Methotrexate Cytotoxicity for L5178Y/Asn^- Lymphoblasts: Relationship of Dose and Duration of Exposure to Tumor Cell Viability

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ABSTRACT

To determine the relative contribution of dose and duration of exposure to methotrexate (MTX) cytotoxicity, suspension cultures of L5178Y/Asn^- murine leukemic cells were exposed to 0.1 to 100 \( \mu M \) MTX for 3 to 42 hr. Viability was determined by cloning in soft agar. While there was a linear relationship between dose and MTX cytotoxicity for exposure durations of 3 and 6 hr \((r = -0.66)\), there was a pronounced flattening of this curve at exposure durations of 18 to 42 hr \((r = -0.45)\). Furthermore, there was an excellent correlation \((r = -0.85)\) between MTX cytotoxicity and durations of exposure for 6 to 42 hr (dose range, 1 to 100 \( \mu M \)). Using the linear least-squares method, a best-fit equation for the kinetics of MTX cytotoxicity was determined to be: log viability = 2.25 — 1.76 (log duration) — 0.31 (log dose). In practice, this equation predicts that a 1-log increase in duration of exposure results in almost a 2-log increase in cytotoxicity, whereas a 1-log increase in dose results in only a 0.3-log increase in cytotoxicity. The clinical utility of these data suggest that protracted infusions of lower doses of MTX would be equally as useful as or more useful than short-term high-dose infusions.

INTRODUCTION

Despite more than 30 years of research and clinical use, the optimal doses and schedules for MTX remain to be determined. Although the exact mechanism of cytotoxicity remains to be determined, the primary biochemical target appears to be DHFR. To maximally inhibit DHFR, intracellular MTX must be present in an osmotically active, unbound form. This requirement for unbound MTX may be necessary to ensure complete binding of MTX to DHFR (14), to compete with the natural substrate dihydrofolate for the enzyme (26), or to inhibit DHFR isozymes with a lesser affinity for MTX and other possible low-affinity sites (4–6, 33).

Clark (11, 12) recognized the relationship between pharmacological effect and the dose or concentration \( C \) and the duration of drug exposure or time \( t \) from which he formulated the concept that \( C^t \times t = k \). His work has subsequently been extended by others (31, 35, 38, 46, 48). Studies of cell cycle kinetics suggest that MTX is a cell cycle-dependent drug and that its toxicity varies with the proliferative status of the cell population (13, 24, 25). Thus, protracted exposure of tumor and normal cell populations to MTX is likely to result in an enhanced cytotoxic effect to either cell population.

Clinical trials with MTX have been conducted with doses ranging from mg to g (9, 15–17, 27, 28). In spite of this substantial increase in dosage, toxicity to normal organs can be minimized by terminating the effect of the antifolate with a suitable antidote (2). However, there appears to be a sharp cut-off for host toxicity relative to duration of drug exposure, both in the mouse (52, 53) and in humans (30).

In studies of the relative contribution of dose and duration of drug exposure to MTX toxicity, L5178Y/Asn^- lymphoblasts were exposed in vitro to concentrations of MTX achievable in various low-dose, moderate, and high-dose clinical protocols for exposure durations ranging from 3 to 42 hr.

MATERIALS AND METHODS

Drugs. MTX was obtained from Lederle Laboratories (Pearl River, N. Y.). Stock solutions of MTX (25 mg/ml) were stored at room temperature and were diluted immediately prior to use.

Cells. All experiments were performed with suspension cultures of L5178Y/Asn^- cells in exponential growth (approximately 2 to 5 x 10^6/ml). This line has a doubling time in vitro of approximately 12 hr. Cells were maintained in continuous exponential growth in Fisher's medium supplemented with L-asparagine and 10% horse serum.

Cloning. The viability of L5178Y/Asn^- cells following exposure to MTX was determined by the soft-agar cloning technique of Chu and Fischer (10). The average cloning efficiency for control cells was 58.3%, which was normalized to 100%. Each condition was cloned in triplicate, and each data point represented the mean of at least 2 experiments.

Statistical Analysis. The significance of the relationship between dose, duration of exposure, and tumor cell viability was determined with the aid of the SPSS and SAS statistical packages. Model fitting was performed using linear and weighted least squares to examine power and interaction terms.

RESULTS

Dose-Time Relationships for MTX Cytotoxicity. L5178Y murine leukemic cells were exposed to MTX concentrations ranging from 0.1 to 100 \( \mu M \) for 3 to 42 hr. Results are depicted in Chart 1. For short periods of drug exposure, i.e., 3 and 6 hr, there was a distinct dose-response relationship. However, with increasing duration of exposure, there was a maximal or ceiling dose beyond which a further increase in dose did not result in any further cytotoxicity. As the duration of drug exposure increased, the maximally effective dose shifted to the left, clustering around 1 \( \mu M \). Thus, exposure of the cells at 1 \( \mu M \) MTX for 36 or 42 hr was significantly more effective than shorter exposure periods of 3 or 6 hr at 100 \( \mu M \).

Dose Relationships for MTX Cytotoxicity. The contribution of dose effect to cytotoxicity is shown in Chart 2. Cytotoxicity was explored over a 100-fold dose range from 0.1 to 10 \( \mu M \) as a function of short-term exposure, i.e., 3 to 6 hr, or long-term
Effect of dose and duration of exposure to MIX on cytotoxicity to L5178Y cells. L5178Y cells were exposed to MTX for varying doses and durations of exposure. Following drug exposure, cells were washed free of drug and cloned in drug-free media plus agar with 15% horse serum. Each experiment was performed in triplicate and each data point represents at least 2 experiments.

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Chart 2. Dose-time relationships for cytotoxicity. Experimental points are identical to those in Chart 1.

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Best-Fit Equation for the Kinetics of MTX Cytotoxicity. Computer-generated models using linear least-squares analysis and weighted least-squares analysis were explored to analyze the dose-time interrelationships relative to MTX cytotoxicity. This analysis explored linear, log and power functions, and dose-time interactions. The simplest best-fit equation generated by the linear least-squares analysis over the entire range of dose and durations was:

\[ \log \text{viability} = 2.25 - 1.76 (\log \text{duration}) - 0.31 (\log \text{dose}) \]  

where \( F = 125.8; \ p < 0.001 \); and \( r^2 = 76\% \). In practice this equation predicts the following: a 1-log increase in duration of drug exposure results in almost a 2-log increase in cytotoxicity. Conversely, a 1-log increase in drug concentration results in only a 0.3-log increase in cytotoxicity.

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DISCUSSION

The relationship between concentration and duration of exposure in producing a biological effect has been the subject of numerous investigations. Clark (11, 12) quantified the relationship between concentration (C) and duration (t) of exposure in producing a biological effect by stating that \( C^n \times t = k \). Pittillo et al. (38) noted a similarity between bacterial and mammalian cell populations in their susceptibility to anticancer agents.

The induction of a folate-deficient state by MTX is clearly a dose-dependent process. Critical determinants include carrier-mediated transport (22), saturation and inhibition of DHFR (29), or other low-affinity sites (4, 5, 33). The maximal depletion of reduced folate cofactor pools is dependent upon the persistence of free intracellular MTX (20, 21) and the level of thymidylate synthetase activity (36). Dose-dependent cytotoxicity of MTX was investigated by Skipper et al. (47). They determined that MTX cytotoxicity followed first-order kinetics, i.e., the percentage of cell kill, rather than the reduction in absolute cell number was reasonably constant per unit dose. Furthermore, they established a direct proportionality between daily dose and cell kill. Therefore, they reasoned “the potential of a single-dose, high-level schedule is much superior to a chronic maximum daily schedule if one is seeking ‘cures’” (47). Together with the work of Goldin et al. (19), these data form the experimental basis of clinical high-dose MTX therapy with folinic acid “rescue.”

Theoretical and experimental considerations of
bolus or brief infusions of escalating doses of MTX include carrier-mediated transport processes that are not operational at lower drug concentrations (21, 50), inhibition of increased or altered DHFR (6), or increased plasma concentrations of MTX that may be needed to overcome blood-organ barriers such as the central nervous system (49). In consideration of these biochemical effects, high-dose MTX therapy has been tried and found to be of value in the treatment of refractory acute lymphoblastic leukemia (15, 16), lung cancer (17), head and neck cancer (9), and metastatic osteogenic sarcoma (27, 28).

In general, the therapeutic effect of MTX has been related to dose, while toxicity to normal organs has been related to duration of exposure. Intracellular metabolism of MTX to polyglutamate derivatives is receiving increasing attention, because this metabolite may relate to cellular retention of the drug and ultimately to its cytotoxicity. Polyglutamation of MTX appears to be dependent on the presence of free intracellular MTX (40). In this regard, the reaction is dose dependent (41), up to that concentration of MTX which maximally inhibits DHFR, beyond which the extent of polyglutamation is relatively dose independent (40). At this point, the duration of exposure may be of critical interest, in that the continued presence of MTX in the extracellular fluid has been related to the overall accumulation of intracellular polyglutamates (41) as well as to the ultimate chain length of the glutamate residues (40). With the decline of extracellular concentration of MTX, there is controversy regarding the contribution of polyglutamate derivatives to cellular retention of the drug, i.e., its efflux rate. In hepatic cells in culture, both normal and malignant, there is a considerable difference in the efflux rate of MTX compared to its polyglutamate derivatives (1). In contrast, in L1210 cells, the entire intracellular profile of MTX up to the tetr.glutamate appears to efflux at comparable rates (39).

It is evident that MTX exerts its cytotoxic effect in discrete portions of the cell cycle (24). HeLa cells are most sensitive to MTX in early G1, at the G1-S transition, and in mid-S phase (32, 42). Furthermore, it appears that MTX cytotoxicity varies with the proliferative status of the cell population (13, 25). Therefore, cytotoxicity is dependent upon passage of the cells through various “sensitive” phases of the cell cycle. While the mechanism of cytotoxicity has been linked to the induction and duration of a state of “unbalanced growth,” this phenomenon is poorly understood.

In addition to its inhibitory effect on thymidylate biosynthesis, MTX can also interfere with the synthesis of purines and glycine (23, 43, 45). These effects may be self-limiting by slowing cell cycle transit. Wilkoff et al. (51) have suggested that the extent to which MTX inhibits cell cycle transit into sensitive phases of the cell cycle is directly proportional to the deviation of MTX cytotoxicity from first-order kinetics. In other words, if the primary metabolic effect of MTX is to induce a thymineless state in a cell line with no effect on cell cycle transit, then MTX cytotoxicity would follow first-order kinetics. However, if MTX also exerts an effect on purine and/or glycine biosynthesis, such that the rate of entry into the sensitive portions of the cell cycle is not constant, then MTX cytotoxicity will deviate from first-order kinetics in a manner directly proportional to its effect on cell cycle transit.

The data in the present study indicate that duration of drug exposure is relatively more important than drug concentration in determining MTX cytotoxicity to L5178Y/Asn~ lymphoblasts in vitro. A linear regression analysis model predicts a 1-log increase in duration of drug exposure results in almost a 2-log increase in cytotoxicity, whereas a 1-log increase in drug concentration results in only a 0.3-log increase in cytotoxicity.

Other mathematical models dealing with MTX cytotoxicity have been published. Eichholtz and Trott (18), working with Chinese hamster fibroblasts, and Wilkoff et al. (51), working with L1210 cells, have published mathematical models describing MTX cytotoxicity. Eichholtz and Trott (18) demonstrated a varying relationship between concentration, duration of exposure, and survival fraction:

$$ \text{Survival fraction} = 1.5 - e^{-0.1t} \cdot c^{0.15 - 0.027t} $$

where $e$ is base of the natural logarithm; $c$ is MTX concentration (µg/ml); and $t$ is duration of exposure in hr.

The survival fraction has an exponential relation to duration of exposure and a power relation to drug concentration. This model demonstrates a predominant effect of duration of exposure over drug concentration, especially notable at longer durations. Using a Gompertz model, Wilkoff et al. (51) demonstrated that the surviving cell number ($N$) was related to an exponential function of drug concentration that varied across time.

$$ N = N_0 e^{-\frac{t}{\beta}} (1 - e^{-\frac{t}{\alpha}}) $$

where $N$ is number of viable cells present at time $t$; $N_0$ is number of viable cells present at time 0; $t$ is duration of drug exposure; $e$ is base of the natural logarithm; $\beta$ is rate of population reduction; and $\alpha$ is constant which expresses rate of change of $\beta$.

Thus, the model derived by fitting data to the Gompertz equation relates cell survival to changes in 2 parameters ($\alpha/\beta$) that vary by time. The relationship of $\alpha$ to $\beta$ thus tends to increase the contribution of dose at short intervals and, unlike the Eichholtz and present model, still allows for substantial dose contributions at longer exposures.

The correlation between observed and predicted cytotoxicity and dose versus duration of exposure is shown in Table 1. The most divergent observations tend to be in the model of Wilkoff et al. (51), where the predicted values generally underestimate the observed cytotoxicity. This may result from the approach to modeling chosen by the authors, since they fit a theoretical Gompertz model to their data rather than deriving a model, as was done in the other 2 studies. Although growth in cell systems may often follow a Gompertz model, there is little compelling evidence to suggest that cell kill in vitro or in vivo is necessarily inextricably linked to the model. In fact, for any individual set of data, a derived model may be more accurate.

The relative importance of duration of drug exposure in the present model and the one of Eichholtz and Trott (18) can be demonstrated by an examination of observed and predicted values in Table 1. A 5-fold change in dose (1 to 5 µM) at any given duration of exposure results in a relatively small increase in cytotoxicity, whereas a 4-fold increase in duration of exposure at 1 µM (6 to 24 hr) results in a 1.17-log increase in cytotoxicity in the present investigation and a 0.7-log increase in cytotoxicity in the investigation of Eichholtz and Trott (18). However, all 3 models have one characteristic in common. With increasing durations of exposure, the maximally effective dose
shifts to the left for all 3 cell lines, with a consequent flattening of the dose-response curve.

Analogous results have been obtained by other investigators. Sedwick et al. (44) report that the initial inhibitory effects of 5 μM MTX on deoxyuridine uptake and incorporation into DNA can be maintained by 0.1 μM MTX in human lymphoblasts and leukemic cells. Zaharko (52), Mellet (34), and Zaharko and can be maintained by 0.1 μM MTX in human lymphoblasts and may result in an improved therapeutic index for MTX.

The duration of drug exposure, however, must also be in mind, durations of drug exposure for 42 hr may be possible in turn is primarily affected by renal function. With these factors determined by factors referrable to normal organ toxicity, which some of these factors cannot be readily measured at present, concentration of MTX dependent upon tumor type; cellular trans

dose-independent cytotoxicity. However, with increasing duration of drug exposure, cell viability decreases.

The clinical utility of these data suggest that protracted infusions of MTX would be equally or more useful than short-term high-dose infusions. Bleyer et al. (3) have reported that low-dose protracted exposure to MTX was therapeutically equivalent to high-dose short-term therapy both for remission induction and for duration in patients with meningeval leukemia. Furthermore, protracted infusions of MTX were significantly less neurotoxic than were higher bolus doses of the drug.

Therefore, clinical protocols using protracted infusions of MTX that are equal or superior to bolus or brief infusions of escalating MTX doses could be designed using the following criteria: the achievement and maintenance of a plasma concentration of MTX dependent upon tumor type; cellular transport processes for MTX; and sensitivity of DHFR to MTX. While some of these factors cannot be readily measured at present, a plasma concentration of 1 to 10 μM should be sufficient to induce a folate-deficient state in MTX-sensitive tumors, with termination of drug effect by the administration of leucovorin (2). The duration of drug exposure, however, must also be determined by factors referrable to normal organ toxicity, which in turn is primarily affected by renal function. With these factors in mind, durations of drug exposure for 42 hr may be possible and may result in an improved therapeutic index for MTX.

REFERENCES


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