to the schedule shown, followed by incubation in drug-free medium. Surviving cultures were determined after 10–12 days in those tubes showing growth, as indicated by a change in the pH of the medium and by microscopic examination of the cultures. The number of surviving cultures at that time for each of the drug combinations used is shown. The *p* values for the two groups with survivors was determined using Fisher's exact test.

Group	Drug schedule	No. of surviving cultures
I	MTX-MTX	0/21
II	MTX-DDMP	0/21
III	DDMP-DDMP	18/21[a]
IV	DDMP-MTX	7/21[b]

[a] $0.0013 \leq p \leq 0.002$.

[b] $0.01 \geq p$.

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The effect of MTX and DDMP on thymidine and deoxyuridine incorporation into DNA in the cultures in each group was also examined. Inhibition of deoxyuridine incorporation into DNA by MTX and DDMP in a representative group of the surviving cultures is shown in Fig. 1. No significant differences were found in the subsequent drug responses of the cultures as compared with untreated cells. Thymidine incorporation also did not show any significant differences ($p > 0.10$) of the surviving cells as compared with an untreated control group (Table 2).

The results of this selection protocol suggested that prior exposure of the WIL-2 cells to DDMP led to increased survival after exposure to a second antifolate. We therefore decided to compare the effect that exposure to MTX and DDMP had on DHFR levels throughout the course of such a selection protocol (Fig. 2). Examination of cellular DHFR activity at pH 7.0 gives a measure of intracellular free enzyme level (11). In responses to DDMP, the amount of free DHFR activity at pH 7.0 was almost double that of the control group at the first recovery period (days 4–6), as shown in Fig. 2B. In contrast, MTX did not lead to an increase in the free DHFR activity, and in fact even decreased it to below the control level during the initial drug exposure. Addition of MTX to the DDMP group in Cycle II also reduced the free enzyme activity in cells previously exposed to DDMP. The activity at pH 8.5 gives a good estimate of total enzyme activity in the presence of drug (11, 14). Exposure to both MTX and DDMP increased the DHFR activity at Day 6 (Fig. 2A), but DDMP increased DHFR activity up to 2-fold above that caused by MTX treatment.

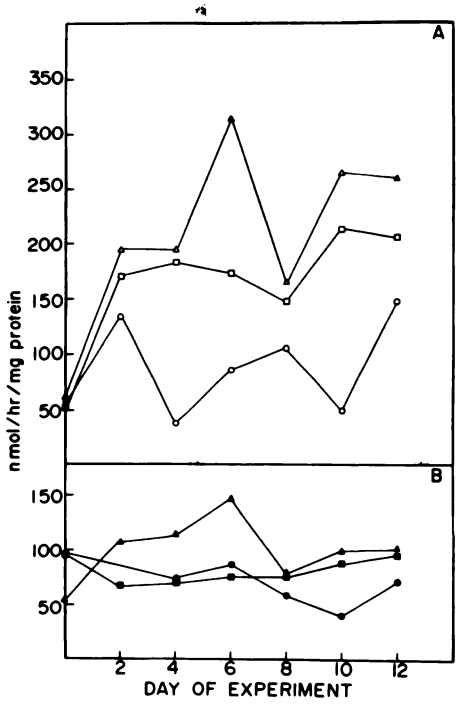

FIG. 1. *Inhibition of deoxyuridine incorporation into DNA in surviving cell cultures*

WIL-2 cells from four of the surviving cultures were examined for the ability of MTX and DDMP to inhibit deoxyuridine incorporation into DNA as compared with untreated, wild-type cells. The cells were preincubated for 45 min in experimental medium at 37°. Cultures are designated by group number as described under Materials and Methods.

A. At time 0, 1 μM MTX was added, and at the times indicated, an aliquot of cells was removed and pulsed-labeled with [^{3}H]deoxyuridine, 2 μCi/ml, for 5 min as described.

B. Samples were treated as described above except that 5 μM DDMP was added at time 0. ●, Control, wild-type cells; ○, Culture III-3; □, Culture IV-4; ■, Culture IV-6; △, Culture III-1.

TABLE 2

Thymidine incorporation into DNA in surviving cell cultures

Cells from four of the surviving cultures were examined for their ability to incorporate thymidine into DNA. An aliquot of cells was incubated for 45 min in experimental medium at 37°. The cells were then pulse-labeled with [^{3}H]thymidine, 2 μCi/ml, for 1 hr and the incorporation of label into DNA was determined. The cultures are designated by group number as described under Materials and Methods. Values are expressed as the percentage of incorporation of label into control (wild-type) cultures, and represent the average of duplicate determinations. Control cultures incorporated 2.4×10^4 cpm/3×10^5 cells/sample.

Culture	Thymidine incorporation
	% control
III-1	123 ± 6.4[a]
III-3	96 ± 15
IV-4	101 ± 3
IV-6	125 ± 10

[a] Mean ± standard deviation.

DISCUSSION

A drug treatment procedure was utilized in these experiments in WIL-2 cells which employed a single concentration of each drug at concentrations that equivalently inhibited UdR incorporation into DNA and cloning efficiency (9, 15, 16). The drug exposure periods used were scheduled to simulate *in vivo* drug regimens. The rationale for this approach was to maximize cytotoxicity by the sequential use of chemotherapeutic agents that act at a single biochemical locus (16, 17). Earlier studies showed a synergistic effect with MTX and DDMP when used simultaneously (18, 19). MTX, because of its higher affinity for DHFR (20), was expected to be more potent than DDMP in killing cells that have a normal transport capacity but which contain high concentrations of target enzyme. DDMP, on the other hand, was expected to be toxic for cells defective in transport of MTX because of its lack of dependence on active transport for cell entry (17). The results showed an unexpected antagonism between DDMP and MTX which allowed cells to survive in the presence of lethal concentrations of MTX after prior exposure to DDMP.

Although this response could be explained phenotypically or genetically, examination of the residual dihydrofolate reductase levels during the selection process supported a phenotypic explanation. DDMP, like MTX, can immediately bind to DHFR and will prevent normal degradation of DHFR in the cell (14, 21, 22). When a drug such as DDMP, which is more reversible in its association with DHFR than is MTX (17), is removed from the medium, the drug apparently elutes from the enzyme and leaves the cell with increased levels of free

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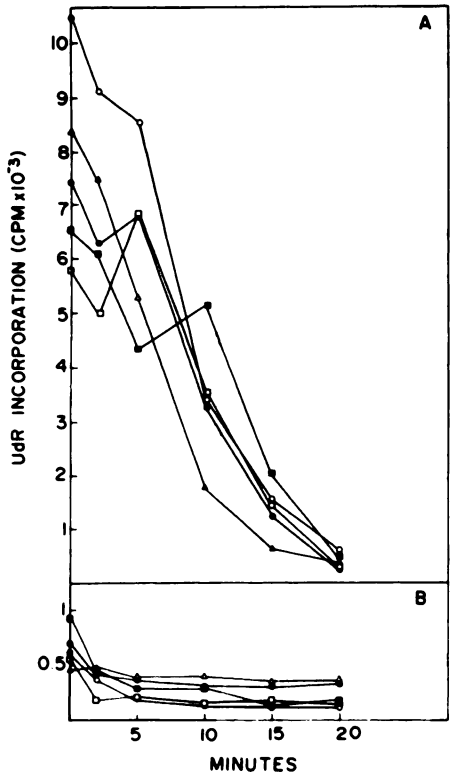

FIG. 2. *Dihydrofolate reductase activity in cells during selection*

An aliquot of cells was removed every other day from cultures incubated with MTX or DDMP as described in Table 1. DHFR enzyme activity was determined for each group as described. Each line represents the average of duplicate determinations, and each experiment was repeated several times with results that showed similar differences between groups. The significance of values obtained for each curve as compared with the control group was determined using Student's *t*-test. Peak values at Day 6 ± standard error are also given below.

A. Dihydrofolate reductase activity was determined at pH 8.5 as described. 0, Control, no drug (86 ± 21); □, MTX-MTX, Group I (173 ± 44) ($p < 0.02$); △, DDMP-MTX, Group IV (317 ± 24) ($p < 0.01$).

B. Dihydrofolate reductase activity was determined as described above except that the enzyme mixture was adjusted to pH 7.0. ●, Control, no drug (86 ± 14); ■, MTX-MTX group (75 ± 3) ($p > 0.1$); ▲, DDMP-MTX group (148 ± 51) ($p < 0.05$).

DHFR (Fig. 2B, Days 4–6). Under these conditions the cell reacts in a manner similar to a DHFR-overproducing mutant when subsequently exposed to an antifolate such as MTX, and some cells survive. By contrast, MTX is very tightly bound to the enzyme (23). Exposure of cells to MTX will cause the cell to accumulate higher levels of DHFR. When MTX is removed from the medium, the bound drug remains within the cell tightly bound to the enzyme (24), keeping most of the enzyme inhibited (25). The small amount of free enzyme that exists is sufficient to provide reduced folates for cellular folate-dependent reactions (24). Under these circumstances, a second exposure to antifolate is still toxic to the cell (Table 1). Our results show that both drugs clearly result in a greater than 2-fold increase in assayable enzyme activity over control cells at pH 8.5. At pH 7.0, which may more closely reflect free intracellular enzyme, the assayable enzyme activities in MTX and control cells were identical. The DHFR activity from the cells treated with DDMP, on the other hand, was increased greater than 2-fold above this level. One cannot discern with the assay used whether there is more active enzyme in DDMP- or MTX-treated cells. However, it has been shown that the increased activity measured at pH 8.5 is due to the presence of increased enzyme levels in the cell as a result of decreased degradation (11, 22). This is consistent with the model discussed above. Related experiments from our laboratory on the time course of inhibition of DNA synthesis by MTX or DDMP, and the recovery of synthesis following removal of the drug, support this concept (16).

This functional resistance to MTX, initially characterized as an altered response to inhibition of deoxyuridine incorporation by the drug, also appears to be transient, since none of the selected cell lines examined have been able to grow in the presence of MTX or DDMP for extended periods (not shown). Additionally, this would suggest no change in the ability of the cells to take up MTX, since inhibition of deoxyuridine incorporation into DNA has been shown to be a good indicator of drug uptake (9, 15).

Previous work from this laboratory has demonstrated that a low dose of MTX or DDMP is sufficient to maintain the toxicity of a brief initial high dose of MTX (9). Curiously, a low dose of MTX or DDMP could not maintain the toxicity of an initial high dose of DDMP.[3] Our work with a gene-amplified cell line resistant to 400 μM MTX suggests that lipid-soluble antifolates such as DDMP may also be useful in obviating MTX resistance due to enzyme overproduction.[4] The drug used in this study, DDMP, has been shown to have antitumor properties, is able to enter "santuary" areas of the body, and is a prototype of newer drugs effective against MTX-resistant cells (25). However, the present experiments show that emphasis must be placed on sequence and timing in the administration of such drugs in a combination chemotherapy regimen.

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[4] M. Hamrell, J. Laszlo, and W. D. Sedwick, unpublished observations.

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Errata

Volume 19, No. 2 (1981), in the article, "The Inhibition of DNA(Cytosine-5) Methylases by 5-Azacytidine: The Effect of Azacytosine-Containing DNA," by Stanley Friedman, pp. 314–320: In Table 4 the fourth and fifth column headings, omitted in the original table, should read 5-MeCyt and 6-MeAde, respectively.

Volume 19, No. 3 (1981), in the article, "Transient Induction of Phenotypic Resistance in Human Lymphoblastoid Cells following Sequential Use of Two Antifolates," by Michael R. Hamrell, John Laszlo, and W. David Sedwick, pp. 491–495: the figure drawings for Figs. 1 and 2 are reversed; the legends and descriptions are correct.

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