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5-Fluorouracil and Methotrexate Combination Chemotherapy: The Effect of Drug Scheduling*

J. H. MULDER,†‡§ T. SMINK† and L. M. VAN PUTTEN†

†*Radiobiological Institute TNO, Rijswijk, The Netherlands,* ‡*Department of Internal Medicine, Rotterdam Radiotherapeutic Institute, Rotterdam, The Netherlands*

Abstract—*The effect of combination chemotherapy with 5-FU and MTX administered either simultaneously or sequentially with time intervals of up to* 24 hr *was studied in the* L1210 *leukaemia, the mouse osteosarcoma* C22LR *and the Lewis lung carcinoma. Tumour growth delay, the number of lung metastases and lung colonies and survival times of leukaemic mice were used as parameters. Sequential treatment with MTX followed by 5-FU was the most effective antitumour schedule in the* L1210 *leukaemia and the osteosarcoma. This sequence of drug administration, however, also resulted in a marked weight loss and in early toxic deaths of the animals, in contrast to the other schedules investigated. The implications of our findings along with an analysis of the experimental data from the literature are discussed in relation to the clinical applicability of this drug combination.*

INTRODUCTION

DRUG schedules are usually based on physician or patient convenience rather than on a biochemical or kinetic rationale. According to experimental data, the clinical application of 5-fluorouracil (5-FU) and methotrexate (MTX) in combination could result in an additive, a synergistic or even an antagonistic effect [1–12]. Since the two drugs are frequently used simultaneously in the adjuvant therapy of operable breast cancer as well as for the treatment of advanced disease, it was decided to determine whether an increase in antitumour effect and a decrease in drug induced toxicity could be achieved by drug scheduling. The effects of treatments were investigated in three tumour cell lines: the L1210 leukaemia, the C22LR osteosarcoma and the Lewis lung carcinoma. This panel of tumour cell lines was used because it was believed that conclusions based on results obtained in a variety of tumours might be more relevant clinically than those derived from results from one tumour cell line. In general, antitumour effects should be compared with the effects of treatment on critical normal tissues. For this purpose, bone marrow stem cell assays as well as whole animal toxicity studies were performed concurrently with the tumour investigations. This strategy has been applied to a variety of two-drug combinations, results of which have been published previously [13–18].

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§Reprints requests to: Dr. J. H. Mulder, Radiobiological Institute TNO, Lange Kleiweg 151, 2288 GJ Rijswijk, The Netherlands.

MATERIALS AND METHODS

Detailed information on materials and methods have been presented in previous reports [13–18]. The most relevant data are given in Table 1.

Tumours and endpoints

The median survival time of L1210 leukaemic mice was used to calculate the percentage increase in survival (% ILS). From the metastasizing Lewis lung tumour and the nonmetastasizing osteosarcoma, single cell suspensions were made and one million tumour cells were inoculated i.m. and s.c., respectively [13]. The endpoint of treatment in both solid tumour lines is the growth delay

Table 1. Biological systems and methods

Biological systems	Endpoints
L1210 leukaemia i.p. in CD2/F1	% increase in life span
Lewis lung carcinoma i.m. in C57BL/Ka or BCBA/F1 (metastasizing)	Growth delay and number of lung metastases
C22LR osteosarcoma s.c. bilaterally (nonmetastasizing) or i.v. in BCBA/F1	Growth delay or number of lung colonies per mouse
Hemopoietic stem cells in BCBA/F1	% surviving cells per femur
Gastrointestinal tract toxicity	Weight loss and early toxic deaths of mice

in days: the displacement in time between the growth curve of the tumours in the control group and the growth curve of the tumours recurring after treatment. The displacement between the growth curves is chosen at a point (usually close to the original tumour volume) where the growth curves for treated and control animals are again parallel. If no original tumour volume is known (e.g. for tumour inocula exposed to treatment before a tumour is palpable), the delay in comparison with the control group to reach an arbitrary volume between 400 and 800 mm^3 is estimated. The endpoint of the lung nodule assay is the number of spontaneous lung metastases for the Lewis lung tumour or percentage of surviving artificially induced lung colonies per mouse for the osteosarcoma. The percentage of surviving hemopoietic stem cells was determined by the spleen colony technique of Till and McCulloch. For the significance calculations, Student's *t*-test was used.

Chemicals and treatment schedules

When 5-fluorouracil (Hoffman–La Roche) and MTX (Lederle Laboratories) were given in combination, the drugs were injected either simultaneously (5-FU + MTX) or with a time interval in between (5-FU → MTX and MTX → 5-FU). The length of time intervals between 5-FU and MTX administration varied from 1 to 24 hrs. 5-FU was given i.p. and MTX s.c. 5-FU in combination with fractionated dosages of MTX was administered at mid-day.

RESULTS

Table 2 presents % ILS data for L1210 bearing mice treated with three different 5-FU/MTX schedules. The effect of MTX $\xrightarrow{24\,hr}$ 5-FU was marginally more effective than 5-FU + MTX and significantly more effective than 5-FU → MTX ($P<0.01$). Early toxic deaths were sometimes observed, resulting from diarrhoea and extreme weight loss. As shown in Fig. 1, the weight loss was highly schedule dependent: the maximum weight reduction after MTX $\xrightarrow{24\,hr}$ 5-FU administration was 28% of the original weight at the start of treatment, which corresponded with approximately 5 g animal weight. To substantiate the suggestion that the observed weight loss and early toxic deaths were likely results of gastrointestinal mucosal damage and not consequences of bone marrow failure, hemopoietic stem cell assays were performed. The data in Table 2 show that there was no indication of increased cytotoxicity after MTX $\xrightarrow{24\,hr}$ 5-FU administration.

Table 2. The effect of 5-FU/MTX drug scheduling in mice bearing leukaemia L1210 *and on normal bone marrow stem cells*

Treatment schedules	L1210 leukaemia % ILS			Hemopoietic stem cells* % surviving cells		
5-FU $\xrightarrow{24\,hr}$ MTX	43			47		
5-FU + MTX	55	N.S.	$P<0.01$	50	N.S.	N.S.
MTX $\xrightarrow{24\,hr}$ 5-FU	59†			34		

L1210 data are means of four separate experiments in which groups of ten CD2/F1 mice were inoculated i.p. with 10^5 L1210 cells on day 0. Treatment was given on days 3 and 4. 5-FU was given i.p. in a dose of 100 $mg.kg^{-1}$ and MTX at s.c. 40 $mg.kg^{-1}$.

*Data are means of two separate experiments. 5-FU was given i.p. in a dose of 500 $mg.kg^{-1}$ and MTX s.c. at 40 $mg.kg^{-1}$.

†Two of 40 mice at risk died of toxicity.

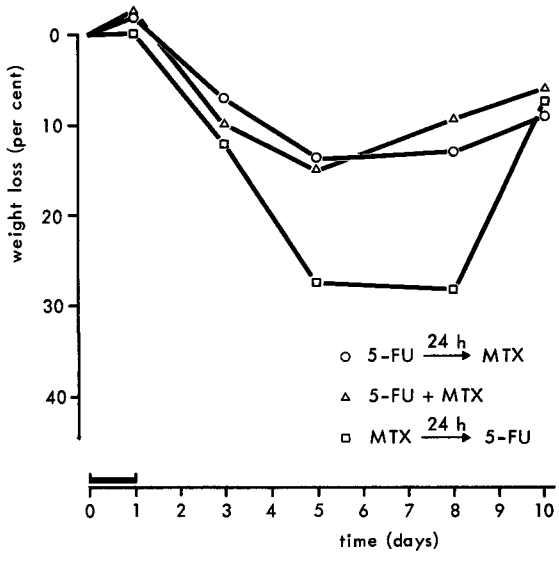

Fig. 1. Schedule dependent weight loss in L1210 *leukaemic mice. Groups of* 10 *CD2 mice inoculated with* L1210 *leukaemia cells on day* 0 *were treated i.p. with* 5-*FU* 100 mg.kg^{-1} *and s.c. with MTX* 40 mg.kg^{-1} *as indicated.*

The treatment of i.m. inoculated Lewis lung carcinoma with MTX given at a dose of 40 mg.kg^{-1} s.c. was completely ineffective. As MTX is a cell cycle phase specific drug, to increase the effect of therapy, the MTX dose was modified from single dose therapy (40 mg.kg^{-1} s.c.) to intermittent administration of four times 2.5 mg.kg^{-1} s.c. every 3 hr. This type of drug administration is comparable to slow infusion of MTX. From the MTX single drug data (not included here), it was concluded that the Lewis lung carcinoma is a MTX-resistant tumour and therefore an inappropriate test system for 5-FU/MTX investigations. The side effects of the treatment, however, proved to be very important. The intermittently given MTX in combination with 5-FU resulted in weight loss and sometimes even in toxic death of the mice bearing Lewis lung tumour. Cumulative data on toxicity from a total of 13 Lewis lung experiments are given in Table 3. In marked contrast to the other two schedules investigated (5-FU + MTX and 5-FU $\xrightarrow{24\,hr}$ MTX), the sequence MTX $\xrightarrow{24\,hr}$ 5-FU was highly toxic to the animals.

The osteosarcoma was sensitive to intermittently administered MTX treatment. To reduce the toxicity of the therapy, the MTX was administered three times instead of four with a 3-hr interval between dosages. As a result of this less protracted MTX administration, no mice died of toxicity and marked weight loss was only observed after MTX $\xrightarrow{24\,hr}$ 5-FU treatment. The results of osteosarcoma experiments are given in Table 4. From the growth delay data as well as those from the lung colony assays, it is concluded that MTX $\xrightarrow{24\,hr}$ 5-FU was the most cytotoxic

Table 3. Early toxic death as a result of sequential administration of methotrexate and 5-*fluorouracil*

Treatment schedules	% early toxic deaths
5-FU $\xrightarrow{24\,hr}$ MTX	1.5
5-FU + MTX	7.7
MTX $\xrightarrow{24\,hr}$ 5-FU	50.7

Lewis lung carcinoma bearing mice were treated i.p. with 5-FU 100 mg.kg^{-1} i.p. in combination with MTX. The s.c. injections of MTX were given either repeatedly over a time period of 12 hr (e.g. 2.5 mg.kg^{-1} every 3 hr ×4) or as a single dose (40 mg.kg^{-1}). The total number of mice at risk per treatment group was 65.

Table 4. The effect of 5-*FU/MTX drug scheduling in* C22LR *osteosarcoma*

Dosages and treatment Exp. No	Growth delay (days) I	II	Per cent surviving lung colonies per mouse III	IV
5-FU mg.kg^{-1} i.p.	100	100	100	100
MTX mg.kg^{-1} s.c.	3×6	3×7.5	40	3×10
5-FU $\xrightarrow{24\,hr}$ MTX	4.7	5.5	84	23
5-FU + MTX	4.2	4.8	130	23
MTX $\xrightarrow{24\,hr}$ 5-FU	5.7	6.8	64	3

In experiments I and II, groups of five BCBA/F1 mice were inoculated bilaterally s.c. with osteosarcoma cells on day 0. MTX was administered every 3 hr in the early stage of tumour growth. The mean growth delay is given. The maximum S.E. was 1.2 days. In the lung colony assay experiments III and IV, groups of five BCBA/F1 mice were injected i.v. with osteosarcoma cells on day 0. Treatment was given on days 3 and 4. The mean per cent surviving lung colonies per mouse is given. The maximum S.E. was 18%. The differences between the highest values and the lowest are statistically significant ($P<0.01$).

schedule in comparison with 5-FU + MTX and 5-FU $\xrightarrow{24\,hr}$ MTX administration.

The influence of shorter time intervals between 5-FU and MTX administration on the antitumour effect in L1210 leukemia was investigated in separate experiments. The results are shown in Table 5. Sequential administration of MTX followed by 5-FU is a more effective treatment schedule than simultaneous administration or treatment with the reverse sequence.

Table 5. The effect of 5-FU/MTX drug scheduling in osteosarcoma and in L1210 *leukaemia bearing mice*

Treatment schedules	Osteosarcoma growth delay	L1210 leukaemia % ILS
5-FU $\xrightarrow{24\,hr}$ MTX	3.2	39
5-FU $\xrightarrow{16\,hr}$ MTX	2.0	38
5-FU $\xrightarrow{3\,hr}$ MTX	2.0	40
5-FU $\xrightarrow{1\,hr}$ MTX	2.7	38
5-FU + MTX	3.3	42
MTX $\xrightarrow{1\,hr}$ 5-FU	3.4	52
MTX $\xrightarrow{3\,hr}$ 5-FU	3.5	42
MTX $\xrightarrow{16\,hr}$ 5-FU	4.2	55
MTX $\xrightarrow{24\,hr}$ 5-FU	5.5	55

Drugs (5-FU 100 mg.kg^{-1} i.p. and MTX 3 × 7.5 mg.kg^{-1} s.c.) were administered in the early stage of osteosarcoma tumour growth. The mean growth delay ($n=10$) is given. The maximum S.E. was 0.6 days. Groups of ten L1210 leukemic mice were treated i.p. on days 3 and 4 with 5-FU at 100 mg.kg^{-1} and MTX at 40 mg.kg^{-1}. The differences between the highest values and the lowest are statistically significant ($P<0.01$).

DISCUSSION

The emphasis in this discussion will be on whether scheduling of 5-FU and MTX leads to an increase in the therapeutic ratio. The antitumour effectiveness of 5-FU/MTX combination chemotherapy was increased by applying an MTX→5-FU schedule. This sequence of drug administration, however, resulted in a concomitant increase in toxicity. In all of the experiments, regardless of whether mice were inoculated with L1210 or were bearing solid tumours, the animals lost weight and many died within 10 days after MTX→5-FU drug administration. Therefore, although MTX→5-FU treatment is more cytotoxic than 5-FU + MTX or 5-FU →MTX, there seems to be no differential effect on tumour cells and normal cells. The results of this study are in accord with the data from the literature as summarized in Table 6. The effect of drug scheduling has been demonstrated by many authors and the patterns described in the literature and presented in our study are in agreement: the sequence in which MTX precedes 5-FU administration is the most cytotoxic schedule. The point to be stressed here, however, is that in order to obtain a therapeutic gain the most cytotoxic treatment schedule should result in a differential cytotoxic effect on tumour and on normal tissues. This, unfortunately, seems not to be the case.

Many investigators have claimed a synergistic antitumour effect of 5-FU/MTX combination therapy, most notably when the MTX preceded 5-FU administration [1–7]. Recently, Bertino extensively discussed the reasons why MTX followed by 5-FU treatment should be more cytotoxic than simultaneously administered treatment [19]. Ternary complex formation between MTX, fluorodeoxyuridine monophosphate and thymidylate synthetase may possibly play a role in the sequence-dependent efficacy of 5-FU/MTX therapy. It is important to note, however, that, in contrast to suggestions of how to explain drug synergism, others have given data and biochemically related arguments purporting to explain the antagonistic interaction of 5-FU and MTX combination chemotherapy [9–11, 20]. It seems beyond the scope of this paper to present the various hypotheses on 5-FU/MTX interaction; moreover, Friedman and Sadee have summarized them in a review on fluoropyrimidines [21].

Conclusions on synergism or antagonism, however, should be based on dose–response curves of the individual drugs. In the osteosarcoma, the dose–response curve for 5-FU is linear, with an initial shoulder below an i.p. dose of 5-FU of 50 mg.kg^{-1}. The curve for MTX reaches a plateau. The 5-FU and MTX curves in L1210 are linear up to approximately 150 and 40 mg.kg^{-1}, respectively, after which a plateau is reached. Therefore, on the basis of the nonlinearity of both dose–response curves, what can be expected is a decrease in efficacy when 5-FU and MTX are given in combination.

How should the preclinical data as summarized in Table 6 be integrated into the clinical management of patients with malignant disease? From our data and from the analysis of data of other investigators, we suggest separate administration of 5-FU and MTX, thereby avoiding any possible adverse drug interaction. Secondly, we would not suggest, as did Bertino [19], an investigation of whether sequential treatment with MTX followed in a few hours by 5-FU administration leads to improved results in human

Table 6. 5-FU/MTX combination chemotherapy in experimental research

Authors (references)	Tumours	Author's conclusion	Comments
Kline, Venditti, Mead *et al.* [1]	L1210	5-FU + MTX synergism	Protracted treatment schedules were employed. Schedule dependency was not investigated
Bareham, Griswold and Calabresi [2]	Plaque forming capacity	MTX $\xrightarrow{1\,hr}$ 5-FU synergism	The other two schedules investigated, 5-FU → MTX and 5-FU + MTX, were less effective than MTX $\xrightarrow{1\,hr}$ 5-FU
Heppner and Calabresi [3]	Mammary carcinoma in mice	The effect of MTX $\xrightarrow{1\,hr}$ 5-FU was greater than that of the other administration sequences	Single drug data are not presented; therefore the results are difficult to analyse for drug interaction
Bertino, Sawicki, Lindquist *et al.* [4]	Sarcoma 180 in mice	Optimal results with MTX → 5-FU at intervals longer than 1 hr	The 5-FU + MTX and especially 5-FU → MTX, however, was less effective than MTX → 5-FU
Lee and Khwaja [5]	Mammary carcinoma in rats	MTX $\xrightarrow{6\,hr}$ 5-FU was the most effective schedule	5-FU + MTX was less effective than MTX $\xrightarrow{6\,hr}$ 5-FU
Brown and Ward [6]	Mammary carcinoma in mice	MTX $\xrightarrow{6-12\,hr}$ 5-FU synergism	5-FU + MTX was less effective than MTX → 5-FU, as determined by the increase in life-span of tumour-bearing animals
Cadman, Heimer and Davis [7]	L1210 *in vitro*	MTX $\xrightarrow{2-4\,hr}$ 5-FU synergism	Data on the effect of 5-FU + MTX not presented
Donehower, Allegra Lippman *et al.* [8]	Human breast carcinoma *in vitro*	MTX $\xrightarrow{2\,hr}$ 5-FU and 5-FU + MTX were additive	Their 5-FU $\xrightarrow{2\,hr}$ MTX schedule was less effective than the other sequences investigated
Tattersall, Jackson, Connors *et al.* [9]	L5178Y *in vitro* and L1210 *in vivo*	5-FU + MTX antagonism	No *in vitro* effect of scheduling was found by this group of investigators. Scheduling *in vivo* not studied
Waxman and Bruckner [10]	L1210, Friend leukemia and human bone marrow	5-FU + MTX antagonism	
Ullman, Lee, Martin *et al.* [11]	L1210	MTX $\xrightarrow{2\,hr}$ 5-FU antagonism	Therapeutic enhancement, according to the authors, was observed with high doses of MTX 2 hr prior to 5-FU administration. Unfortunately, no data of high doses of MTX → 5-FU are given
Maugh [12]	Tumour cells grown in culture	5-FU + MTX additivity	The author refers in his discussion to the work of Santi and Martin; reference not given
Mulder, Smink and van Putten (this paper)	L1210, C22LR osteosarcoma and gastrointestinal tract	MTX $\xrightarrow{24\,hr}$ 5-FU is the most effective antitumour schedule but concomitantly the most toxic one	

cancers sensitive to both drugs. Although encouraging clinical reports of sequential MTX followed by 5-FU therapy have been presented [22–24], an increase in toxicity has also been observed [25, 26]. As the toxicity of this sequential treatment schedule should lead to drug dose reductions, the physician may eventually end up with a less toxic but at the same time also a less effective antitumour treatment schedule. In our opinion, more preclinical research, including experiments on normal tissues, is needed before patients are submitted to a treatment schedule in which MTX is followed by 5-FU administration.

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