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The Effect of Methotrexate Pretreatment on 5-Fluorouracil Kinetics in Sarcoma 180 *in Vivo**

RICHARD HERRMANN,†‡ WERNER KUNZ,§ HANS OSSWALD,§ MARGARETA RITTER † and RÜDIGER PORT§

†*Medizinische Universitätsklinik and* §*German Cancer Research Center, Heidelberg, F.R.G.*

Abstract—*Synergy of sequential MTX and 5-FU has been shown in several* in vitro *and* in vivo *systems. In the present study the influence of time interval between MTX and 5-FU and MTX dose on 5-FU accumulation in tumor cells has been examined in Sarcoma* 180 in vivo. *There was a clear relationship between MTX dose applied and amount of 5-FU detected in the acid-soluble fraction, the RNA fraction and the thymidylate synthase complex fraction. Also, the MTX–5-FU time interval affected clearly the amount of 5-FU detected in all three fractions, the optimum time interval being* 8–12 hr. *The results indicate that for sequential application of MTX and 5-FU selection of an adequate MTX dose and a sufficient time interval is crucial to achieve synergistic action.*

INTRODUCTION

MTX AND 5-FU are anticancer drugs widely used in clinical oncology. In breast cancer, for example, they are part of a commonly used first-line drug combination. Interactions in the mechanism of action can be expected and have been suggested by several investigators based on experimental data [1–4]. *In vitro* as well as *in vivo* studies with a variety of systems have shown synergism between MTX and 5-FU when MTX precedes 5-FU [3–5]. Some studies indicated antagonism when 5-FU precedes MTX [4, 6]. This study was undertaken to evaluate the relative importance of the MTX dose and of the length of the time interval when MTX precedes 5-FU, and to investigate the mechanism of this interaction in an *in vivo* system.

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‡To whom requests for reprints should be addressed at: Medizinische Klinik und Poliklinik, Universitätsklinikum Charlottenburg, Spandauer Damm 130, D-1000 Berlin 19, F.R.G.

Abbreviations—MTX, methotrexate; 5-FU, 5-fluorouracil; TS, thymidylate synthetase; Urd, uridine; dUrd, 2′-deoxyuridine; FUrd, 5-fluorouridine; FUMP, 5-fluorouridine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate; PCA, perchloric acid.

MATERIALS AND METHODS

Chemicals

MTX was obtained from Cyanamid GmbH, Wolfratshausen. 5-FU was generously supplied by Hoffmann-La Roche, Grenzach. [6-^{3}H] 5-FU was purchased from Amersham-Buchler, Braunschweig. Marker substances for column chromatography were purchased from Serva, Heidelberg, except for FUrd, FUMP and FdUMP, which were obtained from Calbiochem, Frankfurt.

Tumor and animals

The tumor used for these experiments was Sarcoma 180 in ascitic form. The tumor ascites was taken from a mouse that had been transplanted i.p. 7 days before, and after counting the cells in a Neubauer counting chamber 10^5 cells in a volume of 0.1 ml were injected i.p. into female Swiss mice weighing 30.5 ± 3 g. Animals were allowed food and water *ad lib.*

Treatment

Treatment was started 7 days post-transplant. Mouse weights at time of treatment did not differ more than 4 g within one experiment. The different schedules of MTX–5-FU combination are listed in Table 1. For each treatment schedule, 20 animals were randomly assigned to two groups of ten animals each. One group received MTX followed by 5-FU, the other group received 5-FU alone and served as control group. All mice

Table 1. Treatment schedules

I. Variation of MTX dose; MTX-5-FU interval 8 hr

(a) MTX 10 mg/kg
(b) MTX 30 mg/kg
(c) MTX 60 mg/kg
(d) MTX 100 mg/kg
(e) MTX 150 mg/kg

II. Variation of MTX-5-FU interval; MTX dose 150 mg/kg.

(f) interval 4 hr
(g) interval 6 hr
(h) interval 8 hr (= e)
(i) interval 12 hr
(k) interval 24 hr

There were ten mice in each group and there was a concurrent control group of equal size with each schedule.

received 65 mg (0.5 mmol) [^{3}H]5-FU (sp. act. 2 mCi/mol) per kg body wt i.p.; MTX was given s.c.

5-FU uptake studies

Two hours after 5-FU injection mice were sacrificed and immediately 1 ml of the ascitic fluid was aspirated with a syringe. Each sample was assayed separately, essentially according to procedures originally described by Schmidt and Tannhauser [7], and by Washtien and Santi [8]. Cells were washed by suspending the ascites samples in 10 ml ice-cold isotonic saline containing 5 mM D-glucose, followed by centrifugation at 600 *g* (4°C) for 5 min. The cell pellets were homogenized in 5 ml 0.6 N PCA and the homogenates kept overnight at 4°C. After washing twice with 3 ml ice-cold 0.3 N PCA and centrifugation (all following centrifugations were carried out at 1500 g for 5 min), the combined supernatants were used for determination of acid-soluble 5-FU metabolites. The acid precipitate was resuspended in 4 ml sodium phosphate buffer (pH 7.3) and incubated for 20 min at 65°C to release [6-^{3}H]FdUMP from the ternary FdUMP-5,10-methylenetetrahydrofolate-TS complex. After cooling the incubate was precipitated with 1 ml 1.8 N PCA and centrifugated; the supernatant was used to determine the radioactivity that had been bound to the TS complex. The pellet was washed twice with 3 ml 0.3 N PCA, resuspended in 3 ml 0.5 N KOH and incubated for 16 hr at 40°C. After precipitation with 0.5 ml 3 N PCA and centrifugation, the resulting supernatant was used to determine radioactivity incorporated into RNA. The remaining pellet was washed twice with 3 ml 0.3 N PCA, resuspended in 5 ml 0.6 N PCA and incubated at 95°C for 15 min. After cooling and centrifugation, the supernatant was used for determination of DNA contents according to the method of Burton [9].

Measurement of radioactivity

One milliliter of liquid samples was dissolved in 10 ml Aqua luma (J. T. Baker Chemicals B.V., Deventer, Holland) and then assayed for radioactivity in a Berthold Betascint BF800 liquid scintillation counter.

Nucleotide pools

The acid-soluble fraction, after the addition of authentic markers, was analyzed by anion-exchange liquid chromatography using a 4 × 280 mm column packed with Aminex-A25 (Bio-Rad, München), with a stepwise gradient of NaCl (72 min 0.1 M, 42 min 0.2 M, 42 min 0.3 M, 60 min 0.4 M) in 0.1 M 2-amino-2-methyl-1-propanol (pH: 9.9) as eluant. The column temperature was maintained at 55°C by a regulated water jacket. The flow rate was 0.6 ml/min at a pressure of 35 bar.

Fractions were collected and analyzed for radioactivity by liquid scintillation counting. Retention times for markers were: Urd 15′, dUrd 18′, FUrd 40′, FdUrd 50.5′, FUMP 97.5′, FdUMP 104′, UDP 125.5′, UTP 142′. The same method was used for analysis of the radioactivity released from the TS-complex.

Statistics

Ninety-five percent confidence intervals for median values of 5-FU incorporation of treatment groups were calculated according to Nair [10].

RESULTS

MTX markedly influenced 5-FU kinetics in Sarcoma 180 cells *in vivo* at certain time intervals and dosages.

The ratio of [^{3}H]5-FU detected in the different fractions of tumor cells was roughly 10:1:1 for the acid-soluble fraction, the RNA fraction and the TS complex fraction respectively and was not changed by MTX pretreatment. Radioactivity in the DNA fraction was negligible. Pretreatment with escalating doses of MTX increasingly facilitated incorporation of 5-FU into the cell fractions studied (Fig. 1). Compared to the controls without MTX the highest incorporation of radioactivity was found following pretreatment with the highest MTX dose employed. While in the acid-soluble fraction the difference is statistically significant ($P < 0.05$) starting at the MTX dose of 100 mg/kg, in the RNA fraction and the TS complex fraction this is the case only for the highest MTX dose. On the other hand [^{3}H]5-FU accumulation in all cell fractions was lower

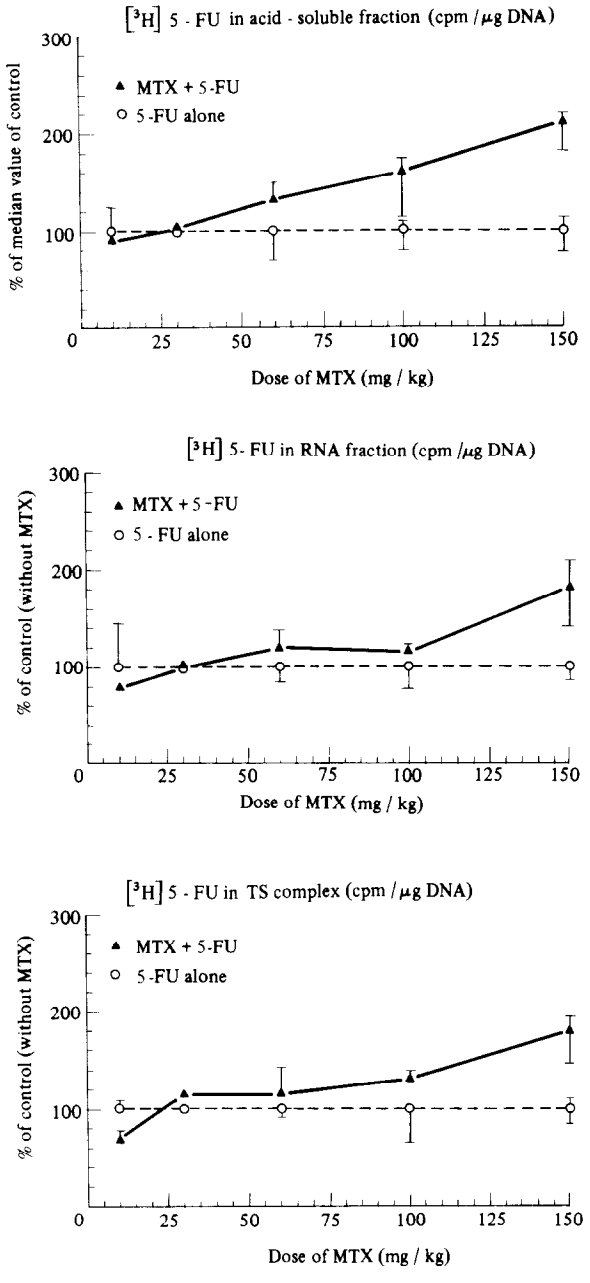

Fig. 1. The influence of different MTX doses on the accumulation of 5-FU in the acid-soluble fraction (a), the RNA fraction (b), the TS complex fraction (c). MTX-5-FU time interval was 8 hr. *Median values of 5-FU incorporation with 95% confidence limits. All values have been expressed as percentages of the median value of the corresponding control group.*

than in the controls after low-dose MTX pretreatment.

Variation of the time interval between MTX and 5-FU at the highest MTX dose revealed the optimum interval to be around 8–12 hr for this tumor (Fig. 2). At a 4-hr interval there was hardly any effect. Extending the interval to 24 hr diminished the effect on [³H]5-FU incorporation, as compared to the 8- or 12-hr interval.

Using anion-exchange liquid chromatography on Aminex A 25 (see Materials and Methods) 5-FU-metabolites in the acid soluble fraction were separated essentially into four peaks. There were two small peaks between 10′ and 20′, most likely representing 5-FU-catabolites and accounting for

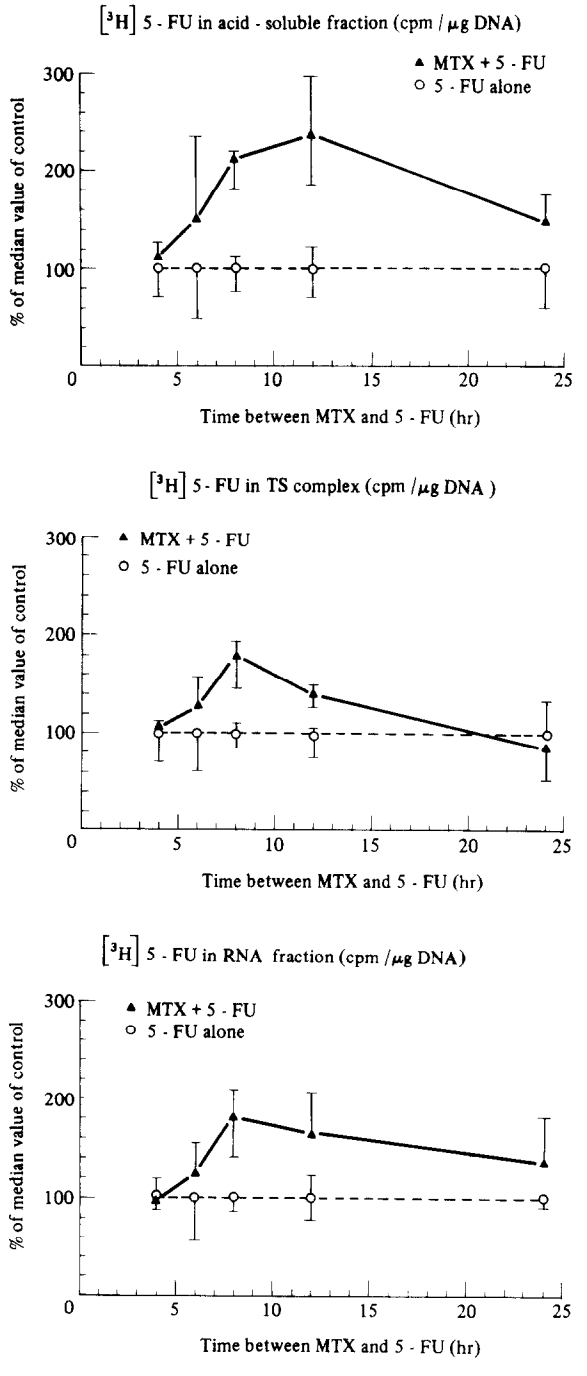

Fig. 2. The influence of different MTX-5-FU time intervals on the accumulation of 5-FU in the acid-soluble fraction (a), the RNA fraction (b), the TS complex fraction (c). MTX dose was 150 mg/kg. *Median values of 5-FU incorporation with 95% confidence limits. All values have been expressed as percentages of the median value of the corresponding control group.*

less than 10% of the total radioactivity. The majority of radioactivity eluted with authentic FUMP after 95–100 min and accounts for 77 ± 4.9% in the MTX pretreated (150 mg/kg, interval 8 hr) and for 64 ± 3.7% in the control group (mean ± S.D. of the analysis of three different samples). The fourth peak eluted at 150′ and did not correspond to any one of the available markers.

Analysis of the TS complex fraction revealed only one peak as expected. Surprisingly its retention time (135′) was distinctly different from that of the FdUMP marker peak.

DISCUSSION

Our results using an *in vivo* experimental model confirms previous findings of MTX-5-FU synergism *in vitro*. In Sarcoma 180 *in vivo*, MTX pretreatment increases 5-FU uptake into the acid-soluble cell fraction, the RNA fraction and the TS complex fraction, provided the MTX dose is sufficiently high and the time interval between MTX and 5-FU is within a certain range.

A correlation has been found between the MTX dose applied and the amount of 5-FU detected in all three fractions. Since similar findings have been reported for other systems [4, 11] this would support the use of high-dose MTX in clinical MTX-5-FU studies.

A variety of time intervals have been used in clinical as well as experimental studies. Overall it is likely that the optimum time interval depends on the tumor studied and may differ significantly from tumor to tumor. The commentary [12] concerning the 1-hr interval of the sequential MTX and 5-FU combination in regard to a synergistic chemotherapeutic action seems to be supported by our experimental findings and the clinical results [13, 14] comparing the intervals used when the dose of methotrexate was sufficient in suppressing the purine synthesis of the tumor [15]. Controlled clinical trials using a 1-hr time interval have failed to demonstrate an advantage of sequential over simultaneous [16] and reverse sequence [17] application. Bertino *et al.* have found a significant survival advantage following treatment even with a 1-hr sequenced MTX 5-FU combination in an experimental model similar to ours [3]. This may be due to the i.p. administration of MTX, which more closely resembles an *in vitro* experiment.

The cytotoxic effects of sequential MTX-5-FU synergism are described by three possible mechanisms with the common prerequisite of transforming 5-FU to the active nucleotide form of the drug. One pathway using PRPP in a one-step conversion to the nucleotide FUMP catalyzed by the enzyme orotate phosphoribosyltransferase occurred in some experimental systems [15, 18, 19]. Inhibitors of the *de novo* purine pathway including MTX in purine synthesis-inhibiting doses increase the intracellular PRPP concentration and by that way enhance 5-FU nucleotide formation, which induces an increased incorporation of FUMP into RNA [20, 21]. Increased FUMP formation and incorporation into RNA have been confirmed in this *in vivo* study.

Other results support the hypothesis that the enzyme thymidylate synthetase may represent the deciding target. The 5-FU metabolite FdUMP accomplishes the inhibition of TS, forming part of a ternary complex with the enzyme and with the cosubstrate 5,10-methylenetetrahydrofolic acid. The MTX-induced depletion of reduced folate pools in DNA synthesizing cells should antagonize the formation of that ternary complex but the loss of reduced folates is accompanied by the increase of the dihydrofolate levels which can compensate for 5,10-methylenetetrahydrofolic acid in forming the ternary complex, and MTX polyglutamates can participate as well in the formation of the ternary complex [22, 23].

While in L1210 cells *in vitro* the amount of FdUMP bound to TS was found to be significantly decreased following preincubation with MTX [24] we found increased 5-FU in the TS complex fraction following MTX pretreatment in Sarcoma 180 *in vivo*. Analysis of the TS fraction revealed only one peak which is likely to represent FdUMP altered during the preparation procedure. The identity of this metabolite is now being investigated.

Finally, another potential pathway of MTX-5-FU synergism may find its base in the incorporation of nucleotide metabolites into the DNA of treated cells [25–27]. Enhanced excision of 5-FU residues from DNA is induced by the presence of MTX and thymidine, which could augment the fragmentation of DNA by cyclic incorporation and removal of 5-FU nucleotides by excision-repair processes [27, 28]. That process of MTX-5-FU synergism requires the presence of high concentrations of thymidine above the usual physiologic levels [28, 29]. In our study the impact of 5-FU incorporated into DNA cannot be determined. However, radioactivity recovered in the DNA fraction was extremely low.

In the past the mechanisms of action of sequential MTX-5-FU has mostly been studied *in vitro*. The relevance of these findings to the *in vivo* situation has been called into question since they are results of studies in non-physiological culture conditions [11]. It is of interest that our results of an *in vivo* study seem to confirm both major hypotheses for the synergism [4, 23].

However, further research is necessary to fully understand this mechanism and to be able to explore it to a higher extent for clinical purposes.

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