Europ. J. Cancer Vol. 16, 1057–1063

0014-2964/80/0801-1057 $02.00/0

Limited Effectiveness of *in Vitro* High-Dose Methotrexate and Leucovorin to Overcome Resistance in L1210 Leukemia Cells with Elevations of Dihydrofolate reductase*

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Abstract—*Two L1210 cell lines, one sensitive to methotrexate (MTX) (L1210S) and the other resistant (L1210/740R) due to high dihydrofolate reductase were exposed to MTX in serial log concentrations from* 10^{-3} to 10^{-8}M *MTX. Maximal* (90%) *inhibition of* de novo *DNA synthesis was produced by* 10^{-6}M *MTX in L1210/S.* 10^{-4} *MTX produced no more than* 40% inhibition in the L1210/740*R line. Leucovorin* [N-5-*formyltetrahydrofolic acid (LV)*] 10^{-4} M *completely rescued L1210S from* 10^{-7} M *MTX and L1210/740R from* 10^{-5} M *MTX. There was a partial rescue by LV of L1210/740R but none in L1210/S from* 10^{-4} M *MTX. LV completely corrected the metabolic defect produced by* 10^{-4} M *MTX when the cells were washed in MTX free media following exposure to* 10^{-4} M *MTX. Thus, high-dose MTX has no apparent major advantage when MTX resistance is due to high dihydrofolate reductase. High-dose MTX can produce a defect involving a low affinity (reversible) site which is not corrected by LV and a high affinity site defect which is completely corrected by LV. LV resue is not directly dependent on the concentration of free MTX alone in extracellular media. The measurement of the antimetabolic effect of different simulated MTX treatment programs in cell lines with different defined mechanisms of resistance to MTX may serve as a method for identifying the biochemical parameters of cells and optimum conditions, if any, for treatment with high-dose MTX and LV.*

INTRODUCTION

METHOTREXATE (MTX) given in various dosages and schedules has a wide spectrum of antitumor activity [1]. New treatment schedules designed to be used with *N*-5 formyltetrahydrofolic acid [leucovorin (LV)] permit 10–1000 times larger than the conventional amounts of MTX to be used to preferentially inhibit tumor growth and rescue normal tissue from MTX [2, 3]. Treatment with high-dose MTX–LV regimens may be superior to conventional treatment in some clinical situations such as osteogenic sarcoma [4], leukemia resistant to conventional doses of MTX, and perhaps some pediatric lymphomas [2, 5]. However, most of the common tumors which sometimes respond to conventional doses of MTX and those resistant to conventional dosage of MTX have not undergone sufficient evaluation with the high-dose MTX schedule. High-dose MTX may not always prove effective or superior to conventional doses. Consequently, it remains to be determined if high-dose MTX will achieve a much broader spectrum of antitumor activity than conventional MTX. Also, the optimum quantitative requirements, effective concentrations of MTX and LV, and their schedule remain to be defined for specific tumor systems.

The mechanism and conditions which may confer an advantage to high-dose MTX compared to conventional doses of MTX have not been demonstrated. It is unknown which type(s) of resistance to MTX can be overcome with high-dose MTX treatment. On

Accepted 25 September 1979.

*This work was supported by the Chemotherapy Foundation, Inc., NCI Contract NO1-CM 43703 and NIH Grant No. 1 RO1-AM16690-03. Requests for reprints should be addressed to Dr. Samuel Waxman, Division of Medical Oncology, Fifth Avenue and 100th Street, New York, NY 10029, U.S.A.

theoretical grounds, it is possible that MTX may overcome transport resistance by diffusion [6], however, whether high-dose MTX will overcome resistance due to elevated dihydrofolate reductase is less predictable.

These experiments were designed to determine if resistance to MTX due to elevations of cellular dihydrofolate reductase can be effectively overcome by high-dose MTX therapy using conditions which simulate *in vitro* pharmacologically definable features of high-dose MTX therapy. The inhibition of *de novo* DNA synthesis by MTX was compared in L1210 cells, a parent line L1210/S sensitive to MTX, and a line, L1210/740R, resistant to MTX because of high intracellular concentrations of dihydrofolate reductase. Studies were designed to test the contribution of various isolated pharmacological conditions for producing inhibition of *de novo* DNA synthesis by MTX and thereby determining the optimum conditions for therapy with MTX for different cell lines. They also test the value of these simulated conditions as new methods for identifying cells which are sensitive and resistant to MTX.

The simulated conditions include: short exposure to high concentrations of MTX in the presence or absence of LV; removing MTX from the extracellular fluid with or without the subsequent addition of LV (simulating the period of decline of the concentration of MTX in serum due to excretion of MTX) and the rescue or protection by LV. The *in vitro* system was suitable for testing controlled concentrations of each drug, conditions of exposure time to MTX, and concentrations of MTX or LV which exceed the concentrations or exposure times to these drugs which have been achieved *in vivo*. Thus, the system may be suitable for testing theoretical limits of manipulating the therapeutic dosage schedules for MTX or LV in MTX sensitive and resistant cell lives.

MATERIALS AND METHODS

L1210 leukemia cells were supplied by Dr. I. Wodinski, A. D. Little Company, Cambridge, Mass. L1210/S and L1210/740R were harvested into Hanks Balanced Salt Solution (HBSS) from the ascitic aspirate of DBA/2J mice 7 days after i.p. inoculation with 1×10^6 cells. The cells were washed three times to remove heparin and ascitic fluid and resuspended in HBSS containing 10% dialyzed fetal calf serum to a concentration of 5×10^7 cells/ml.

The dialyzed fetal calf serum contained less than 1 ng/ml folate and did not bind ^{3}H-MTX or added deoxyuridine (UdR). The cells had not been exposed to MTX for several passages. The antimetabolic effect of MTX and the effect of LV on *de novo* DNA synthesis was measured by the deoxyuridine (UdR) suppression test as previously described [7, 8]. In this system, abnormal *de novo* DNA synthesis produced by MTX is demonstrable by the reduced ability of UdR to suppress incorporation of subsequently added ^{3}H-thymidine (^{3}H-TdR) into DNA. The experiments included cells exposed to ^{3}H-TdR alone, with and without MTX.

For culture, 0.2 ml cells ($11–13 \times 10^6$) were pipetted into siliconized glass tubes after the various radioactive and non-radioactive test components were added in 0.1 ml aliquots, as indicated by the individual experiment. The final volumes were achieved by dilution with HBSS. The cells were first equilibrated with UdR and drugs for 60 min at 23°C, and then the entire experiment was done at 37°C for 3 hr after the addition of 0.1 μCi ^{3}H-TdR (spec. act. 20 Ci/mM, Amersham/Searle, Arlington Heights, Ill.). All reactions were performed in triplicate.

These studies designed to measure the effect of LV were done in two ways. The cells exposed to MTX and UdR for 1 hr at 23°C before LV and TdR test materials were added for a 3 hr incubation to simulate LV "rescue". In separate experiments, LV was added at the same time as MTX 1 hr before TdR to simulate "protection" by LV.

In the studies designed to evaluate MTX washout, following 3 hr exposure to MTX, the cells were washed at 23°C three times with HBSS for a cumulative washing time of 15 min, then UdR and ^{3}H-thymidine were added.

At the end of the incubation time the cells were washed twice with cold 0.9% NaCl solution. Two ml of 10% trichloracetic acid (TCA) was added and the resultant precipitate was washed once with 10% TCA. The acid-precipitable material was dissolved in 1.0 ml NCS reagent (Packard Instruments, Fullerton, Calif.) and counted in 15 ml of a scintillation mixture (toluene containing 30% ethanol, 0.6% PPO and 0.03% dimethyl POPOP) in a Beckman LS250 liquid scintillation system to a counting error of 2% or less. Results are expressed as percentage of radioactivity incorporated where 100% is that amount of ^{3}H-TdR incorporated in the absence of UdR. Each triplicate had a variation

of uptake which was less than 2%. Drugs tested in this system consisted of MTX, LV and folic acid (Folvite), Lederle Laboratories, Pearl River, New York. ^{3}H-MTX (12.3 Ci/mM) and ^{3}H-folic acid (40 Ci/mM) was purchased from Amersham Searle, Arlington Heights, Ill., and was diluted to a concentration of 5 ng/ml in HBSS. The purity of the radioactive compounds used in each experiment, varied from 90–95% as determined by descending paper chromatography in 0.1 M phosphate buffer, pH 7.9.

For measurement of MTX uptake, L1210 cells were incubated for 15 min at 23°C in HBSS. Incubation volumes were brought to 0.9 ml with HBSS and placed at 37°C. The reactions were started with the addition of ^{3}H-MTX (0.5 ng) and stopped at intervals ranging from 0 to 180 min by the addition of cold 0.9% NaCl solution. The pellet was dissolved in 1.0 ml NCS reagent and counted. All experiments were run in triplicate [8].

Dihydrofolate reductase levels in L1210 cells were measured by the reduction of ^{3}H-folic acid to ^{3}H-tetrahydrofolate utilizing the method of Rothenberg [9]. The dihydrofolate reductase preparation was obtained from cells that were washed three times with saline, resuspended in three volumes of 0.20 M sodium citrate and freeze thawed three times. The centrifuged supernatant was assayed for protein and used for the assay. The incubation mixture consisted of 0.25 ml citrate buffer (0.02 M, pH 4.5), 0.05 ml NADPH (0.1 mg), 0.05 ml mercaptoethanol (3.5 mM) and 500 ng folic acid and 0.05 mg ^{3}H-folic acid (0.25 mg). The reaction is started with the enzyme source and the entire incubation mixture incubated at 37°C for 30 min. The reaction is stopped by the addition of 0.2 ml folic acid (12 mg/ml) and precipitated with 0.2 ml 5% $ZnSO_4$-$7H_2O$. No additional precautions for the stabilization of ^{3}H-folic acid were attempted. The levels of dihydrofolate reductase in L1210/S and 740R were measured in simultaneous experiments.

RESULTS

Characterizations of the L1210 leukemia cell lines

The L1210/740R line was characterized as a high dihydrofolate reductase line. It had dihydrofolate reductase activity capable of converting 21.9 nM of folic acid per mg of protein as compared to L1210/S which converts 0.88 mM of folic acid per mg of protein. The two cell lines were identical in their net uptake of ^{3}H-MTX into the cell during the first 30 min. Net uptake of MTX is greater in the L1210/740R line than in L1210/S at 60 min (Fig. 1). Other possible modes of resistance in L1210/740R such as altered K_i for MTX or efflux kinetics for MTX were not assessed.

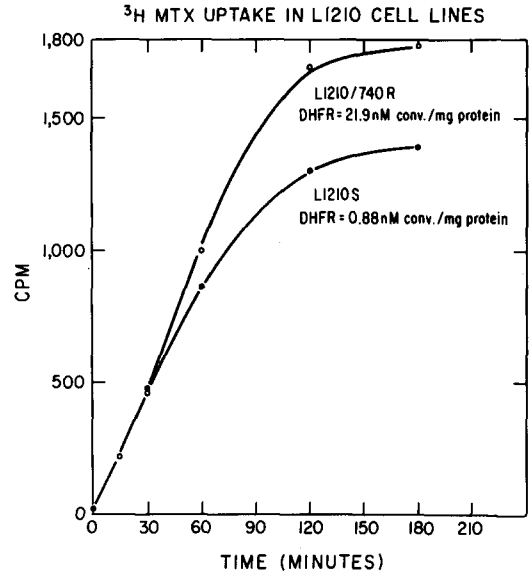

Fig. 1. Characterization of L1210S and L1210/740R with respect to MTX uptake and dihydrofolate reductase activity.

Methotrexate effect on de novo *DNA synthesis*

The antimetabolic effect of MTX in L1210/S is measurable at a concentration of less than 1×10^{-7} M, increases markedly at 5×10^{-7} M, and thereafter higher concentrations show no further increase in inhibition of *de novo* DNA synthesis (Fig. 2). In contrast,

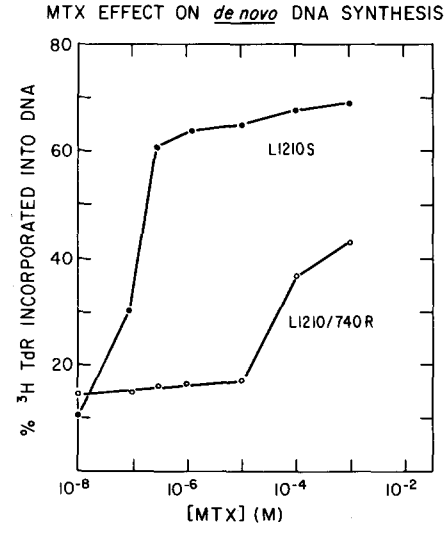

Fig. 2. MTX effect in L1210S and L1210/740R measured by the UdR suppression assay. 0.1 μM UdR added/ml.

in the L1210/740R cell line the antimetabolic effect of MTX is first measurable in concentrations in excess of 10^{-5} M. There is less than 50% inhibition of *de novo* DNA synthesis at 10^{-4} M. MTX at 10^{-3} M in L1210/740R fails to achieve a degree of inhibition of *de novo* DNA synthesis comparable to that in L1210/S achieved with any concentration of MTX above 10^{-6} M.

The effect of methotrexate on ^{3}H-thymidine incorporation into DNA

The effect of MTX serial concentrations on ^{3}H-thymidine incorporation in DNA was measured. MTX increased ^{3}H-thymidine incorporation into DNA except at doses greater than 10^{-3} where ^{3}H-thymidine incorporation was reduced. This direct MTX effect on thymidine incorporation is not sufficient alone to explain the difference between ^{3}H-thymidine incorporation in the L1210/S and L1210R lines in the presence of UdR and MTX. This small degree of inhibition of ^{3}H-thymidine incorporation by 10^{-3} M MTX is more marked in the L1210/740R and was not consistently observed with L1210/S.

Effect of leucovorin

In L1210/S, LV 10^{-4} M partially protected (40%) against the defect in DNA synthesis induced by MTX 10^{-6} M to 10^{-4} M. At 10^{-3} M MTX, the ability of LV to correct inhibition of *de novo* DNA synthesis in L1210/S appears to be almost absent. In contrast, against L1210/740R, 10^{-4} M LV is more protective, correcting the defect in DNA synthesis by 80% at 10^{-4} M MTX and by 40% at 10^{-3} M MTX (Figs. 3 and 4). Results were qualitatively the same when LV was added at the same time (protection) as MTX or at 1 hr (early rescue) after MTX (not shown).

Methotrexate "washout" experiments

The cell lines were exposed to various concentrations of MTX and "washed" to remove the extracellular and loosely bound MTX prior to the UdR suppression assay in order to determine the residual MTX effect on *de novo* DNA synthesis. In L1210/S, washout completely eliminated the inhibition of *de novo* DNA synthesis by MTX concentrations of 10^{-5} M (Fig. 5). In contrast, washout produced complete loss of MTX effect in the L1210/740R line at concentrations as high as 10^{-3} M (Fig. 6). The addition of LV following washout of 10^{-3}–10^{-4} M MTX corrects the MTX induced defect in DNA synthesis which was not correctable by LV prior to washing of the L1210/S cells (Figs. 4 and 5).

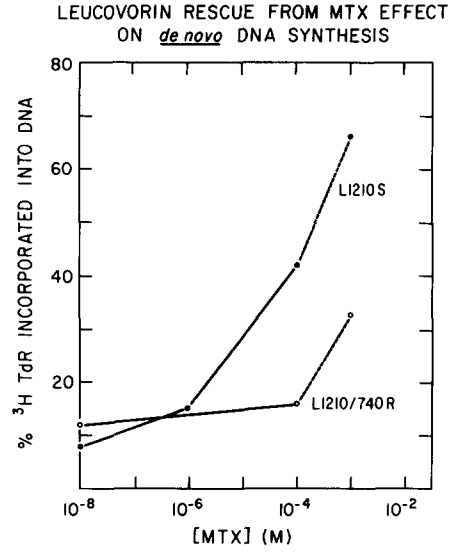

Fig. 3. LV rescue from MTX in L1210S and L1210/740R as measured by the UdR suppression assay. 0.1 μM UdR, 10^{-4} M LV added/ml. (----)=range where incorporation of ^{3}H-thymidine is inhibited directly by MTX.

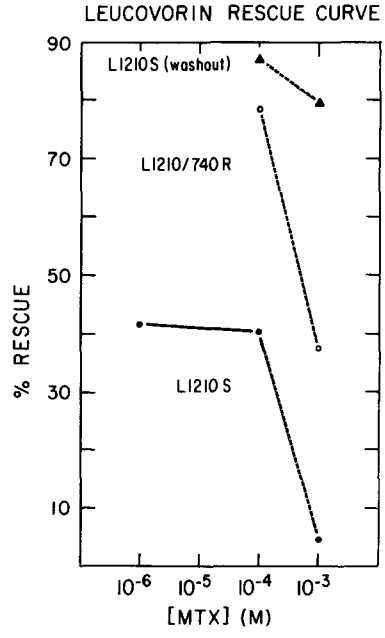

Fig. 4. LV rescue curve in L1210S and L1210/740R as measured by UdR suppression assay. 0.1 μM UdR, 10^{-4} LV added/ml. (----)=range where ^{3}H-thymidine incorporation into DNA is inhibited directly by MTX.

DISCUSSION

High dose MTX produced only a small inhibition of *de novo* DNA synthesis when resistance to MTX appeared to be due to a

twenty-fold increase in the concentration of dihydrofolate reductase. This occurred in spite of a modest net increase in uptake of MTX by the L1210/740R line. The inhibition of *de novo* DNA synthesis in L1210/740R was less than that produced by 10–100-fold lower concentrations of MTX in the L1210/S line.

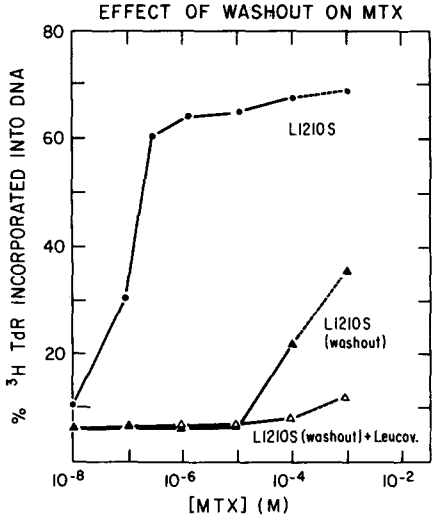

Fig. 5. Effect of washout of MTX in L1210S prior to addition of LV or measurement by the UdR suppression assay.

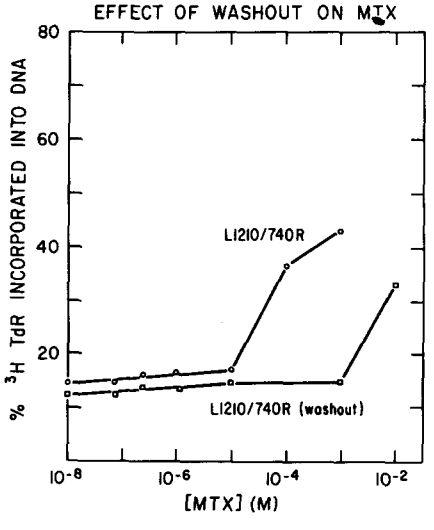

Fig. 6. Effect of washout of MTX in L1210/740R prior to measurement by the UdR suppression assay.

There is more than a proportional increase in concentration of MTX required for effective inhibition of *de novo* DNA synthesis as the cell increases its concentration of dihydrofolate reductase [10]. *More than* double the original amount of intracellular drug is required to inhibit a two-fold increase in enzyme. Experiments reported herein demonstrate this increased requirement for MTX can be further geometrically increased beyond clinically achievable concentrations when DHFR is 20 times normal as in the L1210/740R cell line. The degree of inhibition is less than predicted on theoretical grounds [10]. The degree of increases in the level of dihydrofolate reductase may determine if any clinically achievable concentration of MTX can effectively overcome resistance [11, 12].

Our tests demonstrate two additional reasons why high-dose MTX may not effectively overcome resistance due to elevations of dihydrofolate reductase. When the dihydrofolate reductase was twenty times greater than normal, both protection and rescue from MTX by LV was greater in the L1210R/740R resistant line than the L1210S normal line. Similarly, washing the cells in MTX-free medium also easily reversed inhibition of *de novo* DNA synthesis by high-dose MTX in the L1210/740R. In contrast, washing failed to completely reverse inhibition of L1210S at the high-dose concentration of MTX 10^{-3} of 10^{-4} M. Thus, these pharmacological variables (concentration, LV rescue or washout and rescue) fail to demonstrate the effectiveness of high-dose MTX schedules against such resistant lines. These characteristics would predict limited therapeutic effectiveness for MTX in tissue with marked elevations of dihydrofolate reductase.

The failure of LV to totally reverse the effect of MTX in L1210/S suggests, in contrast to the rescue of L1210/R, that the extracellular concentrations of MTX and LV cannot be the only factors preventing correction of the DNA defect in the L1210/S line by LV. Intracellular factors must also be critical because the extracellular concentrations of both MTX and LV were the same in experiments with L1210/S and L1210/740R. When the dihydrofolate reductase or high affinity sites were greater than normal, as in L1210/740R, correction of *de novo* DNA synthesis by LV was facilitated.

Free or low affinity bound MTX [13] may be largely responsible for the suppression of *de novo* DNA synthesis by high-dose MTX in both L1210/S and L1210/740R lines. Removing free or loosely bound MTX by washing the cells eliminates the inhibition except in the sensitive line at the high-dose MTX concentration of 10^{-3} or 10^{-4} M. This remaining inhibition is reversed by LV. In contrast, LV is unable to eliminate the effect of MTX on

the postulated low affinity sites as evidenced by the requirement for washing before LV effects "rescue". Therefore, LV appears to correct the defect in *de novo* DNA synthesis associated with MTX binding at the high affinity sites. These washout experiments suggest that the low affinity site can be important in the selective efficacy of high-dose MTX therapy for some cells.

The observation that high-dose MTX sometimes inhibits ^{3}H-thymidine incorporation may represent an additional parameter for comparing the effect of high-dose MTX on different cells. Low-dose MTX normally increases ^{3}H-thymidine incorporation into DNA. The inhibition of ^{3}H-thymidine incorporation by high-dose MTX has been observed by others in normal tissue [14] and may indirectly reflect an antipurine effect [15].

The *in vitro* tests may improve the selection of tumors for high-dose MTX therapy. These current tests measure a series of pharmacological and quantitative parameters. In contrast, earlier *in vitro* predictive tests which measured only a single parameter using the suppression of deoxyuridine, provided a partial but not totally reliable correlation with clinical sensitivity to MTX [16–18]. The availability of related, defined genetic mutants, which have many biochemical similarities also define the cellular factors which determine sensitivity or resistance to MTX.

Similar experiments have been conducted in this laboratory using an MTX *transport-resistant* Friend leukemia cell line [19]. High-dose MTX and LV is effective in overcoming transport resistance in contrast to the high dihydrofolate reductase type of resistance. Other antifolates, such as diaminopyrimidines which utilize different mechanisms of entering the cell, may also overcome transport resistance [20]. Thus, resistance due to dihydrofolate reductase may be a biochemical contraindication to effective therapy with MTX or other antifolates [21]. Methods are available for histochemical determination of dihydrofolate reductase levels [22], but these have not yet had an adequate clinical trial. Measurement of dihydrofolate reductase in tumor tissue prior to treatment may eliminate the cells which will not benefit from treatment with MTX or other antifolates.

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