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7-Hydroxymethotrexate Cytotoxicity and Selectivity in a Human Burkitt's Lymphoma Cell Line versus Human Granulocytic Progenitor Cells: Rescue by Folinic Acid and Nucleosides*

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Abstract—*The cytotoxicity of 7-hydroxymethotrexate (7-OH–MTX), the primary plasma metabolite of methotrexate (MTX) in humans, was assessed by inhibition of colony formation in agar, using human bone marrow granulocyte-macrophage stem cells (CFU) from healthy volunteers and RAJI cells, a human Burkitt's lymphoma cell line. After a* 2 hr *exposure of cells to 7-OH–MTX, the concentrations necessary to produce a* 50% *inhibition of colony formation were* 180 μM *and* 10 μM *for bone marrow cells and for RAJI cells respectively. A continuous incubation with* 20 μM *folinic acid (CF) protected the RAJI cells from 7-OH–MTX cytotoxicity at concentrations below* 5 μM *but was not able to completely reverse 7-OH–MTX effects at higher doses. Continuous incubation of 7-OH–MTX-preloaded cells* (2 hr, ID_{90}) *with the end products of folate-dependent reactions, adenosine* (100 μM) *and thymidine* (10 μM), *completely rescued RAJI cells from the 7-OH–MTX cytotoxic effects. Moreover, while thymidine alone had no effect on the 7-OH-MTX response curve, both adenosine alone or CF–adenosine combination produced* 75% *and* 90% *protection respectively. CF and adenosine concentrations necessary to achieve* 90% *protection were* 20 *and* 100 μM *respectively. This study demonstrates that* (i) *7-OH–MTX can exhibit a cytotoxic selectivity for this human Burkitt's lymphoma cell line as compared to human bone marrow stem cells and* (ii) *the cytotoxicity of 7-OH–MTX cannot be reversed by CF alone. These data suggest that 7-OH–MTX and/or its polyglutamylated derivatives may play an important role on different enzyme(s) involved in the interconversion of tetrahydrofolate cofactors necessary for the* de novo *purine biosynthesis.*

INTRODUCTION

THE FOLATE antagonist methotrexate‡ (MTX) is rapidly converted to 7-hydroxymethotrexate (7-OH–MTX) in humans after injection of high, medium and also low doses of MTX [1–3]. This catabolite was considered as an inactive detoxification product because of its low affinity for the purified MTX target enzyme, dihydrofolate reductase (DHFR) [4]. Recently, Fabre *et al.* [5] first demonstrated the conversion of 7-OH–MTX to 7-OH–MTX polyglutamates in MOLT 4 cells. This metabolic conversion was further demonstrated in Ehrlich ascites tumor [6], K-562 cells [7] and also in a cell-free system with partially purified folylpolyglutamate synthetase [8], the enzyme responsible for the conversion of folate and antifolates to polyglutamyl derivatives [9, 10].

In addition, Fabre *et al.* [6, 7] analyzed the formation, retention and the selective binding to DHFR of 7-OH–MTX tetraglutamate and correlated these parameters to the cytotoxicity of 7-OH–MTX in K-562 cells. These studies showed

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‡Abbreviations used are: A, adenosine; H, hypoxanthine; MTX, methotrexate; 7-OH–MTX, 7-hydroxy-methotrexate; CF, Citrovorum Factor, 5-formyltetrahydrofolate, folinic acid; CFU, colony-forming unit, granulocytic prognenitor cells; DHFR, dihydrofolate reductase; T, thymidine; ID_{50}, concentration necessary to produce 50% inhibition of colony formation.

that 7-OH–MTX produced its cytotoxic effects, at least in part, by inhibiting the enzyme DHFR. These data suggested that 7-OH–MTX, as did MTX, depleted intracellular levels of reduced folate cofactors required for *de novo* purine, thymidylate and protein biosynthesis [11–14].

In this study, we present the dose–response curves for 7-OH–MTX in a human tumor cell line (RAJI) and in human granulocytic progenitor cells from healthy volunteers by clonogenic assay in agar, as compared to the parental compound MTX. To assess further the role of 7-OH–MTX on the folate-dependent reactions, the effects of folinic acid and/or adenosine and/or thymidine on cells pre-exposed to high doses of 7-OH–MTX were evaluated.

MATERIALS AND METHODS

Chemicals

MTX was obtained from Lederle Laboratories and was purified by DEAE–cellulose chromatography [15]. 7-OH–MTX was prepared in our laboratory and purified according to the method of Jacobs *et al.* [16]. Its purity was confirmed by UV absorption and HPLC [5]. Adenosine, hypoxanthine and thymidine were purchased from Sigma Chemical Co (St Louis, MI). CF was kindly supplied by Lederle Laboratories.

In all experiments the folinic acid (CF) concentration referred to the racemic d-l mixture.

Drug exposure

Two modes of drug exposure were investigated: a brief (2 hr) exposure and a continuous exposure. For the short term mode of exposure, an incubation mixture of 5 ml consisting of a cell suspension in RPMI 1640 medium (Roswell Park Memorial Institute; flobio-Gibco), 5 mM L-glutamine and the drug solution were incubated for the selected period of time at 37° C in 7% CO_2 and humidified air. Thereafter, cells were washed twice in cold medium and plated in agar as described below. For the continuous mode of exposure, the drug solution was added directly in the plating mixture.

Collection of human bone marrow cells and clonogenic assay

Bone marrow was aspirated from the sternum or the posterior iliac crest of healthy volunteers. Mononuclear cells were separated from the whole marrow samples by centrifugation on Ficoll–Hypaque for 20 min at 800 × ***g*** at room temperature. The cells were collected and washed twice with ice cold RPMI 1640. The culture system was adapted from the technique described by Pike and Robinson [17]. Harvested cells were resuspended in α-MEM medium (Eurobio) supplemented with 2 mM L-Glutamine, 53.6 mM $NaHCO_3$, 107.5 units per ml penicillin, 100 μg per ml streptomycin and 10% heat-inactivated dialyzed fetal calf serum containing 0.35% Bacto-Agar (Difco, Detroit, MI). Bone marrow cells were originally plated as a single cell suspension in 0.35% agar, on top of freshly prepared feeders (10^6 peripheral blood cells in 0.50% agar). Cells were plated in triplicate, in 1 ml aliquots, into 35 mm 6-well plates (Nunclon, Delta Danemark) and incubated for 10–14 days at 37° C, in 7% CO_2 humidified atmosphere.

Colonies, defined as aggregates of more than 50 cells, were counted. Viability was determined as follows:

$$\text{viability} = \frac{\text{number of colonies in drug-treated cultures}}{\text{number of colonies in control cultures}} \times 100.$$

Results are expressed as the mean ∓ S.D. of four and five experiments (four to five volunteers) for MTX and 7-OH–MTX respectively.

Tumor cell cultures and cell cloning assay

The RAJI cell line, originally derived from a patient with a Burkitt's lymphoma, was maintained in continuous culture in RPMI 1640 medium supplemented with 15% heat-inactivated undialyzed fetal calf serum and 2 mM L-glutamine. Cells were harvested during exponential growth and resuspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 30% heat-inactivated dialyzed fetal calf serum, and 0.35% Bacto–Agar. Two ml of this medium were added to 35 mm 6-well plates and incubated as described above for 7–10 days.

Each experiment was performed in triplicate and repeated at least three times.

RESULTS

Cytotoxicity studies

Freshly isolated human bone marrow stem cells from healthy volunteers, prepared as described in "Materials and Methods", were exposed for 2 hr to either 7-OH–MTX or MTX. The cells were washed twice, cloned in soft agar and cytotoxicity was determined as the ability of the drug to prevent clonal growth compared to an untreated control. The results presented in Fig. 1 and in Table 1 illustrate the respective abilities of 7-OH–MTX and MTX to prevent the clonal growth of marrow stem cells. Results are the mean ∓ S.D. obtained with cultures derived from four to five different healthy volunteers. 7-OH–MTX did produce cytotoxicity at concentrations above 20 μM but was markedly less cytotoxic than was MTX. After a 2 hr exposure, 7-OH–MTX exhibits an ID_{50} of 180 μM compared to a MTX ID_{50} of 6 μM. ID_{50}

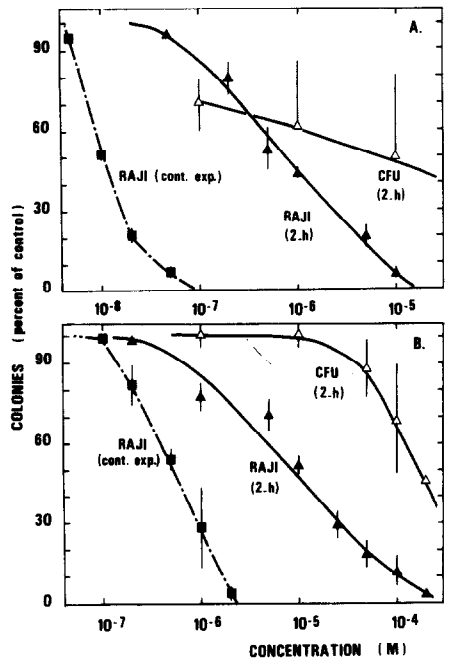

Fig. 1. Cytotoxicity of MTX (A) *or 7-OH-MTX* (B) *to RAJI cells and human granulocytic progenitor cells (CFU). The different cells were exposed for* 2 hr *or continuously to different MTX* (A) *or 7-OH-MTX* (B) *concentrations and plated in agar as described in Materials and Methods. Symbols, mean percentage of control colonies in three to five different experiments; bars, S.D.*

Table 1. Cytotoxicity (ID_{50}) *of MTX and 7-OH-MTX. Cells were exposed to various concentrations of MTX or 7-OH-MTX for the indicated times, following which cells were plated on soft agar and colony formation was assessed* 10–14 days *later as described in Materials and Methods. Data are expressed as the mean of at least three different experiments.*

	MTX ID_{50}	7-OH-MTX ID_{50}	Ratio $\frac{ID_{50}\ 7\text{-OH-MTX}}{MTX}$
CFU	6.00 μM	180.0 μM	30
RAJI (2 hr)	0.70 μM	10.0 μM	14
RAJI (cont. exp.)	0.01 μM	0.7 μM	70

were determined graphically and summarized in Table 1. Continuous exposure of bone marrow cells to MTX or 7-OH–MTX was not investigated since these compounds presented also a cytotoxicity on the feeder-layer cells.

Cytotoxicity was also assessed in a Burkitt's lymphoma cell line. RAJI cells were exposed to increasing MTX (A) or 7-OH–MTX (B) concentrations, continuously or for 2 hr, and effects on colony formation were monitored (Figs. 1A and 1B). A continuous exposure to 1 μM 7-OH–MTX produces a marked suppression of clonal growth of these tumor cells. This result is of great interest in relation to the observations that (i) 7-OH–MTX plasma levels up to 100 μM are currently achieved after a 4–24 hr infusion of high-dose MTX regimen, and (ii) its plasma half-life is long (10–24 hr; [1–3]).

It can be seen from these data (Fig. 1, Table 1) that 7-OH–MTX was less cytotoxic than MTX in these two cell systems. These results are in agreement with those reported by McGuire *et al.* [8] in different human leukemia cell lines and by Fabre *et al.* [7] in K-562 cells. Of particular interest is the observation that 10 μM 7-OH–MTX produced, after a 2 hr exposure, a marked suppression of clonal growth of RAJI cells (ID_{50}) while the clonal growth of human bone marrow stem cells was unaffected at the same concentration. These results establish that 7-OH–MTX may exhibit a selective cytotoxicity for leukemia cells compared to human bone marrow stem cells.

Rescue of 7-OH–MTX cytotoxicity by folinic acid

The effect of CF on 7-OH–MTX cytotoxicity was first studied in human bone marrow stem cells. Cells were exposed for 2 hr to 200 μM 7-OH–MTX, washed twice and then cloned in agar in the continuous presence of 20 μM CF. Under these conditions CF reverses 7-OH–MTX cytotoxicity from 66.2 ∓ 18.3% to 15.3 ∓ 15.2% ($n = 3$).

Rescue of 7-OH–MTX cytotoxicity by CF was also investigated in RAJI cells. Cells were exposed for 2 hr to the 7-OH–MTX ID_{90}, 100 μM. Then cells were washed twice and cloned in agar in the presence of increasing CF concentrations. CF concentrations up to 1 μM have weak effect in reversing the 7-OH–MTX cytotoxicity. However, increasing rescue appeared between 1 and 20 μM

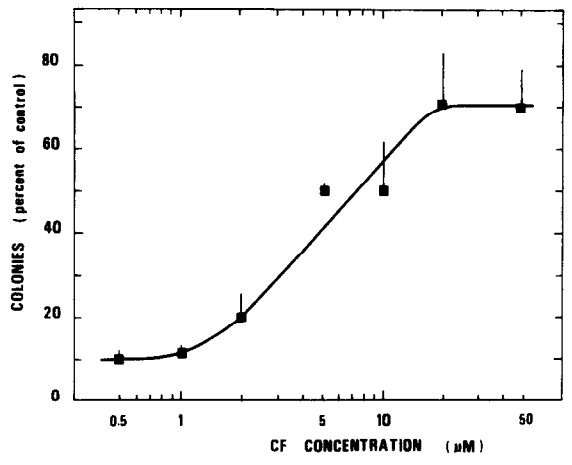

Fig. 2. Effect of folinic acid on colony formation of RAJI cells after exposure to 7-OH–MTX. Cells were exposed for 2 hr *to* 100 μM *7-OH–MTX* (ID_{90}), *washed twice, then cloned in agar in the presence of increasing CF concentrations as described in "Materials and Methods". Symbols, mean percentage of control colonies of three different experiments; bars, S.D.*

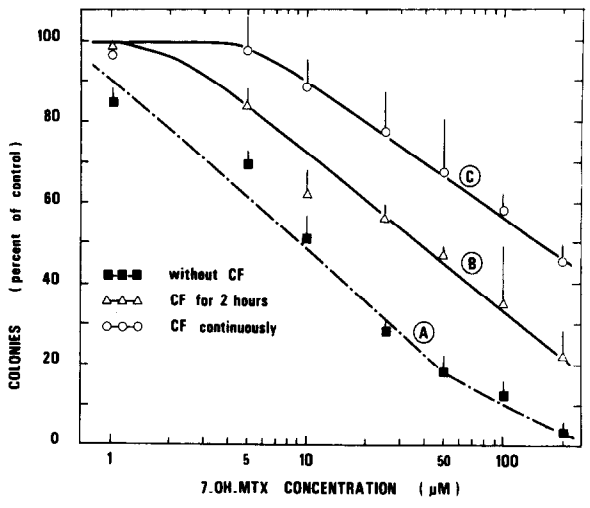

Fig. 3. Effect of folinic acid on colony formation of RAJI cells after exposure to 7-OH-MTX. Cells were exposed for 2 hr *to increasing 7-OH-MTX concentrations, washed twice, then resuspended for* 2 hr *in CF-free medium (curve A) or in medium containing* 20 μM *CF (curve B). Cells were washed again twice and plated in agar. For curve C, cells exposed to 7-OH-MTX as described above, were washed twice and plated in agar in the continuous presence of* 20 μM *CF. Symbols, mean percentage of control colonies of three different experiments; bars, S.D.*

with a maximum of approx. 70% recovery between 20 and 50 μM (Figure 2). Higher CF concentrations, up to 200 μM (concentration which exhibits no cytotoxicity), did not increase the reversal effects (data not shown). Similar results were reported by different authors [12, 18, 19] in different cell lines for the parental compound, MTX. These results raise the possibility that the mechanism of action of 7-OH-MTX is similar to that of MTX, and that CF competitively reverses 7-OH-MTX cytotoxicity as was demonstrated for MTX.

Figure 3 illustrates the effects of 20 μM CF on 7-OH-MTX cytotoxicity. RAJI cells were exposed for 2 hr to increasing 7-OH-MTX concentrations, washed twice and then incubated for 2 additional hr in CF-free medium or in medium containing 20 μM CF (Fig. 3, curves A and B respectively). Cells were washed twice, then cloned in agar for 7–10 days. For curve C, cells pre-exposed to 7-OH-MTX as described above, were washed twice then plated in the continuous presence of 20 μM CF. For each incubation condition, control plates were performed in parallel to correct the eventual loss of cells during the washing steps. When cells were incubated with CF for a 2 hr exposure, the cytotoxic curve for 7-OH-MTX shifted to the right, illustrating the ability of this agent to reverse the drug effects for low doses, i.e., below 1 μM. When CF is present during the incubation period (7–10 days), 7-OH-MTX cytotoxicity was completely reversed with concentrations up to 5 μM, although antifolic effects were expressed for higher doses of 7-OH-MTX.

The ID_{50} for 7-OH-MTX after a 2 hr exposure was progressively increased from 10 μM in control cells to 35 μM after a 2 hr period of rescue and to 140 μM in the continuous presence of CF during the incubation period.

Effect of different nucleosides and/or folinic acid on 7-OH-MTX cytotoxicity

As reported before for both MTX [12–14, 20–24] and 7-OH-MTX [7], the GAT (glycine, adenosine, thymidine) combination protects the cells from the cytotoxicity of antifolates.

In Fig. 4 is illustrated the differential rescues of 7-OH-MTX cytotoxicity by nucleosides and/or CF. Cells were exposed for 2 hr to 100 μM 7-OH-MTX (ID_{90}), washed twice, then cloned in agar supplemented by different nucleosides, adenosine (100 μM) and/or thymidine (10 μM) and in the presence or in the absence of 20 μM CF. These different nucleoside concentrations exhibit no cytotoxic effect on cell clonogenicity.

As reported above in Fig. 3, CF alone caused only a partial recovery of colony formation, while adenosine plus thymidine completely protect the cells from the cytotoxicity of 7-OH-MTX by providing the end products of folate-dependent reactions. Thymidine alone has no effect on 7-OH-MTX cytotoxicity and the combination thymidine–CF does not improve the efficacy of CF alone. Adenosine alone reverses in part the 7-OH-MTX cytotoxicity (from ID_{90} to 26.8 ∓ 1.9%; $n = 4$). Associated to CF, adenosine causes an almost complete recovery of colony formation (95.1 ∓ 4.1%; $n = 3$).

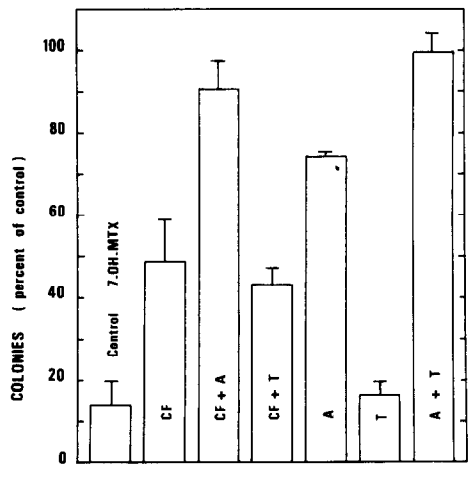

Fig. 4. Effects of folinic acid and/or nucleosides on colony formation of RAJI cells pulsed with 7-OH-MTX. Cells were exposed for 2 hr *to* 100 μM *7-OH-MTX* (ID_{90}), *washed twice, then plated in agar in the continuous presence of CF* (20 μM) *and/or adenosine* (100 μM) *and/or thymidine* (10 μM). *Symbols, mean percentage of control colonies of three different experiments; bars, S.D.*

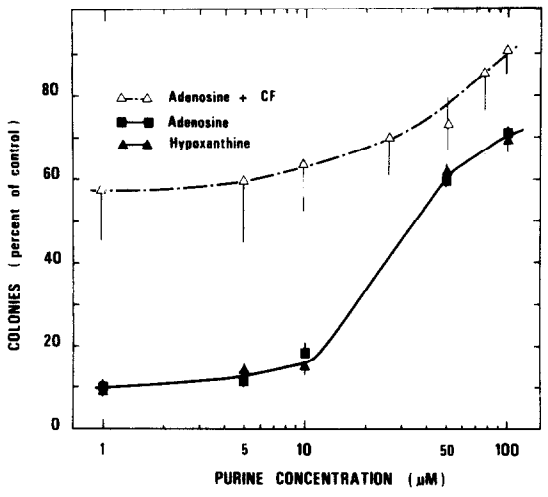

Fig. 5. Effect of purines in the absence or the presence of folinic acid on colony formation of RAJI cells after exposure to with 7-OH-MTX. Cells were exposed for 2 hr *to* 100 μM *7-OH-MTX* (ID_{90}), *washed twice, then plated in agar in the continuous presence of increasing purine concentrations, in the absence or the presence of* 20 μM *CF. Symbols, mean percentage of control colonies of six determinations in two different experiments; bars, S.D.*

The effects of increasing adenosine or hypoxanthine concentrations, alone or in combination with 20 μM CF after a 2 hr exposure to 100 μM 7-OH–MTX are illustrated in Fig. 5. Cells were exposed to 7-OH–MTX for 2 hr, washed twice, then plated in agar with increasing adenosine or hypoxanthine concentrations in the absence or the presence of 20 μM CF. Reversal effects of adenosine and hypoxanthine on 7-OH–MTX cytotoxicity were similar and dose-dependent. Purine concentrations below 10 μM have little effect in reversing 7-OH–MTX inhibition. There is an increasing rescue between 10 and 100 μM to achieve a maximum value of 60% recovery at 100 μM. The presence of CF improves the purine treatment, allowing to achieve a maximum value of 90% recovery. These data suggest that purine pathways are the principal site(s) of action of 7-OH–MTX and/or its polyglutamates in this cell line.

DISCUSSION

In an earlier study [5], we reported the ability of 7-OH–MTX to be polyglutamylated in MOLT 4 cells. This biotransformation process was confirmed by others using different cell lines and in cell-free systems [6–10]. The cellular pharmacology of the 7-hydroxy catabolite of MTX was also characterized in Ehrlich ascites tumor cells in comparison to the situation observed with MTX [3, 25, 26]. These studies demonstrated a rapid transport of 7-OH–MTX mediated by the MTX-tetrahydrofolate cofactor carrier and the inhibitory effect of 7-OH–MTX on the rate of MTX polyglutamylation in cells, at least in part a consequence of the inhibition of MTX transport. These data suggested that 7-OH–MTX could modulate MTX activity. In addition, while 7-OH–MTX is only a weak inhibitor of the DHFR, and has been considered inactive, its tetraglutamyl derivative was a much better inhibitor of dihydrofolate reductase than the monoglutamate, raising the possibility that the intracellular 7-OH–MTX-G_3 may have a pharmacologic activity. This was further demonstrated in K-562 cells and was correlated with the binding of the 7-OH–MTX-G_3 to the DHFR [6] and possibly to another site(s) [7].

Our studies confirmed the 7-OH–MTX cytotoxicity in a Burkitt's lymphoma cell line and extended those to both (i) the evaluation of the selectivity of the catabolite in a human Burkitt's lymphoma cell line vs immature mononuclear cells from healthy human bone marrow and (ii) the rescue of that cytotoxicity by CF and/or purine and/or pyrimidine. The range of 7-OH–MTX concentrations studied corresponds to plasma concentrations currently achieved after high dose MTX therapy; indeed 7-OH–MTX plasma levels up to 100 μM have been observed in the treatment of osteosarcoma by methotrexate (12 g/m^2 over 6–8 hr).

Colony formation of RAJI cells was inhibited by approx. 80% after a 2 hr exposure to 50 μM 7-OH–MTX, while formation of granulocyte-macrophage colonies was only slightly affected under the same conditions (88.4 ∓ 10.6% of the control). These data indicate that 7-OH–MTX as MTX [24] selectively kills tumor cells grown long in culture.

Recent studies [24, 27–33] strongly suggest that formation of MTX polyglutamyl derivatives may be an important step in drug selectivity. Indeed, tumor cells form large amounts of polyglutamates exceeding the dihydrofolate reductase binding capacity. As reported earlier [24, 28], mouse granulocytic progenitor cells did not form detectable amounts of polyglutamates. Koizumi *et al.* [33] showed that granulocytic progenitor cells from normal human bone marrow produce small quantities of MTX polyglutamates, which, although selectively retained, did not result in prolonged inhibition of the DHFR activity, and did not produce cytotoxicity after the extracellular drug was removed. As demonstrated for MTX [24], a recent study reported that polyglutamyl derivatives of 7-OH–MTX were responsible for the cytotoxicity of 7-OH–MTX in tumor cells [7].

Extensive polyglutamylation of 7-OH–MTX was also demonstrated in RAJI cells. After a 2 hr exposure to 100 μM 7-OH–MTX, a concentration for which cytotoxicity is expressed (ID_{90}), RAJI

cells accumulate 6.47 ∓ 4.15 nmol/g dry wt ($n = 3$) far above the DHFR binding capacity (around 0.4 nmol/g dry wt; data not shown). Unfortunately and because of the small amount of cells, we could not analyze the ability of bone marrow stem cells to synthesize polyglutamates. However, although the relative rate of formation of polyglutamyl derivatives of 7-OH–MTX exceeded that for MTX [6], 7-OH–MTX has a lower affinity for the transport carrier [3, 25, 26]. Based upon these different observations, we suggest that the selectivity of 7-OH–MTX in RAJI cells can be accounted for by a low level of synthesis and accumulation of 7-OH–MTX polyglutamates with chain length longer than two additional glutamate moictics in human bonc marrow cells.

Like MTX, 7-OH–MTX and/or its polyglutamyl metabolites are thought to exert their cytotoxicity by causing depletion of intracellular reduced folates required as cofactors for the *de novo* purine and thymidylate synthesis [35]. While biochemical activities of MTX and 7-OH–MTX can be reversed by providing cells with the end products of folate dependent reactions (thymidine and purines), folinic acid a one-carbon carrier, failed to reverse the cytotoxicity of high 7-OH–MTX concentrations. These data are in agreement with those reported by different authors for MTX [18, 20, 21, 36–42]. Both CF and adenosine or adenosine–thymidine are necessary to achieve a complete rescue of the RAJI cells from 7-OH–MTX effects. Our data demonstrate that sustained 7-OH–MTX cytotoxicity cannot be reverted by CF alone in tumor cells, while a complete rescue of its cytotoxicity in bone marrow can be achieved for 7-OH–MTX concentrations up to 100 μM. This suggests that the mechanism of action of 7-OH–MTX is different in tumor and normal cells and/or that the CF-requirement of normal and tumor cells for protection against 7-OH–MTX cytotoxicity are different. As was demonstrated earlier for MTX [43–46], the lack of 7-OH–MTX polyglutamylation could be responsible for this selective protection in normal cells.

Recent work by Allegra *et al.* [46, 47] demonstrated the ability of MTX polyglutamates, but not of the monoglutamate, to inhibit the aminoimidazole–carboxamide ribonucleotide transformylase (AICAR transformylase), involved in the *de novo* purine biosynthesis, and the thymidylate synthesis. This would suggest that at high 7-OH–MTX concentrations, the large amounts of 7-OH–MTX polyglutamates formed in RAJI cells, would be able to inhibit other enzyme(s) involved in the *de novo* purine biosynthesis. Indeed, Allegra *et al.* [46] reported that although purine synthesis might be inhibited indirectly by depletion of the reduced folate pool, the direct inhibition by MTX polyglutamates might be important, in that it could explain the failure of leucovorin treatment.

Earlier studies from this and other laboratories have demonstrated that 7-OH–MTX could play a role in modulating MTX action by its inhibitory effects on MTX transport and net cellular MTX and MTX polyglutamate accumulation [3, 25, 26]. Moreover, cytotoxicity of 7-OH–MTX was reported in many malignant cell lines [7, 8]. Although 7-OH–MTX has a weaker potency than MTX to inhibit RAJI cells colony formation, there was a relative similarity of 7-OH–MTX to MTX.

This study demonstrates, in addition, (i) a selective cytotoxicity of 7-OH–MTX in RAJI cells compared to human bone marrow cells and (ii) a preferential rescue of 7-OH–MTX cytotoxicity by purines suggesting that 7-OH–MTX and/or its polyglutamates may have potent inhibitory effects on other folate-dependent enzyme(s), such as those involved in purine biosynthesis or folate interconversions.

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