The Effect of Vincristine on Methotrexate Uptake and Inhibition of DNA Synthesis by Human Lymphoblastoid Cells

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SUMMARY

Vincristine (VCR) has been shown to enhance the transport of methotrexate (MTX) in animal systems and in acute myelogenous leukemia cells in vitro. In this report, the uptake of MTX and the effect of VCR were studied in two human lymphoblastoid cell lines grown in tissue culture. The effect of VCR on MTX suppression of deoxyuridine incorporation into DNA by these cell lines was also investigated.

The data indicate that an extracellular VCR concentration achievable following conventional single i.v. doses used clinically (0.1 μM) failed to enhance total intracellular MTX accumulation in either cell line at either conventional (1 μM) or “high-dose” (100 μM) extracellular MTX concentrations. An extracellular VCR concentration not achievable clinically (10 μM) did enhance total intracellular MTX at both MTX concentrations in both cell lines. However, neither VCR concentration (0.1 or 10 μM) enhanced the ability of MTX to suppress the incorporation of deoxyuridine into DNA, thought to be of vital importance to the antitumor effect of MTX. However, maximal suppression of deoxyuridine incorporation into DNA by MTX alone may have precluded detection of a VCR effect.

The results suggest that individual tumor thresholds exist for the MTX-VCR interaction and suggest that the empiric addition of VCR to clinical trials MTX, especially in the high-dose protocols currently being investigated, may be unwarranted. Further studies with a variety of human tumors are indicated to define more clearly the MTX-VCR interaction and its applicability to clinical trials.

INTRODUCTION

The transmembrane movement of MTX² and other naturally occurring folates has been investigated in a number of mammalian cells, and studies suggest that the intracellular accumulation of MTX occurs via a carrier-mediated transport mechanism (13, 16, 17). Cellular uptake of MTX may be altered by other folates, especially folic acid, by organic anions, such as cephalothin and hydrocortisone, and by a variety of other drugs, including other antineoplastic agents, particularly VCR (2, 3, 9, 19). VCR has been shown to augment the intracellular accumulation of MTX in human myeloblast cells in vitro at an extracellular MTX concentration of 1.0 μM with VCR concentrations of 10.0 and 0.1 μM. Furthermore, recent work suggests that 10.0 μM augments intracellular MTX in Ehrlich ascites tumor cells at concentrations achieved by high-dose MTX protocols (12).

Because of a limited number of studies on MTX transport in human tumor cells and recent reports suggesting efficacy of MTX doses which result in plasma concentrations of 100 μM or greater, this study was undertaken to determine the possible effects of VCR on MTX transport at conventional and high dose levels, using human lymphoblast cells grown in tissue culture. The effect of VCR on the MTX-induced blockade of UdR incorporation into DNA was also investigated.

MATERIALS AND METHODS

Chemicals. [3',5',9(n)-3H]MTX was obtained from Amersham/Searle Corp., Arlington Heights, Ill. Purification was carried out as previously described (14) on a DEAE-cellulose column with the use of a linear gradient elution with ammonium bicarbonate buffer. The final specific activity was 0.27 mCi/μmole. VCR and “cold” MTX were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, Md. [6-3H]UdR with a specific activity of 23 Ci/μmole was obtained from Amersham/Searle Corp.

Cells and Media. Two human lymphoblast cell lines were obtained from the H.E.M. Research, Inc., Rockville, Md. One of these, known as CEM, is a T-cell lymphoblast (8), while the other, known as NC-37, is a B-cell lymphoblast (7). The cells were grown in Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal calf serum and were harvested at a density of 1 to 2 × 10⁶ cells/ml. Cells were collected by centrifugation at 750 × g for 5 min, and the final cell pellet was resuspended at a cell density of 1 to 2 × 10⁶ cells/ml in 4° Eagle’s minimal essential medium without serum or folates. Viability was assessed by trypan blue exclusion. All preparations had greater than 95% viability by this technique.

Incubation Techniques. The cell suspensions were placed in glass incubation flasks immersed in a 37° water bath. VCR was added to bring the final concentrations to either 10 or 0.1 μM, while simultaneous control flasks contained cells suspended in media alone. Suspensions were continuously agitated by a mechanical stirrer. A stream of

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² The abbreviations used are: MTX, methotrexate; VCR, vincristine; UdR, deoxyuridine; TCA, trichloroacetic acid; [MTX]L, extracellular methotrexate concentration; [MTX], intracellular methotrexate concentration.

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warm, humidified 95% O₂-5% CO₂ was continuously passed over the cell suspension, and the mixture was preincubated for 5 min prior to the addition of [³H]MTX. During an experimental period of up to 180 min, the pH of the medium remained stable, cell clumping was negligible, and viability remained at greater than 90%.

**Measurement of MTX Uptake.** The method of determining MTX uptake has been previously described in detail (2). Sufficient [³H]MTX was added to achieve a concentration of either 1 or 100 μM for each respective VCR concentration and for each of the 2 cell lines studied. Simultaneous controls containing MTX alone were always included with each experiment. Incubation was carried out for 1 to 80 min, samples (2 to 4 ml each) were withdrawn at regular intervals, and uptake was terminated by rapid centrifugation and two 4° 0.85% NaCl solution washes to remove extracellular MTX. The resulting cell pellet was drawn up into the tip of a Pasteur pipet and gently extruded onto a polyethylene tare. After overnight drying at 70°, the cell pellet was weighed on a Cahn RG autoelectrobalance with digital readout (Cahn Instruments, Inc., Paramount, Calif.). Pellets were 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 (2) were added and the vials were counted in a Sequin Mark III liquid scintillation system (Searle Analytic, Inc., Chicago, Ill.). The tritium counting efficiency was 26% and the quench variation between samples was negligible. Results were expressed as nmoles of MTX per g, dry cell weight, of the cell pellet. Replicate experiments were performed on at least 3 different days with duplicate determinations at each experimental point.

**Measurement of MTX Efflux.** Efflux experiments at 37° were performed as previously described (2) by preloading the cells with [³H]MTX for 80 min to allow cells to reach a steady state. Following uptake, cells were centrifuged and washed in 4° Eagle’s medium, and an aliquot of the packed cells was removed for the preefflux determination. The cell pellet was then resuspended in a large volume of MTX-free Eagle’s medium at 37°. Samples were removed at designated time intervals and processed as described above, with 1 additional washing. The exchangeable intracellular MTX level was determined by subtracting the noneffluxable intracellular MTX level from the total intracellular MTX. The extracellular water content was determined as previously described (13) by measuring the [¹⁴C]inulin space and subtracting this from the difference between the wet and dry cell pellet weights. This allowed determination of an intracellular water to dry cell pellet weight ratio for each cell line at each MTX concentration which provides an estimate of intracellular volume (13).

**UdR Studies.** The incorporation of [³H]UdR into cellular DNA was measured for both cell lines by incubating cell suspensions with unlabeled MTX at concentrations of 0, 1, or 100 μM with or without VCR at 0.1 or 10 μM for 80 min to achieve steady state conditions. [³H]UdR was then added at a final concentration of 0.10 μM. Aliquots of the cell suspensions (2 ml) were then removed at regular intervals, and incubation was terminated by the addition of an equal volume of 4° 20% TCA. Following centrifugation, the supernatant was aspirated, and 2 additional washes with cold 5% TCA were performed. The washed cell pellets were drawn into Pasteur pipets, extruded onto polyethylene tares, and dried overnight as described above. Following processing similar to that described in "Measurement of MTX Uptake," scintillation counting was performed, and the results were expressed as dpm/mg, dry weight, of the TCA precipitate.

Preliminary studies determined that the incorporation of [³H]UdR was linear over the experimental period of 30 min for both cell lines with the use of a method previously reported (10). Furthermore, a comparison between the radioactivity incorporated into the TCA precipitate and that incorporated into DNA as determined by the perchlorate extraction method (10) confirmed previous reports (10) of the reliability of TCA precipitation as an indicator of deoxyuridine incorporation into DNA with greater than 90% of the tritium label recovered in the TCA precipitate incorporated into DNA.

**RESULTS**

**Uptake of [³H]MTX by Human Lymphoblast Cells.** The uptake of MTX proceeded rapidly over the 1st 20 min with a steady state reached by 60 min (Charts 1 and 2). Further incubation, from 60 to 80 min, showed no significant increase in the intracellular MTX levels for either of the cell lines studied. However, the total intracellular MTX accumulated at either 1 or 100 μM [MTX]ₚ was greater in the CEM cell line.

Using the ratio of intracellular water to cell dry weight (4.22 ± 0.29 and 3.98 ± 0.20 for CEM cells and 4.11 ± 0.12 and 3.92 ± 0.14 for NC-37 at 1.0 and 100 μM [MTX]ₚ, respectively) and subtracting the tightly bound, nonexchangeable fraction of drug, the distribution ratios [MTX]ₚ/[MTX]ₖ were determined. The tightly bound fraction was 4.3 nmoles/g, dry cell weight, at both 1.0 and 100 μM [MTX]ₚ when the CEM cell line was studied. The distribution ratios were, therefore, 1.45 and 0.109 for 1 and 100 μM [MTX]ₚ, respectively. For the NC-37 cell line, the tightly bound fraction was...
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Chart 2. The time course of MTX uptake for NC-37 lymphoblasts. Two extracellular MTX concentrations, 1 and 100 µM, were studied. Each point represents the mean ± S.E. of duplicate determinations on at least 3 different days.

3.9 and 4.2 nmoles/g dry cell weight at [MTX]e of 1 and 100 µM, respectively, resulting in distribution ratios of 1.30 and 0.15.

Enhancement of [3H]MTX Uptake by VCR. The effects of 0.10 and 10 µM VCR on [3H]MTX uptake were studied at 2 [MTX]e (i.e., 1 and 100 µM). The results are shown in Charts 3 and 4. For the CEM cell line, when VCR at 0.1 µM was included in the incubation mixture, no enhancement of MTX uptake was observed at either [MTX]e. When VCR at 10 µM concentration was included in the bathing medium, total intracellular MTX was enhanced by 25% at 1 µM [MTX]e and by 24% at a 100 µM [MTX]e. This enhancement was statistically significant with p values of <0.05 (Chart 3), as determined by paired t test analysis.

When the effect of VCR on MTX uptake was studied using NC-37 lymphoblasts, similar effects were observed (Chart 4). The inclusion of VCR at 0.1 µM failed to enhance MTX uptake for a [MTX]e of 1 or 100 µM. Statistically significant enhancements of total intracellular MTX of 15% for a [MTX]e of 1 µM and of 22% for a [MTX]e of 100 µM were observed when 10 µM VCR was included in the incubation mixture.

Effect of VCR on MTX Inhibition of [3H]UdR Incorporation into DNA. As mentioned above, [3H]UdR incorporation with time was linear for both cell lines studied. MTX alone at [MTX]e of 1 and 100 µM inhibited UdR incorporation by 58 and 93%, respectively, for the CEM cell line. For the NC-37 cell line, 59 and 86% inhibitions were noted for MTX alone at 1 and 100 µM [MTX]e, respectively. The effect of the inclusion of VCR on the suppression of [3H]UdR incorporation by MTX is demonstrated for the CEM cell line in Chart 5 and for the NC-37 cell line in Chart 6. No further enhancement of the ability of MTX to suppress [3H]UdR incorporation was noted for either VCR concentration in either cell line. For the CEM cell line, when 1 µM MTX was used, VCR addition at either 10 or 0.1 µM resulted in a 24 or 7% increase in [3H]UdR incorporation, compared with MTX controls. When 100 µM MTX was studied, a 10% increase in [3H]UdR incorporation was observed in the presence of 10 µM VCR, and a 4% increase for 0.1 µM VCR was noted. Similar results were seen for the NC-37 cell line, with 1 µM MTX and 10 or 0.1 µM VCR resulting in a 16 or 28% increase in [3H]UdR incorporation compared with MTX-alone controls, respectively. For 100 µM MTX, 98% of control values

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Chart 3. Enhancement of MTX uptake by VCR in CEM lymphoblasts. Two extracellular MTX concentrations and 2 extracellular VCR concentrations were studied. Shaded area, percentage (mean ± S.E.) of the control level of MTX taken up by CEM cells in the presence of each concentration of VCR at the 60-min or steady state point; p values are determined by paired t test analysis.

Chart 4. Enhancement of MTX uptake by VCR in NC-37 lymphoblasts. Two extracellular MTX concentrations and 2 extracellular VCR concentrations were studied. Shaded area, percentage (mean ± S.E.) of the control level of MTX taken up by NC-37 cells in the presence of each concentration of VCR at the 60-min or steady state point; p values are determined by paired t test analysis.
were observed for both 10 and 0.1 µM VCR. The p values were not statistically significant for any VCR-induced augmentation of [3H]UdR incorporation in either cell line. VCR-alone controls run simultaneously with the above-described experiments did not suppress [3H]UdR incorporation but rather enhanced it by 2% for the CEM cell line and by 17% for the NC-37 cell line at 10 µM VCR.

DISCUSSION

The effects of 2nd agents on the in vitro cellular transport of MTX were first described by Zager et al. (19) in 1973. At doses considered to be approximately comparable to achievable peak blood levels in man after conventional single i.v. doses, VCR and vinblastine at 10 µM concentrations enhanced intracellular MTX accumulation in L1210 leukemia cells by 66 and 49%, respectively. The VCR enhancement was still noted at concentrations as low as 0.001 µM where a 2.4% enhancement of MTX uptake was observed in their cell line. The inclusion of VCR did not alter the nonexchangeable level of MTX, implying no effect on the amount of MTX bound to dihydrofolate reductase. Fyfe and Goldman (9) further characterized the MTX-VCR interaction and found that 10 µM VCR slowed MTX efflux from Ehrlich ascites tumor cells, resulting in an increased level of exchangeable intracellular MTX. The vincristine effect was also correlated with increased inhibition of UdR incorporation into DNA (11), compared with MTX alone, an effect thought to be crucial to the antitumor activity of MTX. Subsequent studies have confirmed the effect of 10 µM VCR on net MTX accumulation at [MTX]₀ of 10, 100, or 250 µM in Ehrlich ascites cells (12). In vitro studies with acute myelogenous leukemia cells (2) revealed enhancement of intracellular MTX accumulation by 54 and 33% at VCR concentrations of 10 and 0.1 µM, respectively. Since the original observations that “high-dose” MTX produced responses in drug-refractory childhood leukemia (4, 5, 18), attempts have been made to lessen MTX toxicity by using folic acid “rescue” (1, 14). Studies suggesting VCR augmentation of the antitumor effect of MTX have generated uncontrolled clinical studies utilizing VCR in combination with high-dose MTX and folic acid (15).

Our data suggest that the total steady state intracellular MTX accumulated by the human lymphoblasts used in this study at a [MTX]₀ of both 1 µM (5 to 15 nmoles/g dry cell weight) and 100 µM (40 to 50 nmoles/g dry cell weight) is comparable to that achieved in human myeloblasts (2) and in Ehrlich ascites cells (12) at these respective concentrations. Furthermore, an exchangeable intracellular MTX concentration of ~11 µM at a [MTX]₀ of 100 µM is similar to a value of ~6.5 µM in Ehrlich ascites at a [MTX]₀ of 85 µM (12). The distribution ratios (i.e., the ratio of the intracellular MTX concentration to the extracellular MTX concentration) for these cell lines are also comparable to results reported in animal systems (10). The lack of enhancement of MTX uptake at a clinically achievable VCR concentration of 0.1 µM in human lymphoblastoid tumor cells may reflect individual tumor thresholds for the MTX-VCR interaction, since previous investigations in human myeloblasts report enhancement at this concentration (2).

The important question to be answered by any study reporting enhanced intracellular MTX accumulation is the effect on the antitumor activity of MTX. This is reflected in the ability of MTX to suppress [3H]UdR incorporation into
DNA. Goldman documented the ability of MTX to inhibit DNA synthesis in L-cell mouse fibroblasts (10) and subsequently correlated VCR-enhanced intracellular accumulation of MTX with enhanced suppression of DNA synthesis (11). He suggested that these data might justify the inclusion of VCR into MTX chemotherapy protocols. We found no enhanced suppression of [3H]UdR incorporation into DNA when VCR was included in experimental systems in either lymphoblastoid cell line at a [MTX] of 1 or 100 μM, despite a 15 to 25% increase in total intracellular MTX in the presence of 10 μM VCR. It is possible that 1 or 100 μM MTX produced a maximal suppression of UdR incorporation, thereby precluding detection of a VCR effect. If this was the case, then a lower MTX concentration might permit detection of a VCR effect on UdR incorporation. However, in an attempt to simulate the clinical setting, MTX concentrations of 1 and 100 μM were chosen. As these already maximally inhibit UdR incorporation into DNA, the relevance of a VCR effect might be questioned. Under these circumstances, it is unlikely that VCR could be expected to have a further beneficial cytotoxic effect.

In summary, our observations in 2 human lymphoblastoid cell lines failed to show enhancement of the intracellular accumulation of MTX when a clinically achievable concentration of VCR (i.e., 0.1 μM) was included in the incubation mixture. This was noted for either conventional (i.e., 1 μM) or high-dose (i.e., 100 μM) MTX concentrations. Furthermore, the augmentation of MTX uptake noted for 10 μM VCR in both cell lines at either MTX concentration did not correlate with enhanced suppression of DNA synthesis. These data strongly suggest the existence of individual tumor thresholds for the MTX-VCR interaction, and suggest that the empiric addition of VCR to clinical trials in which MTX is used may be unwarranted.

REFERENCES

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