Vol. 187, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1992 Pages 522-528

METHOTREXATE DECREASES THYMIDINE KINASE ACTIVITY

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Received July 20, 1992

SUMMARY: MTX cytotoxicity is not fully explained by its well-known inhibition of dihydrofolate reductase activity which leads to a decrease in the dTMP synthase reaction, since TdR kinase which converts TdR to dTMP could readily circumvent MTX action through this salvage activity. TdR kinase is of particular significance, since in various types of carcinoma cells its activity is orders of magnitude higher than that of dTMP synthase. To throw light on this problem, we tested the hypothesis that the impact of MTX treatment might in fact involve an inhibition or decrease in TdR kinase activity. Injection in rat of MTX (i.p.) decreased TdR kinase activity in a time- and dose-dependent fashion in liver ($t_{1/2}$ = 46 h; IC_{50} = 95 mg/kg), bone marrow ($t_{1/2}$ = 10 h; IC_{50} = 5 mg/kg) and rapidly growing transplantable hepatoma 3924A ($t_{1/2}$ = 56 h; IC_{50} = 5 mg/kg). Injection in rat of cycloheximide (15 mg/kg, i.p.), an inhibitor of protein biosynthesis, rapidly decreased TdR kinase activity in the hepatoma ($t_{1/2}$ = 3.6 h); activities of other purine and pyrimidine synthetic enzymes, dTMP synthase, IMP dehydrogenase, GMP reductase and GMP synthase, declined at a markedly slower rate ($t_{1/2}$ = 11, 11.6, 12 and 22 h, respectively). MTX, by curtailing purine and pyrimidine biosynthesis, limits production of TdR kinase which is more sensitive to unopposed protein degradation than other enzymes of nucleic acid biosynthesis. TdR kinase is a newly discovered target of MTX treatment.

MTX administration inhibits dihydrofolate reductase activity (1) leading to a decrease in the conversion of dUMP to dTMP, resulting in a block in the de novo biosynthesis of dTMP (2). However, this could not eliminate dTMP production, since TdR kinase (EC 2.7.1.21) by converting TdR to dTMP provides this salvage activity (Fig. 1). Moreover, the activity of TdR kinase in normal tissue and various types of cancer cells is orders of magnitude higher than that of dTMP synthase (3,4). The biological significance of the TdR salvage pathway was demonstrated by coadministration of acivicin (an inhibitor of de novo biosynthesis of purines and

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Abbreviations: TdR, thymidine; dTMP, thymidylate; MTX, methotrexate; $t_{1/2}$, the time required for 50% decrease in activity; APRT, adenine phosphoribosyltransferase; IC_{50}, dose required for 50% decrease in activity; GPRT, guanine phosphoribosyltransferase; s.c., subcutaneously; i.p., intraperitoneally; AZT, azidothymidine, zidovudine; DH, dehydrogenase.

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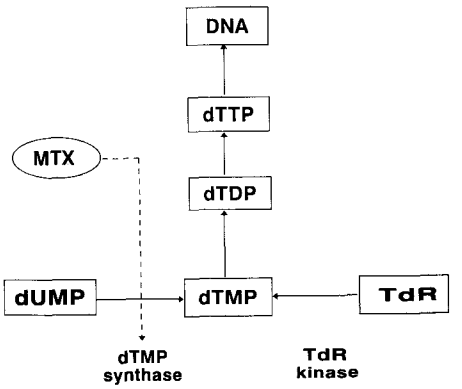

FIG. 1. The pathways of dTMP biosynthesis: 1. de novo through dTMP synthase action, and 2. salvage through thymidine kinase activity.

pyrimidines) and dipyridamole (which blocks transport of TdR and other nucleosides into carcinoma cells) (3,5). Under these conditions in tissue culture dipyridamole was synergistic with various inhibitors of de novo purine and pyrimidine biosynthesis (3,5,6). The importance of salvage was also supported by our observation that AZT (a competitive inhibitor of TdR kinase) with MTX resulted in synergistic cytotoxicity (7,8). The need to inhibit salvage was further revealed in our recent report that AZT with dipyridamole provided the most marked synergism we have observed with MTX (9). Nevertheless, tissue culture studies and clinical observations show that MTX alone can provide effective cytotoxicity in sensitive cancer cells. Therefore, in this investigation, we tested the hypothesis whether MTX treatment might lead to a decrease in TdR kinase activity in the cancer cells. The results we now report indicate that injection of MTX in rat markedly decreased TdR kinase activity in a time- and dose-dependent fashion in liver, bone marrow and transplanted hepatoma. Our investigations reveal that MTX action entails an until-now-unreported mechanism: a decrease in TdR kinase activity. This mechanism of MTX action is apparently based on the short half-life of TdR kinase.

MATERIALS AND METHODS

Chemicals and supplies. Methotrexate, cycloheximide and ATP were purchased from Sigma (St. Louis, MO). Radioactive isotope ^{14}C-TdR was from DuPont (Boston, MA). Other chemicals were also of the highest purity available, purchased from Sigma.

Biochemical studies. Wistar and ACI/N inbred male rats (190-210 g) were kept in individual cages. Rapidly growing hepatoma 3924A was s.c. transplanted; drugs were injected i.p. Rats were killed by stunning and decapitation. The livers and hepatomas were rapidly excised, femurs were cut and bone marrow was suctioned out within 90 sec of decapitation. From the tissues 20% homogenates were made in 0.15 M KCl, then 100,000 x g supernatant fractions were prepared by centrifugation of the homogenates for 30 min at 4°C. Protein concentration was determined by a routine method using crystalline bovine serum albumin as a standard.

TdR kinase activity (^{14}C-TdR + ATP→dTMP + ADP) was measured by the PEI cellulose plate method (3). Enzymic activities were calculated in nmol substrate

metabolized per h per mg protein, as specific activity; data are also expressed as percentages of controls. Results were subjected to statistical evaluation by the t-test; differences between means yielding a probability of < 5% were considered statistically significant.

Bone marrow. Cell counts were determined in a hemocytometer.

RESULTS AND DISCUSSION

Effect of various doses of MTX on TdR kinase activity in rat liver, hepatoma and bone marrow. Rats carrying s.c. transplanted hepatoma were given a single i.p. injection of MTX and tissues were removed 48 h later. Table 1 shows that TdR kinase activities in liver, bone marrow and hepatoma were 3, 91.7 and 15.2 nmol/h/mg protein, respectively. Thus, liver activity was the lowest and it increased 5-fold in the hepatoma and over 30-fold in the bone marrow. MTX doses varying from 5 to 100 mg/kg had little effect on TdR kinase activity in liver. However, the activities in the bone marrow were depressed to 11% or lower. By contrast, TdR kinase activity in the hepatoma decreased by 5 mg/kg to 48% and further increases of MTX up to 60 mg/kg depressed it further only down to 36% of the control values. These data suggest that the sensitivity to MTX of TdR kinase activity varied inversely with the absolute enzymic activities of the tissues before treatment (Table 1). These results agree with clinical observations that the bone marrow is highly sensitive to MTX treatment.

Sequence of events in MTX treatment on TdR kinase activity in rat liver, hepatoma and bone marrow. Rats carrying s.c. transplanted hepatomas received

Table 1
Effect of different doses of MTX on TdR kinase activity in rat liver, bone marrow and hepatoma 3924A

Dose mg/kg	TdR kinase activity nmol/h/mg protein %		
	Liver	Hepatoma	Bone marrow
Saline control	3.0 ± 0.09	15.2 ± 1.0	91.7 ± 15.9
0	100	100	100
5	-	48*	-
15	127*	44*	11*
30	103	40*	6*
60	73*	36*	4*
100	87	-	1*

Single MTX injection (i.p.) was given and rats were killed 48 h later. Means ± S.E. of 3 or more rats per group are shown and % of controls.

***Significantly different from controls ($p < 0.05$).**

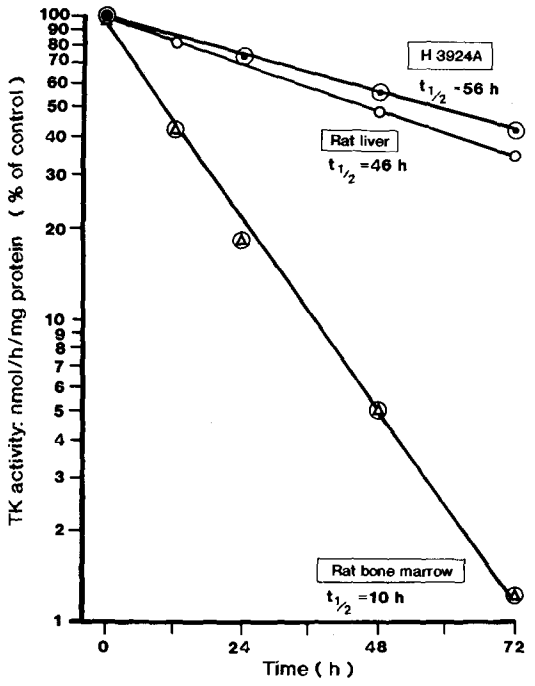

FIG. 2. Sequence of events after a single injection of MTX (150 mg/kg) in rats carrying s.c. transplanted hepatomas. Three or more rats were killed at each time period and hepatomas were removed for enzyme assays.

single i.p. injections of MTX (150 mg/kg) and tissues were removed at intervals during a 72-h post-injection period. The pre-injection control values for liver, hepatoma and bone marrow were 3.7 ± 0.4, 11.4 ± 1.0 and 101.6 ± 0.4 nmol/h/mg protein which are in agreement with those in Table 1. Figure 2 shows that TdR kinase activity of the bone marrow steadily decreased and was markedly more sensitive to MTX than the activity in liver or hepatoma. Under these experimental conditions MTX yielded for liver, hepatoma and bone marrow $t_{1/2}$ = 46, 56 and 10 h, respectively. At this high dose the difference in sensitivity to MTX observed for liver and hepatoma at lower doses (Table 1) has disappeared and they show only slightly different $t_{1/2}$ values. The sensitivity to MTX of the bone marrow is clearly borne out in this time sequence study where 50% of the TdR kinase activity is lost in 10 h and nearly 90% is absent at 72 h.

Effect of MTX treatment on bone marrow cellularity. The marked impact of MTX on TdR kinase activity in the bone marrow could be due to the destruction of bone marrow cells. We tested this idea by determining the rate of decrease of bone marrow cellularity which then can be compared with the rate of decrease of TdR kinase activity in the bone marrow. The results (not shown) indicate that the bone marrow cellularity decreased with a $t_{1/2}$ of 30 h when rats were injected with MTX (150 mg/kg, i.p.) under the same conditions as in Figure 2. Since $t_{1/2}$ of TdR kinase

Table 2
Effect of cycloheximide treatment on purine and pyrimidine enzymic activities in hepatoma 3924A

Hours after inj.	TdR kinase	dTMP synthase	IMP DH	GMP synthase	GMP reductase	APRT	GPRT
Saline	21.8 ± 2.0	0.142 ± 0.01	12.2 ± 0.8	96.9 ± 1.9	9.24 ± 0.34	92.1 ± 2.0	380 ± 42
control	100	100	100	100	100	100	100
0.5	72*	71*	93	94	94	89	66
3	57*	89	80*	90	88	86*	60
4	30*	36*	77*	90	81*	93	42*
6	34*	87*	57*	85*	68*	105	47*
8	22*	59*	70*	70*	62*	88	53*

Means ± S.E. of 3 or more rats per group are given as nmol/h/mg protein and % of control values. Rats were injected (15 mg/kg, i.p.) and killed at various intervals. Enzymic activities were measured as cited (3,4,7,8) and expressed in nmol/h/mg protein and as % of the control values.

*Significantly different from controls ($p < 0.05$).

activity was 10 h, the decline in enzyme activity markedly preceded the decrease in bone marrow cellularity.

Effect of cycloheximide on enzymic activities in hepatoma 3924A. Adult male Wistar rats (190-220 g of weight) were injected with cycloheximide (15 mg/kg) and killed at various time periods after treatment. Table 2 shows the behavior of activities of key enzymes of de novo and salvage biosynthesis of dTMP (dTMP synthase and TdR kinase) and of purine nucleotides (de novo biosynthesis, IMP dehydrogenase, GMP synthase and salvage, APRT, GPRT) and the activity of GMP reductase which recycles GMP to IMP. The data illustrate statements above that the activity of TdR kinase in transplantable hepatoma is markedly higher (154-fold) than that of dTMP synthase. Enzymic capacities in purine biosynthesis show that the activity of the salvage enzyme of guanylate production, GPRT, is 31-fold higher than the activity of IMP dehydrogenase, the rate-limiting enzyme of de novo GTP biosynthesis.

Cycloheximide treatment inhibits protein biosynthesis and permits observation of the rate of unopposed degradation of the various enzymes. In the group of purine and pyrimidine synthesizing enzymes, the degradation rate of TdR kinase is the most rapid, yielding a $t_{1/2}$ of 3.6 h. In comparison, the $t_{1/2}$ of dTMP synthase, IMP dehydrogenase, GMP reductase and GMP synthase are 11.0, 11.6, 12.0 and 22.0 h, respectively. The activity of APRT decreases little in 8 h, but that of GPRT has a half-life of about 9 h.

These results reveal that among these enzymes TdR kinase has the shortest half-life when the processes of protein biosynthesis are curtailed. MTX administration does lead to a decrease in the availability of purine and pyrimidine nucleotide precursors which play a part in the biosynthesis of various enzymes.

These observations might explain, at least in part, the preferential decline in TdR kinase activity after MTX treatment. Thus, the chemotherapeutic impact of

MTX entails a decrease in the activity of the TdR salvage enzyme which could circumvent the action of MTX by providing dTMP. That this is an important part of the cytotoxic impact of MTX is emphasized by the observation (Table 2) that the inhibition of the low dTMP synthase activity would amount to very little had the markedly higher TdR kinase activity not decreased.

Comparison of action of MTX, actinomycin D and cycloheximide on $t_{1/2}$ of TdR kinase in hepatoma. Incubation of MTX, actinomycin D or cycloheximide in vitro with tissue extracts did not affect TdR kinase activity (not shown). Adult rats carrying transplanted hepatomas were killed at various periods after injection of MTX (150 mg/kg) or actinomycin D (100 μg/kg) or cycloheximide (15 mg/kg). For MTX, actinomycin and cycloheximide $t_{1/2}$ = 56, 24 and 3.6 h, respectively, were observed in the hepatoma. In these studies where MTX curtails purine and pyrimidine biosynthesis, actinomycin D inhibits DNA-directed RNA production and cycloheximide blocks protein biosynthesis, the most effective agent was cycloheximide. These investigations reveal that TdR kinase which has a relatively short half-life is sensitive to agents that block macromolecular biosynthesis.

Role of TdR kinase activity in the overall impact of MTX. The role of TdR salvage in the clinical pharmacology of MTX has received attention (2). The novel observations communicated here illuminate the metabolic relationship of the enzymology of de novo and salvage biosynthesis of dTMP. The new observation that MTX treatment results in a decrease of TdR kinase activity provides an explanation, in part at least, for the overall cytotoxicity of this drug. The significance of TdR salvage was recently shown by producing synergism of MTX with AZT, an inhibitor of TdR kinase, in human pancreatic carcinoma cells (8). A more marked synergism with MTX was observed when dipyridamole, an inhibitor of TdR transport, was given in addition to AZT (9). These observations and the present data indicate that combination of MTX which, as we now show, decreases TdR kinase activity and inhibitors of TdR kinase activity and TdR transport (AZT, dipyridamole) should improve the clinical impact of MTX therapy. A brief abstract of these results was published (10).

ACKNOWLEDGMENTS

This investigation was supported by USPHS Outstanding Investigator grant CA-42510 to G.W. and, in part, by a Fellowship from a Cooperation and Exchange Agreement of Indiana University School of Medicine, Indianapolis and the Semmelweis Medical University, Budapest, to M.A.

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