Relative Biochemical Aspects of Low and High Doses of Methotrexate in Mice

Daniel S. Zaharko, Wing-Pun Fung, and Kin-Hal Yang

Laboratory of Chemical Pharmacology, National Cancer Institute, NIH, Bethesda, Maryland 20014

SUMMARY

During low infusion rates of methotrexate (1.0 μg/hr/mouse; plateau plasma concentration, 2 × 10⁻⁸ M), [³H]deoxyuridine incorporation into DNA was inhibited to a significant degree in small intestine and femur marrows. However, incorporation of [³H]thymidine into intestinal DNA was stimulated at this low infusion rate. During high infusion rates of methotrexate (10 μg/hr/mouse; plateau plasma concentration, 4 × 10⁻⁷ M), inhibition of the incorporation of [³H]deoxyuridine at the steady state levels of plasma methotrexate in both the small intestine and femur marrow was significant. In contrast to stimulation at the low infusion rate, incorporation of [³H]thymidine into intestinal DNA at this high infusion rate was inhibited to a significant degree. Inhibition was not statistically significant in femur marrow DNA. The inhibition of [³H]thymidine into intestinal DNA could be reversed by the simultaneous infusion of thymine. Thus, in the in vivo system, an antipurine effect on DNA synthesis at high methotrexate plasma concentration in the small intestine was observed. This antipurine effect was not apparent at the lower concentrations. The lower concentration, however, could still inhibit [³H]deoxyuridine incorporation into intestinal and femur marrow DNA to a significant enough degree that, if prolonged, it would result in lethality to the mice. The thymineless state can be maintained for at most 60 hr in mice without lethal toxicity, whereas the antipurine state can be maintained for no longer than 18 hr in mice without some lethal toxicity. These data have important implications in rescue studies using thymidine or leucovorin.

INTRODUCTION

Several in vitro studies have indicated that MTX in some cell types has an antipurine effect as well as an inhibitory effect on de novo thymidylate and glycine synthesis (2, 4–6, 12). This antipurine effect, although it might be expected because of the known dependence of de novo purine synthesis on reduced folate coenzymes (1), is poorly understood in vivo for normal tissues where little is known about the biological significance of purine salvage (7). Furthermore, the successful use of thymidine rescue regimens to achieve a selective tumor response with MTX implies that the antipurine effect of MTX is unimportant in normal tissues, because salvage mechanisms compensate for the block of de novo synthesis (10, 11). It has been suggested that the antipurine effect is dependent on the concentration of MTX (2, 13). Since with single or multiple injections of this drug in vivo, the plasma concentrations change rapidly with time, we undertook to study the potential antipurine effects of MTX under conditions in which the plasma concentrations of MTX are relatively constant.

The purpose is to investigate quantitative biochemical effects of MTX action on the small intestine and femur marrow of mice to gain further understanding of this drug's differential action at low and high concentrations in different normal tissues.

MATERIALS AND METHODS

MTX (sodium salt) and dl-leucovorin (calcium salt) were obtained from the National Cancer Institute, Drug Development Branch, Bethesda, Md. Inosine, thymidine, and deoxyuridine were obtained from Sigma Chemical Co., St. Louis, Mo., and [³H]TdR (5 to 15 Ci/m mole) and [³H]UdR (15 to 30 Ci/m mole) were obtained from New England Nuclear, Boston, Mass.

Male BALB/c × DBA/2F₁ mice (hereafter called CD2F₁) were implanted s.c. with MTX infusion cells. These cells are able to infuse MTX at a constant rate and maintain plasma concentrations at several desired steady state levels as previously described (3, 9, 14). At set times after beginning the infusion of MTX, mice were given i.v. injections of [³H]UdR or [³H]TdR, 0.4 μg/g and 5 μCi/g of mouse, in a volume of 0.005 ml/g of sterile 0.9% NaCl solution. Twenty min later, the mice were sacrificed. Plasma and tissues were obtained and frozen in liquid nitrogen and assayed, respectively, for MTX and tritium content in DNA by methods described earlier (14). In these studies, 2 mice were sacrificed per time point. This enabled us to have a minimum number of 6 mice for statistical analysis during steady state plasma levels of MTX.

In those experiments in which inosine was infused, the infusion was at the rate of 2.6 mg/hr in a volume of 0.21 ml/hr of sterile 0.9% NaCl solution. This was accomplished with a Model 975 Harvard infusion pump and 5-ml syringe attached by polyethylene tubing to a round-headed stainless steel pin which was inserted s.c. on the back of the mouse and sutured firmly. The mice were then individually

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caged. In these experiments, all mice were sacrificed at one time point (24 hr), and a minimum of 6 mice per time point were used for statistical analysis. We chose a longer time period of infusion for this experiment because increased exposure time to MTX gives a more pronounced inhibitory effect with which to test our reversal hypothesis. All control animals were treated identically as experimental animals except they neither received a MTX infusion cell nor were they infused with inosine. Inosine was used rather than hypoxanthine as the purine source because of its greater solubility in water.

Thymidine and calcium dl-leucovorin were infused with a minipump inserted s.c. (ALZET; Alza Corp., Palo Alto, Calif.).

RESULTS

Results of the infusion of MTX at around 1.0 μg/hr/mouse are shown in Chart 1. The plasma concentration of MTX is maintained at a steady state 7 hr after the initiation of the infusion until the infusion is stopped at 48 hr; then the plasma MTX concentration drops rapidly. Tritium incorporation into DNA as a result of [3H]UdR injection at timed intervals is inhibited in the intestine during the steady-state period of plasma MTX (7 to 48 hr). Statistical analysis of these data with the Student t test from 7 to 48 hr (14 mice), compared with controls (14 mice) without MTX infusion, indicated a significant inhibition \( p \leq 0.01 \). In contrast, tritium incorporation into DNA as a result of [3H]TdR injection is stimulated above that of controls in the intestine during a similar 7- to 48-hr period, \( p \leq 0.01 \). For comparative purposes, simultaneously collected femur marrows show, during this 7- to 48-hr MTX plasma steady state, a significant inhibition of [3H]UdR incorporation, \( p \leq 0.01 \) but an insignificant change in [3H]TdR incorporation from controls, \( p \geq 0.85 \). During the time period after the infusion (48 to 96 hr), there is a significant overshoot above controls, \( p \leq 0.01 \), for both [3H]UdR and [3H]TdR in the femur.

With MTX infusions of 10 μg/hr (Chart 2), the plasma concentration is maintained at steady state from 5 to 17 hr, at which time the infusion is stopped. Tritium incorporation into DNA as a result of [3H]UdR injection is once again inhibited in the intestine and femur during the steady-state period of plasma MTX (5 to 17 hr), \( p < 0.01 \), 6 experimental mice versus 6 controls. In contrast to the results in Chart 1, tritium incorporation into DNA of intestine as a result of [3H]TdR injection is inhibited significantly compared to controls during the steady state period of plasma MTX, \( p < 0.01 \); simultaneously collected femur data indicate an insignificant difference of [3H]TdR incorporation compared to controls, \( p \geq 0.25 \), during the steady-state period, but after the 40-hr time period, there is a significant overshoot of both [3H]UdR and [3H]TdR incorporation into femur DNA, \( p \geq 0.01 \).

We repeated the experiment with [3H]TdR and MTX (10 μg/hr) infusion, but with an additional dose of MTX (25 mg/kg i.p.) at the beginning of the infusion. This raised the plasma MTX at 5 and 17 hr about an order of magnitude (actual values, 5 hr: 1.1 and 1.1 \( \times 10^{-6} \) M, 2 mice; and 17 hr: 1.3 and 0.6 \( \times 10^{-6} \) M, 2 mice) but had little effect on the plasma concentrations after cessation of infusion. The results of incorporation of [3H]TdR into DNA of both intestine and femur were statistically indistinguishable during the infusion phase from the previous experiment with MTX infusion (10 μg/hr), \( p \geq 0.85 \). These data support the contention that we have reached a maximum early effect of MTX on purine depletion and it is a matter of time of exposure that will manifest a more severe depletion of purines.

In Table 1, data indicate that the infusion of inosine simultaneously with MTX reverses the inhibition of [3H]TdR incorporation into DNA. Tritium incorporation into DNA returns to control levels in the small intestine and to levels substantially above control in the femur.

In Table 2, the toxicity data in mice by both weight and lethality are shown with time of MTX infusions at low and high infusion rates. It can be seen that a substantially longer time of infusion is required at MTX of 1 μg/hr than at 10 μg/hr in order to cause equivalent weight loss and lethality. The time is disproportionately longer than anticipated from the MTX plasma pharmacokinetics. The differences in infusion times required for equal toxicity at 1 and 10 μg/hr infusion rates cannot be explained simply by assuming that toxicity is proportional to the time of drug exposure above a minimum critical concentration. The higher infusion rate requires the lesser exposure time above a critical concentration of \( 2 \times 10^{-9} \) M.

In Table 3, the toxicity data in mice indicate that calcium leucovorin but not thymidine will reverse the toxicity caused by the infusion of MTX at 10 μg/hr for 48 hr. At a MTX infusion rate of 1 μg/hr, concurrent thymidine infusion at 50 μg/hr partially reverses the toxic effects of MTX.

DISCUSSION

Previous experiments in mice have also shown that with s.c. MTX infusions of around 1 μg/hr, plasma MTX concentrations of about \( 2 \times 10^{-6} \) M can be reached in 10 hr and then maintained at a steady state (14). Under these conditions, [3H]UdR incorporation into DNA of small intestine declines over a 10-hr period to a nadir of 10% of control. If the infusion is continued for longer than 100 hr, all the mice die, presumably a thymineless intestinal death, since, on autopsy, these mice are virtually devoid of intestinal mucosal villi (14). However, if the infusion is stopped at 48 hr, all the mice survive (13). The results in Chart 1 indicate that purines are not limiting under such conditions for the incorporation of thymidine into intestinal DNA. However, since [3H]UdR incorporation into DNA is almost completely inhibited, endogenous dUMP conversion to TMP must also be greatly inhibited because of the inhibition of dihydrofolate reductase and the depletion of reduced folate coenzymes necessary for thymidylate synthetase activity. Therefore, the size of the endogenous thymidylate pools must be smaller than normal. Under these conditions, when [3H]TdR is injected, there should be a stimulation of ¯H uptake into DNA above controls because of lesser dilution of the exogenously derived radioactive thymidylate products in the endogenous pools, if purine or cytidine nucleotides are not
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Chart 1. A, plasma MTX following infusion of 1 μg/hr for 48 hr; B, incorporation of 3H into DNA of small intestine following MTX infusion and [3H]TdR (●) or [3H]UdR (○) injection; C, same as B, but for femur marrow. Vertical standard error bars, range of the 2 mice used per point. At least 6 controls were used for each experiment. Two femurs and the 1st 10 cm of the small intestine were used from each mouse. The [3H]UdR values for femur beyond 60 hr remained above 300% of control.

rate limiting. This happens in the small intestine, but the femur marrow is less responsive, and stimulation is statistically insignificant at this MTX concentration and exposure time in mice.

When the infusion rate of MTX is increased to around 10 μg/hr, the plasma MTX concentration rises proportionally to around $4 \times 10^{-7}$ M. Under these conditions, the infusion cannot be maintained for longer than about 20 hr without the danger of losing some animals (Table 2). The plasma MTX concentrations shown in Charts 1 and 2 indicate that the higher infusion rate maintains a plasma MTX concentration above $2 \times 10^{-8}$ M for a time period no longer than the
lower infusion rate. We know that a plasma concentration of \(2 \times 10^{-8} \text{ M}\) MTX or below can be maintained for weeks in mice with no lethal toxicity (15), whereas a steady state plasma concentration of \(2 \times 10^{-8} \text{ M}\) cannot be maintained for much more than 60 hr without some subsequent lethality. Together, these observations suggest to us that a biological effect in addition to inhibition of thymidylate synthesis is contributing to some early event during the high infusion rate of MTX (10 \(\mu\)g/hr) not present during the same time interval at the low infusion rate (1 \(\mu\)g/hr). This event is obviously important, since, when it persists for a time period beyond 20 hr, it is lethal (Table 2). If it is absent but inhibition of \([^{3}H] UdR\) incorporation is still significant (e.g., at 1 \(\mu\)g/hr of MTX infusion, 90% inhibition occurs), this degree of DNA synthesis inhibition can be tolerated for time periods at least twice as long. One such biological effect is the inhibition of purine synthesis that would lead to various degrees of RNA and protein inhibition and that may result in irreversible toxicity earlier than if DNA synthesis alone were inhibited. If this is the case, when \([^{3}H] TdR\) is given, there
should be an inhibition of the incorporation of \(^3\)H into DNA because of purine limitations for DNA synthesis during the steady state plasma concentration of \(4 \times 10^{-7} \text{ M} \) MTX. Statistical analysis of the data in Chart 2 supports this hypothesis for the small intestine but not for the femur marrow. There is, however, a statistically significant (p < 0.05) overshoot phenomenon exhibited by the femur beyond 40 hr. Further study of this phenomenon has suggested that it is due to the recruitment of marrow cells into the cell cycle (8).

These data concerning tracer incorporation into intestinal DNA indirectly indicate that the purine nucleotide pools are depleted less drastically than the thymidylate pools \textit{in vivo} and require a substantially higher concentration of plasma MTX before this depletion begins to occur. When it does occur, rescue with thymidine infusions or injections would be highly inadequate to prevent toxicity. However, as can be seen in Table 3, simultaneous infusion of calcium leucovorin can prevent the toxicity resulting from infusions of MTX of \(10 \mu\text{g/hr}\).

If our explanation of the data to this point is correct, then an exogenous supply of purines provided during an MTX infusion of \(10 \mu\text{g/hr}\) should return \(^3\)H-TdR incorporation into DNA toward control values in the intestine. Results shown in Table 1 confirm this notion, as \(^3\)H-TdR incorporation into DNA is within control values for the small intestine when inosine is infused simultaneously with MTX. It is, however, significantly above control values for the femur marrow. These data for the femur marrow suggest that the rate of thymidine incorporation into DNA in the presence of MTX may be limited by the availability of endogenous salvage purines to the bone marrow. This explanation is in agreement with the suggestion of Murray (7) that bone marrow cell division depends primarily on the salvage of purines. Although we have shown (8) that marrow cells can form clones in an \textit{in vitro} system without a source of exogenous purines, which means they are capable of \textit{de novo} purine synthesis, perhaps their rate of division \textit{in vivo} under normal conditions is limited by the availability of purine nucleosides being delivered to the marrow by the blood stream.

These biochemical findings suggest that the toxic response of MTX \textit{in vivo} in CDF, mice at low concentrations (plasma around \(2 \times 10^{-6} \mu\text{g/hr}\) MTX) is due to a lack of thymidylate available to the dividing intestinal mucosal cells. At a plasma MTX concentration about 10 times higher (\(4 \times 10^{-5}\)), in addition to the lack of thymidylate, there is also a shortage of purine nucleotides for the conversion to purine deoxyribonucleotides and for RNA synthesis. We speculate that there is a preferential use of the folate coenzymes for purine synthesis at the lower MTX plasma concentrations at which thymidylate synthesis is inhibited. Thymidine rescue would therefore be appropriate to reduce toxicity that results from the persistence of low plasma MTX for a long time. However, in cases in which toxicity is due to the persistence of a plasma MTX at around \(10^{-7} \mu\text{g/hr}\) or greater, thymidine rescue of toxicity would be highly inappropriate, whereas calcium leucovorin use would be appropriate.

### REFERENCES


### Table 1

<table>
<thead>
<tr>
<th>Infusion rates</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX (10 µg/hr)</td>
<td>14 ± 1&lt;sup&gt;a&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>MTX (10 µg/hr) and inosine (2.6 mg/hr)</td>
<td>86 ± 20</td>
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<tr>
<td>Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100 ± 2</td>
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</table>

<sup>a</sup> Mean ± S.E.; n = 6 to 8.
<sup>b</sup> Significantly different from control; p < 0.05.
<sup>c</sup> Small intestine 1857 ± 66 and femur 1172 ± 117 dpm/µg DNA.

### Table 2

<table>
<thead>
<tr>
<th>Infusion rate/mouse (µg/hr)</th>
<th>No. of mice</th>
<th>Time of infusion (hr)</th>
<th>Maximum wt loss (%)</th>
<th>Lethality&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>40</td>
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</table>

<sup>a</sup> Earliest death recorded was 5 days and latest death was 8 days after the start of infusions.
<sup>b</sup> LD<sub>50</sub>, dose lethal to 50% of animals.

### Table 3

<table>
<thead>
<tr>
<th>Infusion rate/mouse</th>
<th>No. of mice</th>
<th>Maximum wt loss (%)</th>
<th>Lethality&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
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<tr>
<td>MTX, 10 µg/hr, for 48 hr</td>
<td>10</td>
<td>24</td>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
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<td>MTX, 10 µg/hr, for 48 hr and thymidine, 50 µg/hr for 67 hr</td>
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<td>23</td>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>MTX, 10 µg/hr, for 48 hr and calcium l-leucovorin, 1 µg/hr, for 48 hr</td>
<td>10</td>
<td>1</td>
<td>LD&lt;sub&gt;9&lt;/sub&gt;</td>
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<tr>
<td>MTX, 1 µg/hr, for 108 hr</td>
<td>10</td>
<td>24</td>
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<td>MTX, 1 µg/hr, for 108 hr and thymidine, 50 µg/hr, for 118 hr</td>
<td>10</td>
<td>19</td>
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<sup>a</sup> LD<sub>50</sub>, dose lethal to 50% of animals.


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