

Alteration of Methotrexate Uptake in Human Leukemia Cells by Other Agents

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SUMMARY

The uptake of methotrexate (MTX) and the effect of drugs known to either inhibit or enhance MTX transport in L1210 murine leukemia were studied in man using blast cells from patients with acute myelogenous leukemia *in vitro*.

MTX uptake was found to proceed slowly, requiring at least 160 min for cells to reach a "steady state" when extracellular MTX concentrations were 1 μ M. Efflux of MTX from preloaded cells required 80 to 120 min and the nonexchangeable or tightly bound fraction was 40% of the total intracellular drug.

Utilizing doses that are estimates of achievable peak blood levels following single i.v. injection, cephalothin (21 μ g/ml) and hydrocortisone (20 μ g/ml) inhibited net MTX accumulation by 20 and 28%, respectively. Vincristine sulfate at 8.3 and 0.083 μ g/ml enhanced MTX uptake by 54 and 33%, respectively, by inhibiting MTX efflux, thus increasing the level of intracellular drug in excess of the tightly bound fraction. The potential clinical implications of using MTX in combination with the aforementioned drugs for cancer chemotherapy are discussed.

INTRODUCTION

MTX¹ has been used both as a single agent and in the combination chemotherapy of the acute leukemias (1, 2, 12). In addition, patients being treated with MTX often receive other pharmacologically active compounds concomitantly. The administration of other "drugs" has been shown to both inhibit and enhance the cellular uptake of MTX in L1210 murine leukemia (15); however, similar studies in human leukemia are lacking.

To examine this, VCR, the most effective enhancer of MTX uptake in L1210 leukemia cells, was chosen for study, as were KEF and HC, 2 of the most effective inhibitors. Inhibition or enhancement of a similar degree to that in L1210 leukemia cells were also found by *in vitro* studies of human AML blast cells.

¹ The abbreviations used are: MTX, methotrexate; VCR, vincristine sulfate; KEF, sodium cephalothin; HC, hydrocortisone sodium succinate; AML, acute myelogenous leukemia; [MTX]_e, extracellular methotrexate concentration; [MTX]_i, intracellular methotrexate concentration.

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MATERIALS AND METHODS

Chemicals. [3',5'-³H]MTX was obtained from Amer-sham/Searle Corp., Arlington Heights, Ill., or Dhom, North Hollywood, Calif. Purification was carried out as previously described (7) on a DEAE-cellulose column using linear gradient elution with ammonium bicarbonate buffer. The KEF, HC, and VCR used in these experiments were all the commercially available clinical preparations.

Cells and Media. Leukemic cells were obtained from 7 patients with AML in relapse whose peripheral blood contained greater than 90% blast cells. None of the patients had received any chemotherapy within 2 weeks prior to study nor had any patient received MTX at any time during their illness. Peripheral blood was aspirated into plastic syringes containing no anticoagulant and immediately expelled into 10 volumes of 4° bicarbonate-buffered 0.85% NaCl solution adjusted to pH 7.4. The cells were then collected by centrifugation at 750 \times g for 5 min at 4°. The cell pellet was resuspended in 4° bicarbonate-buffered 0.85% NaCl solution and freed of erythrocytes by a 60-sec hypotonic lysis. After lysis, the leukocytes were washed twice in 4° bicarbonate-buffered 0.85% NaCl solution and concentrated by centrifugation at 400 \times g for 5 min. The slower centrifuge speed tended to prevent cell clumping; however, if clumps did occur the suspension was then filtered through 2 layers of silk filter cloth (average pore size, 0.04 sq. mm; Joymar Scientific, New York, N.Y.). The white cell pellet was then resuspended in 4° Eagle's minimum essential medium without serum or folic acid and viability assessed by trypan blue exclusion. All preparations had greater than 90% viability by this technique. Smears of the cell suspensions were also prepared at this time using a Cyto-Centrifuge (Shandon Southern Instruments, Inc., Sewickley, Pa.) from which differential cell counts were made. Cell suspensions were kept at 4° until ready for use; this interval never exceeded 3 hr and viability was always reassessed before use.

Incubation Techniques. The cell suspension was adjusted to a cell count of 3 to 4 \times 10⁷/ml, and a volume of suspension was added to a like volume of Eagle's medium containing the test drug at twice the desired concentration. The addition thus resulted in a cell suspension of 1 to 2 \times 10⁷/ml bathed in experimental drug at the desired concentration, or no drug as in control studies. The concentrations of drugs used were estimates of levels achievable in man following single i.v. doses (*i.e.*, KEF, 21 μ g/ml; HC, 20 μ g/ml; and

VCR, 0.083 $\mu\text{g/ml}$) with the exception of VCR at 8.3 $\mu\text{g/ml}$ which is not clinically achievable. The cell suspension was then placed in a glass incubation flask suspended in a water bath at 37° and continuously agitated by a mechanical stirrer. A stream of warmed, humidified 95% O_2 -5% CO_2 was passed over the incubation mixture, and the mixture was preincubated for 10 min prior to the addition of [^3H]MTX. During the 160-min incubation period, the pH of the medium remained stable and cell clumping was negligible.

Measurement of MTX Uptakes. An uptake experiment was begun by the addition of sufficient [^3H]MTX to the cell suspension to achieve a final MTX concentration of 1×10^{-6} M. Serial samples (2 to 4 ml each) were then withdrawn at designated time intervals and expelled into 13-ml centrifuge tubes, and the cell pellets were rapidly isolated by centrifugation and aspiration of the supernatant material, an aliquot of which was saved for determination of $[\text{MTX}]_e$. The pellet was washed twice with ice-cold (2–4°) 0.85% NaCl solution to remove extracellular MTX. Studies to follow suggest that less than 5% of the [^3H]MTX was lost from the cells during this procedure, a result similar to the observations of Goldman *et al.* (7) and Kessel and Hall (9). The resulting cell pellet was drawn up into the tip of a Pasteur pipet and gently extruded onto a flexible polyethylene disc. The cell pellet was then dried overnight at 70° to constant weight, removed from the oven, immediately separated from the disc, and weighted on a Cahn RG autoelectrobalance with a digital readout. Correction for weight increase during exposure to room temperature air was done by serial weight determinations and interpolation to the time of removal from the oven. Dry pellet weights ranged from 0.5 to 3.0 mg. The pellet was then placed in the bottom of a scintillation vial and digested in 1 N KOH at 70° for 1 hr. After cooling to room temperature, 18 ml of a methanol-toluene scintillation fluid (700 ml of toluene, 300 ml of methanol, 3 g of PPO, and 100 mg of POPOP) were added and the vials were counted in a Beckman LSC-230 liquid scintillation counter. The tritium counting efficiency was 21% and the quench variation between samples was negligible.

Measurement of MTX Efflux. Efflux experiments at 37° were performed by preloading the AML cells with [^3H]MTX for 120 min, when cells were approaching a steady state. The cells were then centrifuged and washed 2 times in 4° Eagle's medium and an aliquot of the cold, washed, packed cells removed for the preflux determination. The cell pellet was then immediately resuspended in 36 ml of 4 or 37° MTX-free Eagle's medium. Efflux studies in the presence of VCR were performed in 1×10^{-5} M VCR solutions at 37° following 120-min incubations. Samples were removed at designated intervals and processed as described above, with 1 additional washing.

Efflux studies at 4° were performed on 3 patients to confirm the observations already reported for L1210 murine leukemia (7) and for human leukocytes (9). Incubations carried out to 120 min reveal less than 5% loss of intracellular MTX during 4° incubations when compared to preflux determinations. Such data suggest that washing

the blast cells at 4° does not result in any appreciable loss of intracellular MTX.

Identification of Accumulated Material. To determine the extent of cell metabolism of [^3H]MTX during the longest experimental procedure, suspensions of AML cells were incubated with 1 μM [^3H]MTX for 160 min. The cell fraction was separated by centrifugation, washed twice with ice-cold 0.85% NaCl solution, and resuspended in 0.1 M ammonium bicarbonate. The cells were then freeze-thawed 3 times and centrifuged at $9,000 \times g$ for 20 min at 4° to remove cell debris. The sediment was washed once with 0.1 M ammonium bicarbonate and this material added to the initial supernatant following recentrifugation. Following the addition of a small amount of purified, nonradioactive MTX to serve as a carrier, the supernatant fluid was fractionated on a DEAE-cellulose column as previously described. By this technique, more than 95% of the [^3H]MTX was found in the MTX peak. Less than 1% of the total radioactivity was present in the $9,000 \times g$ sediment. This observation suggests that metabolism of the [^3H]MTX was negligible during the experimental periods in this study.

RESULTS

Uptake of [^3H]MTX by AML Blast Cells. The time course of [^3H]MTX uptake by AML cells was measured in replicate experiments in 7 patients with duplicate determinations at each time point. $[\text{MTX}]_e$ ranged from 0.87 to 1.12 μM . Cellular MTX is used to define the total intracellular drug including both "nonexchangeable" and "exchangeable" fractions (7). The nonexchangeable fraction is presumably bound to the enzyme, dihydrofolate reductase, although tight binding to other intracellular sites has not been excluded. The time points recorded were chosen after preliminary experiments suggested them to be optimal for purposes of this study. The uptake of MTX proceeds rapidly over 80 min following which cells begin to reach a steady state (Chart 1). The change in intracellular drug level from 80 to 160 min is only 10% of the steady state value. Further incubation from 160 to 240 min showed no significant

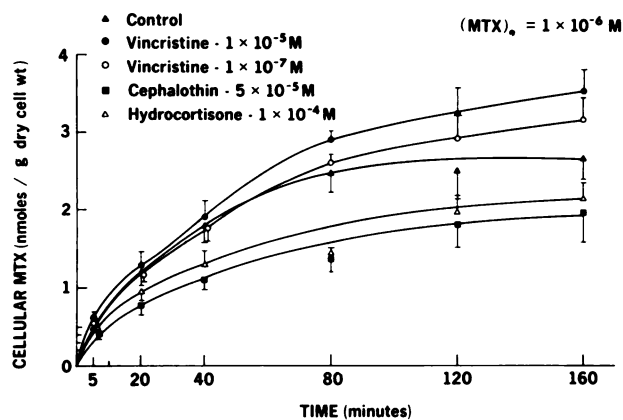


Chart 1. Effect of chemotherapeutic agents on MTX uptake in AML blast cells *in vitro*. Points, mean \pm S.E. of at least 4 experiments.

increase in intracellular MTX, but after 240 min there was a fall in cell viability.

Using the ratio of intracellular water to cell dry weight as determined by Goldman *et al.* (7) for L1210 murine leukemia and subtracting 40% of intracellular drug that constitutes the tightly bound, nonexchangeable fraction (see chart 3), a determination of $[MTX]_i/[MTX]_e$ is possible. When $[MTX]_e$ is $1 \mu M$ then the ratio is 0.42 at steady state.

Inhibition of $[^3H]$ MTX Uptake in AML Blast Cells. The

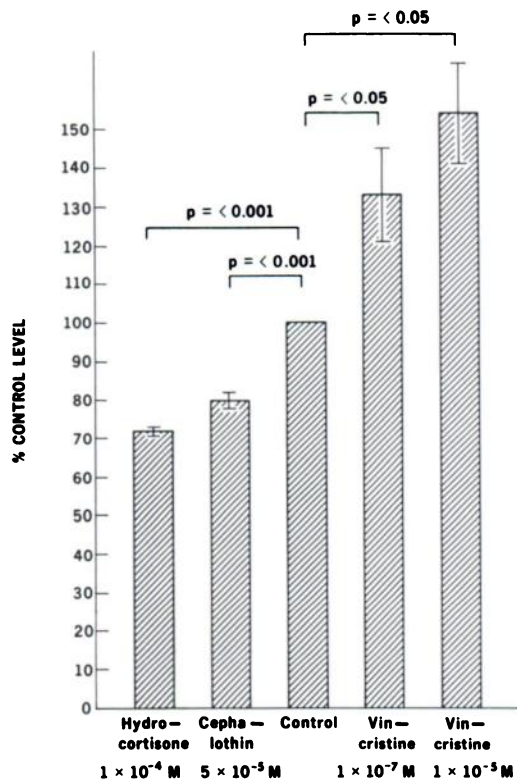


Chart 2. Inhibition and enhancement of MTX uptake by chemotherapeutic agents in AML blast cells *in vitro*. Shaded area, mean percentage \pm S.E. of the control level of MTX taken up by AML cells in the presence of each "drug" at the 160-min or steady-state point. *p* values are determined by paired *t* tests analysis.

effects of KEF on $[^3H]$ MTX uptake were studied in 5 patients at a MTX concentration of $1 \mu M$ (range, 0.93 to $1.08 \mu M$). The time course of MTX uptake in the presence and absence of a number of agents is indicated in Chart 1. The effect of these agents on total intracellular MTX was quantitated by taking the percentage of difference between control and treated cells at 160 min and subjecting these values to a paired *t* test analysis, as illustrated in Chart 2. KEF inhibited MTX uptake by 20% ($p < 0.001$). In 4 patients HC inhibited MTX uptake by 28% ($p < 0.001$).

Enhancement of $[^3H]$ MTX Uptake in AML Blast Cells.

The effects of VCR on $[^3H]$ MTX uptake were studied at 2 different VCR concentrations (1×10^{-6} and $1 \times 10^{-7} M$) in 5 patients. The uptake curve for both concentrations may be found in Chart 1. An enhancement of total intracellular MTX of 54% ($p < 0.05$) and of 33% ($p < 0.05$) was noted, respectively. The uptake and subsequent efflux of MTX in the presence and absence of VCR were studied in 3 patients (Chart 3). Similar to the report by Fyfe and Goldman (3) in Ehrlich ascites tumor cells, there was no change in the initial uptake phase, but the initial efflux of radiolabel was significantly slower in the presence of VCR. With continued incubation the intracellular level of MTX in VCR-incubated cells tended to approach that of control, but due to decreased cell viability with continued incubation, an equivalence point was never reached. However, if VCR-incubated cells were washed free of VCR and resuspended in MTX- and VCR-free media, rapid efflux of MTX occurred so that a control level was reached by 80 to 100 min.

DISCUSSION

MTX transport in human leukocytes is compatible with a carrier-mediated process (8, 9). Similarly, studies in leukemic blast cells (10) suggest that MTX uptake is nonconcentrative. Although a distribution ratio for exchangeable MTX of 0.42 was determined for AML cells in this report, it is not certain whether this reflects an energy-dependent process since the electrochemical potential for intracellular MTX was not established.

The data indicate that VCR at 0.1 and $10 \mu M$ enhances the transport of MTX, increasing MTX distribution ratios to

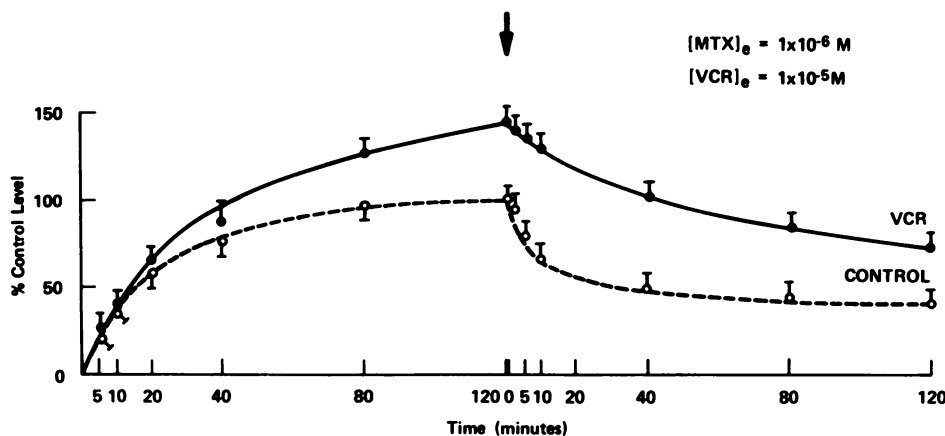


Chart 3. MTX uptake and efflux in the presence and absence of VCR. AML cells were incubated in the presence of MTX and VCR (VCR curve) or MTX alone (control curve) at 37° . After 120 min (bold arrow), cells were washed twice and resuspended in medium with or without VCR, respectively, and the efflux curves plotted. Points, mean \pm S.E. of 3 experiments.

0.51 and 0.57, respectively. Conversely, HC and KEF inhibit the uptake of MTX and diminish the distribution ratios to 0.32 and 0.35, respectively. The mechanism of the VCR-induced augmentation of MTX uptake has been related to inhibition of an energy-dependent efflux "pump" (3) and/or enhanced binding of MTX to low-affinity intracellular binding sites (6). We have demonstrated that VCR has a similar effect on MTX efflux from human leukemia cells, but cannot comment on its possible alteration of low affinity intracellular binding. However, VCR does not influence the number of high affinity binding sites in that VCR-incubated cells efflux MTX down to control levels once extracellular, and presumably intracellular, VCR has been removed. The effect of VCR on the affinity of MTX for these high affinity sites was not examined.

The mechanism by which HC and KEF inhibit MTX uptake in AML cells is unclear. As both drugs are the sodium salts of weak organic acids, they exist as anions in aqueous solution similar to MTX. Other anions, notably nitrate, sulfate, pyruvate, and organic phosphates, inhibit the influx of MTX in L1210 leukemia cells and Ehrlich ascites tumor cells and reduce the steady-state distribution ratio (4). It has been suggested that anions may compete with MTX for a carrier binding site and thus act as competitive inhibitors for uptake (4); however, additional studies are needed before unequivocal competition can be demonstrated.

The ability of these drugs to influence the MTX distribution ratio may influence the cytotoxic effect of the antifolate. It has been demonstrated that increasing intracellular MTX in excess of the tightly bound fraction enhances the therapeutic efficacy of MTX in L-cell mouse fibroblasts (5) and in Ehrlich ascites tumor cells (6). It appears that this component of intracellular MTX is necessary for effective inhibition of DNA, RNA, and protein synthesis in these cell types. Further, studies by Zager *et al.* (15) have demonstrated that VCR enhances net MTX uptake in L1210 leukemia cells *in vitro* and that pretreating mice bearing L1210 ascites tumor with VCR prior to administering MTX prolongs their survival. It would seem likely that these *in vitro* observations may represent the basis for the demonstrated synergism between MTX and VCR *in vivo*. Recent studies by Sirotnak *et al.* (13) suggest an alternate explanation, but they will require confirmation and further clarification. Since VCR does not appear to alter the influx of MTX or the enzyme-MTX interaction, it seems likely that the ability of VCR to influence MTX cytotoxicity is related to the increase in the level of intracellular MTX in excess of the tightly bound fraction that is induced by this agent. A further understanding of the nature of the interaction between VCR and MTX may help to exploit the potential clinical advantages of this drug combination.

Conversely, the transport inhibitors, HC and KEF, might limit the chemotherapeutic efficacy of MTX by reducing the rate of influx to such an extent that saturation of high-affinity intracellular binding sites does not occur during the period of exposure to MTX, and/or reducing the intracellular MTX level in excess of the tightly bound fraction

following saturation of the high-affinity binding sites. The possible effect of these drugs on the cytotoxic activity of MTX that may accrue from their decreasing $[MTX]_i$ remains to be evaluated. Based upon the increased time required for AML blast cells to reach a steady state as compared to animal tumors previously studied, the data suggest that human leukemia cells are less permeable to MTX. Hence, during therapy, plasma levels of MTX may need to be maintained at desired concentrations for several hr to ensure maximum uptake by neoplastic cells and saturation of high-affinity binding sites.

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