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Deoxyuridine Incorporation as a Useful Measure of Methotrexate and Metoprine Uptake and Metabolic Effectiveness

W. DAVID SEDWICK,[1] MARY JO FYFE, OLIVER E. BROWN, THOMAS A. FRAZER, MARC KUTLER, AND JOHN LASZLO

Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710

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SUMMARY

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This paper contrasts the inhibition of deoxyuridine incorporation by the lipid-soluble antifolate, DDMP, and methotrexate in the human lymphoblastoid cell line, WIL-2. It shows how deoxyuridine incorporation can be used as an indirect tool for assay of drug uptake and facilitates interpretation of experiments with labeled drugs. Whereas 1 μM methotrexate takes 15-30 minutes to maximally inhibit deoxyuridine incorporation, DDMP maximally inhibits incorporation within five seconds of addition to the cell culture. These protocols expand the potential of deoxyuridine-based assays in analysis of antifolate action and may be useful for *in vitro* testing of potential tumor responses to antifolates.

INTRODUCTION

Assays of UdR[2] incorporation into the DNA of mammalian cells are useful in detecting resistance to folate antagonists, for comparing differential cellular responses to these drugs, and for determining their relative potencies (1). Here we have used the measurement of UdR incorporation into the DNA of a human lymphoblastoid cell line, WIL-2, to compare the inhibitory properties of two pharmacologically distinct antifolates. Methotrexate (MTX), a prototypic active transport-dependent, tight-binding inhibitor of dihydrofolate reductase, is compared with metoprine [2,4-diamino-5-(3'4'-dichlorophenyl)-6-methylpyrimidine; DDMP], a lipid-soluble antifolate (2), which is also an inhibitor of dihydrofolate reductase.

Although inhibition of UdR incorporation into DNA is often used as a sensitive measure of the *in vitro* metabolic activity of folate antagonists (3), this assay does not necessarily correlate with *in vivo* antitumor effects (4, 5). While it is evident from these and other studies (6, 7) that a variety of assays must be utilized in order to approach a high probability of success in predicting *in vivo* response of tumors, assay of UdR incorporation still provides a straightforward way to assess the relative response of cells to antifolates.

This paper contrasts the kinetics of UdR inhibition by DDMP and MTX. It shows

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[1] Reprint requests should be addressed to Dr. W. David Sedwick, Box 3835, Duke University Medical Center, Durham, N.C. 27710.

[2] The abbreviations used are: UdR, deoxyuridine; MTX, methotrexate; MEM, minimal essential medium; PBS, phosphate-buffered saline.

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that *in vitro* UdR incorporation can be used as an indirect assay of drug uptake, a tool which increases the pharmacologic significance of uptake studies with labeled drugs. It is hoped that the approach developed will extend the general utility of UdR-based assays for *in vitro* prediction of *in vivo* antitumor effects.

MATERIALS

Deoxyuridine (6-^{3}H) (24.2 Ci/mmole) was obtained from the New England Nuclear Company. MTX, 3',5',9(n)-^{3}H was kindly provided by NCI, Contract No. 1-CM-67121, and repurified before use as described by Goldman *et al.* (8). DDMP and ^{14}C DDMP were kindly furnished by Dr. C. A. Nichol of Burroughs Wellcome Co. and silicon oil (DOW 702 diffusion pump fluid) was obtained from DOW-Corning. The cell line, WIL-2, originally isolated by J. Levy *et al.* (9), was obtained from Dr. M. Hershfield, Duke University Medical School, Department of Medicine. The MEM used to support the growth of WIL-2 cells was the autoclavable formulation for suspension cultures sold by GIBCO. Fetal calf serum, obtained from GIBCO, was dialyzed for three days against three changes of ten volumes of saline solution prior to use in incorporation and drug uptake experiments.

METHODS

Cell maintenance. WIL-2 cells were maintained in suspension cultures which were continuously agitated on a rotary shaker at 37° at a density of 1.5 to 8 × 10^5 cells/ml. MEM used for cell growth experiments was supplemented with 10% fetal calf serum.

Pulse labeling protocol. Cells used in pulse labeling experiments were first incubated for 30 minutes in fresh medium (MEM) containing 10% dialyzed fetal calf serum. Cells were then pulse labeled with ^{3}H-deoxyuridine by transferring 0.2 to 0.3 ml of cells to a tube containing ^{3}H-deoxyuridine (5 μCi/ml). The reaction was stopped by addition of ice-cold PBS, followed by rapid centrifugation and removal of the supernatant. The cells were then resuspended in 100 μl of PBS and transferred to Whatman filter paper discs. The discs were further processed for scintillation counting as previously described (1).

Rapid assay of UdR incorporation. The procedure used for transport studies was modified from Marz *et al.* (10). Total uptake of UdR by cells was assayed by injecting 0.3 ml of cells at the same time with 0.3 ml of labeled UdR at 37° into tubes containing a 0.2 ml layer of mineral oil (Squibb) and diluted silicon oil (30:70), and centrifuging the samples for 30 seconds in a microcentrifuge as further described for the MTX uptake assay. Samples were taken at intervals varying from two minutes to two seconds before centrifugation in a Brinkmann-Eppendorf Centrifuge 3200, a procedure which terminates further incorporation of UdR by removing cells from the aqueous layer into a pellet beneath the oil layer. Three milliliters of aquasol were then added to each vial, and the samples were shaken vigorously prior to scintillation counting.

Samples obtained for measurement of TCA precipitable counts were pipetted into glass tubes and immersed in an ice bath with simultaneous ten-fold dilution by PBS to stop the incorporation of deoxyuridine into DNA. Samples were immediately centrifuged, spotted on filters, treated with trichloroacetic acid as previously described (1) and counted by scintillation spectroscopy under identical quench conditions to those used for determination of total UdR uptake.

Assay of methotrexate uptake. Uptake of MTX by WIL-2 cells was measured by rapidly mixing equal volumes of 5 × 10^6 cells/ml in MEM with medium containing two times the desired final concentration of MTX. Samples of 0.3 ml were periodically transferred to microcentrifuge tubes containing a mixture of silicon oil (70%) and heavy mineral oil (30%) and centrifuged for 30 seconds in a microcentrifuge. The top layer containing unabsorbed drug in aqueous solution was removed and the walls of the tube above the oil layer washed by filling the tube twice with deionized water. One molar sucrose (0.5 ml) buffered by PBS was then added to each tube. The tubes were again centrifuged, and the floating oil layer discarded. The cells were then suspended in the remaining 0.5 ml of PBS-

buffered sucrose and transferred to scintillation vials. The remaining material in the microcentrifuge tubes was washed into the scintillation vial by two washes with 1 ml Aquasol (New England Nuclear Corp.). Three milliliters of Aquasol were then added to each vial, and the samples were shaken vigorously prior to scintillation counting.

Assay of DDMP uptake. Cells were collected from growth medium, resuspended in fresh MEM containing dialyzed fetal calf serum, and incubated in a shaking water bath. After ^{14}C-DDMP was added, 0.5 ml samples were periodically removed, suspended in ten volumes of ice-cold Tris-buffered saline and immediately collected on Whatman GFC filters. Filters were washed two times with 5 ml of the saline solution, taking care not to allow the filter to dry between washes. The filters were then dried, solubilized with Protosol for three hours at 37° and counted in toluene-based scintillation fluid, containing 4 g PPO and 0.05 g POPOP/liter of toluene.

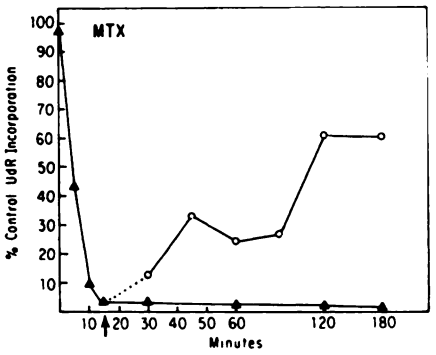

FIG. 1. *The kinetic response of UdR incorporation to the addition and removal of MTX from the cell incubation medium*

1 μM MTX was added to a culture of WIL-2 cells which was in logarithmic growth. Cells were subsequently pulse labeled with 1 μCi/ml of ^{3}H-UdR for *10* minute intervals following drug addition to the medium (▲——▲). Cells were also removed from MTX-containing medium by centrifugation and resuspension of cells in cold medium two times followed by incubation at 37° in fresh drug-free medium. The recovery of the cellular ability to incorporate UdR was also monitored by pulse labeling with ^{3}H-UdR (○——○). Incorporation into drug-free cells was constant during the course of this experiment at 2.3×10^{4} CPM/5×10^{5} cells/sample.

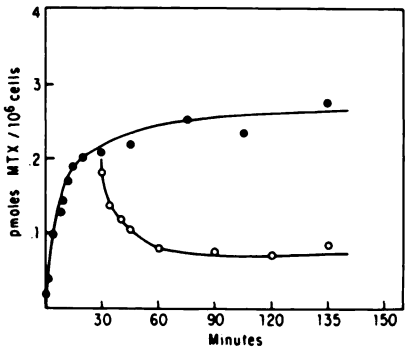

FIG. 2. *The time course of uptake and unidirectional efflux of MTX in WIL-2 cell cultures*

1 μM MTX was added to cells and samples were subsequently taken at the indicated times to monitor the uptake of ^{3}H-MTX (●——●) by cells as described in METHODS. At 30 minutes after drug addition, cells were removed from the medium containing labeled MTX, washed two times with cold medium and resuspended in drug-free medium at 37°. The rate of loss of MTX from cells was then measured as an indication of the drug efflux rate (○——○).

RESULTS

MTX uptake experiments with labeled drug only delineate the apparent rate of entry and efflux of MTX from the cell; however, the pulse labeling experiments with UdR provide an apparent rate of drug entry, a quantitative estimate of metabolic impact, and the rate of recovery of cells from the antimetabolic effect of the drug. Figures 1 and 2 show that the kinetics of inhibition of DNA synthesis by 1 μM MTX, as obtained from pulse labeling experiments with UdR, closely correlate with MTX uptake kinetics obtained directly with labeled drug. The time course for unidirectional free drug efflux from the cells is observed over a 10–15 minute period after resuspension in drug-free medium, whereas maximum recovery of the cells' ability to incorporate UdR does not occur until at least two hours after washout of drug.

Assays of UdR incorporation in the presence of DDMP established both the rapidity of drug entry and its effect on UdR incorporation. The kinetics of inhibition of UdR incorporation shown in Fig. 3 are compared with the kinetics of DDMP uptake shown in Fig. 4. Within 20 seconds, labeled DDMP associates significantly with WIL-2 cells (20 pmols/10^{6} cells); thereafter DDMP

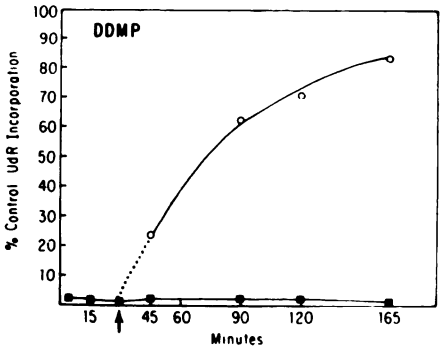

FIG. 3. *The kinetic response of UdR incorporation to the addition and removal of DDMP from the incubation medium*

5 μM DDMP was added to a culture of log phase WIL-2 cells. Cells were subsequently pulse labeled with 2 μCi/ml of ^{3}H-UdR (■——■) for ten minutes at intervals following drug addition. A portion of the cells was removed from DDMP-containing medium by centrifugation at 30 minutes, then washed two times with cold medium, resuspended in fresh drug-free medium and incubated at 37°. The recovery of the capacity of cells to incorporate UdR was then monitored by five minute pulse labeling as described in Fig. 1 (○——○). Control incorporation increased from 9 to 15 × 10^4 CPM/2.5 × 10^5 cells/sample. Percent control was calculated relative to drug-free samples at each time period.

continues to be taken up over a period of 3–10 minutes until it approaches saturation at a concentration one to two-fold greater than its initial association with the cells. These measurements were made as rapidly as possible using the filtration technique (METHODS).

Although DDMP continues to concentrate in the cell over a period of three minutes or longer, a steady state of UdR incorporation in the presence of DDMP is established within about five seconds of DDMP exposure (Fig. 5, Panel A). This inhibition by DDMP of UdR incorporation is not due to an inhibition of UdR uptake; rather, the presence of DDMP increases the overall rate and amount of UdR uptake by WIL-2 cells (Fig. 5, Panel B).

Removal of DDMP from the incubation medium results in rapid release of labeled drug, leaving a cell-bound fraction that cannot be decreased by washing the cells in ice-cold medium or by incubating them at 37° in drug-free medium for periods as long as four hours (not shown). After 15 minutes of exposure to 5 μM DDMP cells irreversibly bind 7 pmoles drug/10^6 cells, whereas cells exposed to 1 μM MTX irreversibly bind about 0.07 pmoles/10^6 cells of this drug. The bound fraction is saturated within two minutes of exposure to concentrations of DDMP ranging from 0.5 to 50 μM, whereas 0.1 to 5 μM MTX takes approximately 15–30 minutes before it saturates cells with a concentration dependent kinetic.

The ability of cells to incorporate UdR into DNA after a 30 minute exposure to DDMP, as in the case of MTX, recovers maximally about two and one half hours following drug removal (not shown). The kinetics and degree of recovery from DDMP inhibition of UdR incorporation is directly dependent upon the concentration of drug in the incubation medium. In contrast, MTX achieves the same level of inhibition in a time-dependent manner, at all concentrations of drug from 0.5 μM to 5.0 μM (Fig. 6, Panels A and B). Although not shown, treatment with 0.1 μM MTX will also inhibit UdR incorporation by more than 95% after a period of exposure of several hours.

DISCUSSION

Drug uptake studies with mouse and hu-

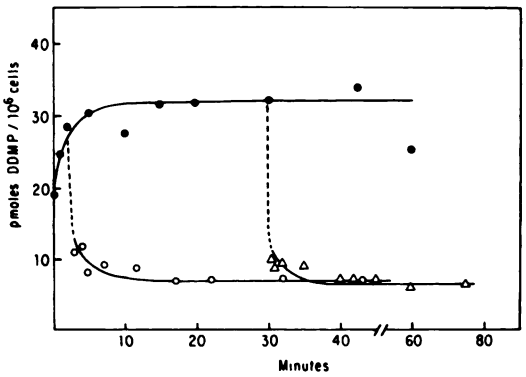

FIG. 4. *Time course of uptake and unidirectional efflux of DDMP in WIL-2 cell cultures*

5 μM ^{14}C-DDMP was added to cells and samples subsequently taken to monitor uptake of DDMP (●——●). A portion of the cells was removed from DDMP-containing medium at 2 (○——○) and 30 minutes (△——△) after drug addition, washed once with ice-cold MEM and suspended in drug-free MEM at 37°. The rate and extent of dissociation of labeled drug from cells was then determined.

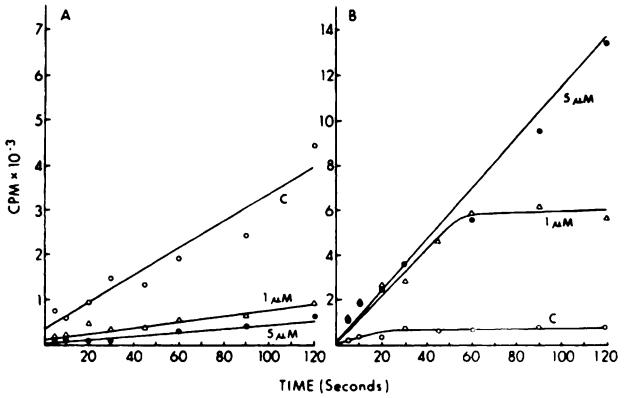

FIG. 5. *UdR uptake and incorporation into DNA in DDMP-treated cells*

DDMP and ^{3}H-UdR were added to cells at time 0 and samples subsequently measured by the rapid kinetic assay described in METHODS. The amount of UdR incorporation into DNA is indicated in Panel A. The amount of total label in the cells at each drug concentration after subtraction of the counts incorporated into DNA is indicated in Panel B [Control (O——O), 1 μM DDMP (Δ——Δ), 5 μM DDMP (●——●)].

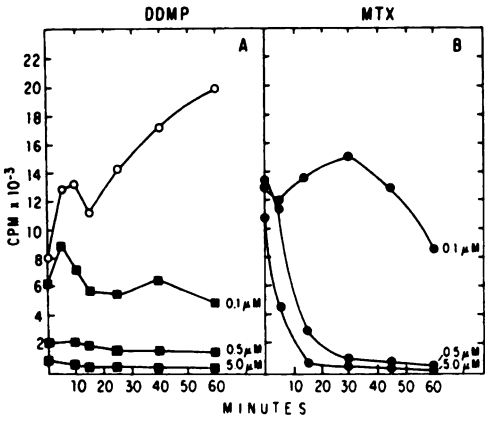

FIG. 6. *Comparison of the cellular kinetic response of UdR incorporation into DNA to different concentrations of MTX and DDMP*

DDMP (Panel A) and MTX (Panel B) were added to cells and the cells subsequently pulse labeled as described in Figs. 1 and 3. 0.1 μM drug; 0.5 μM drug; 1 μM drug; and 5 μM drug are designated in both Panels A and B.

man cells have shown good correlation between low MTX uptake and resistance to the antitumor effect of MTX (11, 12). The pulse labeling protocol used in this study measures the inhibition of UdR incorporation into DNA and provides the basis for an indirect kinetic analysis of drug uptake. At concentrations of 1 μM or greater, the kinetics of inhibition by MTX closely followed the kinetics of uptake with labeled MTX. At lower drug concentrations (0.1 μM), the metabolic inhibition observed lagged behind the drug uptake. However, at low drug concentrations the kinetic comparison is difficult to interpret, as has been shown to be the case in other cells (13). One of the earliest effects of MTX on WIL-2 cells is to reduce the thymidine triphosphate pool, which in turn leads to an initial apparent stimulation of UdR incorporation.[3] At higher drug concentrations, however, the UdR pulse labeling technique seems to give a reliable indirect measure of MTX uptake, as well as an indication of the sensitivity of cells according to the extent of inhibition of UdR incorporation into DNA.

Drug uptake studies with ^{14}C-DDMP gave results that did not correlate well with the observed inhibition of UdR. The inhibition of UdR incorporation caused by DDMP is at an apparent steady state within five seconds of drug addition. DDMP usually concentrates one to two-fold more during the several minutes following its initial high concentration association with WIL-2 cells. In some experiments this lack of correlation was even more marked. For example, when 5 μM DDMP was used, association increased over a period of 1-2 hours, whereas UdR

[3] Brown, O. E. and W. D. Sedwick, unpublished observations.

inhibition was always immediate and constant, or even decreasing, over a two hour period. However, close inspection of Fig. 6 shows that at low concentrations of DDMP (0.5 μM or less), an increasing amount of UdR inhibition can be demonstrated with time. Therefore, a facilitated transport mechanism for DDMP could play a pharmacologically important role at low DDMP concentrations. Such a concentration-dependent uptake relationship would be analogous to the transport of MTX, except that the direct diffusion mechanism for MTX appears to be operative at concentrations above 20 μM (14). These concentration-dependent transport differences are currently under further investigation.

From the results of the experiment described in Fig. 5B, it is clear that DDMP does not directly inhibit UdR uptake by the cell: DDMP stimulated the uptake of UdR into the acid-soluble fraction in a concentration-dependent manner. In other (unpublished) experiments we have shown that like MTX, DDMP inhibition leads to massive accumulation of deoxyuridine monophosphate (dUMP) in the cell, particularly if an exogenous source of UdR is provided. It must be assumed that DDMP immediately enters the cell to inhibit its target enzyme(s) and prevents further anabolism into DNA.

Reports on the transport of DDMP in L1210 (15, 16) cells have documented a kinetic for drug uptake of 50 μM DDMP over a period of 15–30 minutes. This drug concentration is five to ten times greater than that which can be maintained in clinical studies (17). Hill *et al.* (15, 16) have also shown that MTX and citrovorum factor (CF) compete with DDMP for cellular uptake; all of these experiments were carried out at 50 μM DDMP. It is difficult to compare our studies carried out with 0.1 to 5.0 μM DDMP in WIL-2 cells with the L1210 experiments. Clearly, however, TMP synthesis is immediately inhibited at the lower drug concentrations. Furthermore, we have not been able to demonstrate competitive interaction between CF or MTX and DDMP at these concentrations (unpublished data). Moreover, it is important to note that many factors influence DDMP uptake which have little or no effect on MTX uptake, so that studies of DDMP uptake should be carried out in medium identical to that used for metabolic studies. Our laboratory has documented a marked effect of constituents of the medium on both uptake and metabolic inhibition of this drug in both WIL-2 and human leukemic cells.[4] These experiments have shown that proteins and other medium constituents such as amino acids can reduce DDMP effectiveness.

The primary target enzyme responsible for DDMP inhibition of UdR incorporation has not yet been convincingly demonstrated. At this time, direct studies on purified DHFR suggest the likelihood that this enzyme is a principal target of DDMP in the cell (18). Since the bound fraction of DDMP is 100 times that observed with MTX which binds quantitatively and specifically with very high affinity only to DHFR in the cell, it is clear that DDMP is binding to other sites in addition to DHFR. Because of its high affinity for lipoproteins, this is an expected observation and it does not necessarily indicate that DDMP has other specific cellular enzyme targets.

The immediate inhibition produced by DDMP, however, implies that there is no excess pool of methylenetetrahydrofolate available to support thymidine monophosphate biosynthesis in the intact cell, or that DDMP exerts a more direct effect on thymidylate synthetase, on one of the pyrimidine kinases, or on another step leading to incorporation of UdR into DNA.

In conclusion, pulse labeling and rapid kinetic protocols with UdR give an indirect but pharmacologically significant indication of both the level and the kinetics of cellular response to antifolates such as MTX and DDMP. Such studies can be utilized to establish the potential for drug inhibition, the differential response to a drug in cells obtained from different sources and, as we will show in further publications, the effect of metabolites, media or plasma components on drug transport and metabolic inhibition. These protocols also may

[4] Fyfe, M. J., O. E. Brown, J. Laszlo and W. D. Sedwick; unpublished observations.

be useful for augmentation of biochemical assays for *in vitro* testing of potential tumor responses to antifolates.

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