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Schedule-Dependent Cytotoxicity of 5-Fluorouracil and Cyclophosphamide in Experimental Cancer Chemotherapy*

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Abstract—*The effect of drug scheduling of* 5-*fluorouracil and cyclophosphamide was investigated in* L1210 *leukaemia, the Lewis lung carcinoma and the mouse* C22LR *osteosarcoma. The optimum antitumour schedule was found to be that in which the drugs were given simultaneously.*

In the treatment of L1210 *and the Lewis lung carcinoma, drug synergism was observed when the two agents were administered simultaneously. A similar trend was less pronounced for the osteosarcoma. The most cytotoxic schedule for bone marrow stem cells was cyclophosphamide followed by* 5-*fluorouracil. As a consequence of this differential effect, the best tolerated and generally also the most effective antitumour schedule was the simultaneous administration of the two drugs. These experimental data should lead to critical testing of clinically used multi-drug combinations, e.g., Bonadonna's CMF regimen in patients with metastatic mammary carcinoma.*

INTRODUCTION

THE TREND in protocol designs has been to utilise greater numbers of drugs in combination and the use of more complex schedules. It has not been clinically established whether, in 5-fluorouracil (5-FU) and cyclophosphamide (Cyclo) combination chemotherapy, the two drugs are best given simultaneously or sequentially. In Bonadonna's Cyclo, Methotrexate (MTX) and 5-FU combination chemotherapy schedule (CMF) in patients with metastatic mammary carcinoma [1], Cyclo is given daily over a period of 14 days. In the same time period, MTX and 5-FU are administered simultaneously on days 1 and 8. Although CMF therapy in the treatment of patients with breast cancer is acknowledged world-wide [2] as an effective drug combination, there have been few systematic studies designed to determine the optimal sequence in which Cyclo, MTX and 5-FU should be administered. The study reported here is limited to schedules without MTX which were designed to determine whether the sequence in which 5-FU and Cyclo are given alters the therapeutic effectiveness of the combination.

Experimental models for the study of synergistic relationships in combinations of two drugs have been used in a number of laboratories. An enhanced antitumour effect associated with increased toxicity for normal tissues will result in a reduced therapeutic index. Therefore, it was decided to investigate whether a differential effect of 5-FU/Cyclo combination chemotherapy could be obtained in a variety of tumour cell lines and in haemopoietic bone marrow stem cells. It was considered that the result of such an approach might provide relevant experimental data for application in the clinical management of patients requiring 5-FU/Cyclo combination chemotherapy.

MATERIALS AND METHODS

These experiments were designed as part of a study on drug scheduling [3–7]. More detailed information on materials and methods have been reported previously [3].

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Tumours

Our Lewis lung carcinoma was obtained from the "Mario Negri" Institute, Milan, Italy in 1973. For the experimental work, 10^6 tumour cells from a single cell suspension were inoculated into the gastrocnemius muscle of $(C57Bl/Rij \times CBA/Rij)F_1$ (hereafter called BCBA F_1) mice. The effect of treatment on lung metastases was assessed by counting the number of macroscopic metastases on the lung surface 30 days after i.m. tumour inoculation. For the assessment of the effect of treatment on artificial lung colonies produced by i.v. injected Lewis lung tumour cells, the number of colonies was counted at day 18 after i.v. inoculation. The strontium-90 induced C22LR osteosarcoma originated in a mouse in our institute in 1957. In the growth delay experiments, 10^6 cells were injected bilaterally s.c. into BCBA F_1 mice. The osteosarcoma does not give rise to spontaneous lung metastases. For the lung colony assay, a specific number of osteosarcoma cells was injected i.v. into one of the tail veins of BCBA F_1 mice. The L1210 leukaemia was obtained from Yale University, New Haven, CT, in 1969. $(BALB/c \times DBA/2)F_1$ mice (hereafter called CD2 F_1) were used in the i.p. inoculated L1210 survival experiments.

Haemopoietic stem cell systems

To determine the activity of drugs against normal cells, haemopoietic stem cells from BCBA F_1 mice were used. The survival of the stem cells was determined by the spleen colony technique of Till and McCulloch. After treatment with a cytostatic drug, the normally resting stem cells will be recruited into cycle and this may result in increased drug sensitivity. Therefore, the effect of treatment on recruited rapidly proliferating normal bone marrow stem cells was also investigated by the technique described previously [3].

Chemicals

5-Fluorouracil, NSC-19893, was kindly supplied by Hoffman La Roche, Basel, Switzerland, and cyclophosphamide, NSC-26271, was a gift from Asta-Werke, Brackwede, Germany. Both drugs were injected i.p.

Treatment schedule

The drug sequences tested were 5-FU and Cyclo given simultaneously (5-FU + Cyclo), 5-FU given 24 hr before (5-FU $\xrightarrow{24\,hr}$ Cyclo) or 24 hr after Cyclo administration (Cyclo $\xrightarrow{24\,hr}$ 5-FU).

Endpoints and statistical evaluation

The endpoint of treatment in both solid tumour lines is the growth delay 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 400–800 mm^3 is estimated. The endpoint of the lung assay is the number of spontaneous lung metastases or number of artificially induced lung colonies per mouse. The results of the Lewis lung survival experiment are expressed in median survival time in days (MdST). Drug induced toxic death is defined as death within 10 days after the end of treatment unless clearly caused by tumour growth. Data of the L1210 survival experimentals are expressed in terms of mean survival time difference to that of control mice. All leukaemic mice died of the disease. The results of the effect of treatment on resting and rapidly proliferating haemopoietic stem cells were calculated as the mean number of colony forming unit (CFU) per donor femur. These results were compared with the results of a simultaneous assay in a group of untreated donor mice to determine the relative CFU survival. Data of the stem cell experiments are expressed in percentage surviving cells. Student's *t*-test is used for the evaluation of the results of treatment with the different sequential combinations.

RESULTS

Table 1 shows the effect of 5-FU and Cyclo in Lewis lung carcinoma when the agents are administered to the mice before tumours are palpable (exp. No. 1); 5-FU given simultaneously with Cyclo was significantly more effective than the algebraic sum of the values for growth delay determined for 5-FU and Cyclo separately, suggesting drug synergism. The sequence 5-FU → Cyclo was the least effective of the schedules tested, indicating a schedule dependent effectiveness of 5-

Table 1. The effect of sequential treatment on tumour volume, number of spontaneous lung metastases and on survival time of Lewis lung carcinoma inoculated mice

Drugs and treatment schedules	Exp. No. 1 Growth delay and survival time		Exp. No. 2 Growth delay and No. of lung metastases per mouse	
Control	0 ±0.4	29 (26–32)	0 ±0.4	66±4
5-FU	2.3±0.7	30 (29–31)	not done	60±8
Cyclo	4.4±0.7	33 (27–37)	not done	27±6
5-FU $\xrightarrow{24\,hr}$ Cyclo	7.5±0.2 } $P<0.001$	36 (29–40) } n.s.	6.9±0.5 } $P<0.01$	33±4 } $P<0.01$
5-FU + Cyclo	13.4±0.6 } n.s.	41 (36–46) } n.s.	10.1±0.7 } n.s.	12±3 } $P<0.01$
Cyclo $\xrightarrow{24\,hr}$ 5-FU	10.6±2.0	39 (34–46)	9.0±0.4	46±12
Expected effect of the combination	6.7	34		

BCBA F_1 mice were inoculated i.m. with 10^6 Lewis lung tumour cells on day 0. In exp. No. 1, groups of 5 mice were treated on day 3 with Cyclo, 100 mg/kg i.p. A dose of 100 mg/kg i.p. 5-FU was given either simultaneously, 24 hr before or 24 hr after Cyclo administration. In exp. No. 2, groups of 10 mice were treated on day 8 with the same treatment schedule as given in exp. No. 1. Lung metastases were counted on day 30, at which time 5 mice in the control group had died from tumour. The means with the standard errors of growth delay and of number of lung metastases per mouse are given. The median survival time in days is given, with the range in parentheses.

FU/Cyclo combination chemotherapy. After the tumour volume measurements were performed, mice were kept until death occurred as a result of primary tumour growth and the development of multiple lung metastases. The results, expressed in median survival time, show the same pattern as described earlier for the growth delay data. In exp. No. 2 of Table 1, the tumour bearing animals were sacrificed at day 30 postinoculation after the tumour volume data were obtained. The effect of drug scheduling with a time interval of 24 hr is clearly demonstrated; in comparison with sequentially given treatments (5-FU⇌Cyclo), 5-FU + Cyclo therapy resulted in a longer growth delay and a significantly smaller number of lung metastases per mouse. The effect of combination chemotherapy was also investigated on artificially induced lung colonies (Table 2). Treatment in which Cyclo was followed at a time interval of 24 hr by 5-FU (Cyclo $\xrightarrow{24\,hr}$ 5-FU) was less effective than simultaneous administration of the two agents. There was, however, a suggestion of an increased number of drug induced toxic deaths as a result of sequentially administered treatment. This tendency of schedule dependent drug toxicity is confirmed by the data in Table 3. Cumulative toxicity data on eight separate Lewis lung carcinoma experiments show simultaneous treatment or 5-FU followed by Cyclo to be less toxic than Cyclo followed by 5-FU administration.

Osteosarcoma inoculated mice, either bearing the 'primary' tumours or showing multiple artificially induced lung 'metastases'

Table 2. The effect of sequential treatment with 5-fluorouracil and cyclophosphamide on Lewis lung carcinoma assayed by the lung colony technique

Drugs and treatment schedules	Exp. No. 1	Exp. No. 2
Dose of 5-FU	75	100
Dose of Cyclo	75	75
	No. of lung colonies per mouse	
5-FU $\xrightarrow{24\,hr}$ Cyclo	100±19	29±3 (1)
5-FU + Cyclo	47±14 (1)	3±2
Cyclo $\xrightarrow{24\,hr}$ 5-FU	83±10 (1)	13±6 (2)

On days 4 and 5 after i.v. injection of 10^6 Lewis lung carcinoma cells, groups of 5 BCBA F_1 mice were treated i.p. with 5-FU and Cyclo in dosages (mg/kg) as indicated. Lung colonies were counted on day 18. In parentheses are the number of deaths per group due to drug toxicity or infection.

Table 3. Percentage early toxic deaths as a result of sequential administration of 5-fluorouracil and cyclophosphamide

Treatment schedules	Early toxic deaths (%)
5-FU $\xrightarrow{24\,hr}$ Cyclo	15.4
5-FU + Cyclo	13.8
Cyclo $\xrightarrow{24\,hr}$ 5-FU	44.3

Cumulative data of 8 separate Lewis lung carcinoma experiments. Drugs were administered in the early stage of the disease or late, approximately on day 12 after tumour inoculation. The dosages of 5-FU and Cyclo varied between 50 and 100 mg/kg i.p. The total number of mice at risk per treatment group was 65.

were submitted to 5-FU/Cyclo combination chemotherapy as shown in Tables 4 and 5. The observed growth delay after 5-FU and Cyclo in Table 4 was marginally longer than what could be calculated by adding the growth delay data for the two drugs separately. The effect of drug scheduling showed 5-FU + Cyclo to be significantly more effective than 5-FU $\xrightarrow{24\,hr}$ Cyclo treatment. Detailed results of a lung colony assay are shown in Table 5. The animals were treated on days 4 and 5 after i.v. injection of a specified number of osteosarcoma cells. Approximately 2 weeks later, the mice were sacrificed and the number of macroscopic nodules on the surface of both lungs was counted. As shown in Table 5, an accurate estimate of the number of colonies was impossible at times, because of infectious lung disease. From the mean number of colonies per 10^6 cells injected, the percentage of surviving cells could be calculated. The data presented suggest drug synergism; the observed percentage was three times smaller than the expected percentage of surviving cells calculated by multiplying the individual survival fractions.

L1210 leukaemic mice were treated with 5-FU and/or Cyclo (Table 6) on days 3 and 4 after inoculation. The observed effect was significantly greater than the algebraic sum of

Table 4. The effect of 5-fluorouracil and cyclophosphamide combination chemotherapy on C22LR osteosarcoma expressed in tumour growth delay

Drugs and treatment schedules		
	Exp. No. 1	Exp. No. 2
Days of treatment	8, 9	2, 3
Dose of 5-FU	100	100
Dose of Cyclo	50	100
No. of mice/group	5	10
	Growth delay (days)	
Control	0 ±0.2	0 ±0.3
5-FU	1.3±0.3	1.2±0.3
Cyclo	3.4±0.1	13.5±0.3
5-FU $\xrightarrow{24\,hr}$ Cyclo	4.6±0.4 } $P<0.05$	13.7±0.3 } $P<0.01$
5-FU + Cyclo	6.5±0.4 } n.s.	15.2±0.3 } n.s.
Cyclo $\xrightarrow{24\,hr}$ 5-FU	6.3±0.6	14.8±0.4
Expected effect of the combination	4.7	14.7

BCBA F_1 mice were injected bilaterally s.c. with 10^6 osteosarcoma cells on day 0. Dosages are given in mg/kg and the route of administration was i.p.

Table 5. Results of a C22LR osteosarcoma lung colony assay: The effect of 5-fluorouracil and cyclophosphamide combination chemotherapy

Treatment	No. of cells injected ($\times 10^5$)	No. of colonies per mouse	Mean number of colonies per mouse	Mean number of colonies per 10^6 cells	Percentage of surviving cells
Control	0.5	32, 12, 23, 19, *	21.5	430	100
	1	43, 6, 13, 50, *	28.0	280	
	2	87, 108, 90, 25, *	77.5	387	
				$366/10^6 =$	
5-FU	5	65, 58, 60, 53, *	53.4	107	29
Cyclo	5	23, 15, 25, 86, 22	34.2	68	19
5-FU $\xrightarrow{24\,hr}$ Cyclo	7.5	31, 37, 38, 44, * } $P<0.01$	37.5	50	14
5-FU + Cyclo	7.5	6, 4, 5, 11, 3 } n.s.	5.8	8	2
Cyclo $\xrightarrow{24\,hr}$ 5-FU	7.5	12, 6, 40, 5, 12	15.0	20	5
Expected effect of the combination					6

On days 4 and 5 after i.v. injection of osteosarcoma cells, groups of 5 BCBA F_1 mice were treated i.p. with 5-FU 100 mg/kg and Cyclo 50 mg/kg. Lung colonies were counted on day 18. An asterisk indicates an animal with lungs in which tumour colonies could not be distinguished macroscopically from infection.

Table 6. The influence of drug scheduling with 5-fluorouracil and cyclophosphamide on the mean survival time of L1210 leukaemic mice

Drugs and treatment schedules	Mean survival time difference to that of control mice				
	Experiment No.				
	1	2	3	4	5
5-FU	5.8	3.4	3.3	3.6	3.2
Cyclo	4.1	4.0	4.2	2.1	2.8
5-FU $\xrightarrow{24\,hr}$ Cyclo	8.2	9.8	9.5	7.7	7.7
5-FU + Cyclo	10.4	12.4	15.6	13.6	14.5*
Cyclo $\xrightarrow{24\,hr}$ 5-FU	7.4	8.3	9.9	6.8	6.2
Expected effect of combination	9.9	7.4	7.5	5.7	6.0

On days 3 and 4 after i.p. inoculation of 10^5 L1210 cells, groups of 10 CD2 F_1 mice were treated i.p. with 5-FU 100 mg/kg and Cyclo 100 mg/kg. These dosages are nontoxic as determined by concurrent nonleukemic toxicity control animals. The maximum standard error of the MST was 1.3 days. The *P*-values according Student's *t*-test between sequentially and simultaneously given treatments were at most 0.005.

*Time intervals of 6 and 12 hr between the administration of 5-FU and Cyclo resulted in mean survival time differences of approx. 10 days.

the individual effects in all but one experiment (exp. No. 1). The *P*-values according to Student's *t*-test for a difference between sequentially and simultaneously given treatments were at most 0.005.

To assess the activity of 5-FU/Cyclo combination chemotherapy against normal cells, the effects of various schedules on resting and proliferating bone marrow stem cells were evaluated (Table 7). In the resting stem cells, the Cyclo $\xrightarrow{24\,hr}$ 5-FU schedule is a synergistically active drug sequence and clearly more toxic in comparison with simultaneous treatment. In the recruited, rapidly proliferating stem cells, however, neither drug synergism nor schedule dependency was observed.

DISCUSSION

The ultimate goal of combining two drugs in a combination chemotherapy schedule is to increase the therapeutic index (the ratio between the effect on the tumour and the effect on normal tissues). The principal question to be discussed in this study is whether optimal scheduling of 5-FU and Cyclo leads to a differential effect and whether an improvement in the therapeutic index is achieved. The discussion as to whether a differential effect has been achieved in various tumour cell lines and in bone marrow stem cells should be separated from the argument of whether 5-FU/Cyclo synergism has been demonstrated.

The antitumour effectiveness of 5-FU/Cyclo combination chemotherapy was evaluated in two solid tumour cell lines and in L1210 leukaemia. Of the three schedules evaluated, the simultaneous administration of 5-FU and Cyclo seems to be the preferred one. The results shown in Table 7 indicate that, for resting haemopoietic stem cells, Cyclo $\xrightarrow{24\,hr}$ 5-FU gives the lowest survival fraction in comparison to other treatment schedules. This schedule dependent effectiveness on resting bone marrow stem cells corresponds with the observed increase in early toxic deaths after Cyclo $\xrightarrow{24\,hr}$ 5-FU treatment of Lewis lung tumour bearing mice (Table 3); simultaneous administration of 5-FU and Cyclo is the least toxic schedule in comparison with sequentially given treatment. To increase the therapeutic index of 5-FU/Cyclo treatment, one should choose the least toxic sequence and the most

Table 7. The influence of drug scheduling with 5-fluorouracil and cyclophosphamide on the percentage of surviving normal haemopoietic bone marrow stem cells

Treatment	Resting BM stem cells					Repopulating BM stem cells		
Exp. No.	I	II	III	IV	V	VI	VII	VIII
Dose of 5-FU (mg/kg)	50	50	50	50	50	25	25	15
Dose of Cyclo (mg/kg)	50	50	50	50	50	25	25	25
	Surviving cells (%)							
5-FU $\xrightarrow{24\,hr}$ Cyclo	10.0	1.9	9.6	18.2	9.9	5.8	6.2	13.5
5-FU + Cyclo	11.1	5.4	11.7	17.5	11.4	3.7	6.3	14.3
Cyclo $\xrightarrow{24\,hr}$ 5-FU	6.2	1.1	2.1	7.4	4.2	5.4	3.9	8.2
Expected effect of combination	16.9	7.5	7.7	17.7	12.5	3.3	4.2	11.8

effective anti-tumour schedule: simultaneous administration of the two drugs.

Is 5-FU + Cyclo a synergistic drug combination? The identification of synergism should be based on dose-response data. Preliminary data for Cyclo, obtained in the osteosarcoma and L1210 leukaemia, shows a linear dose response relationship for growth delay and survival time. For 5-FU, the dose response investigations were complicated by an observed low therapeutic index for the drug. Although a significantly more than additive effect for 5-FU and Cyclo treatment was obtained in many experiments, the main emphasis in this study was not on 5-FU/Cyclo synergism but on the potential benefit to be gained from optimal scheduling, which depends on the relative effects of treatment on tumours and normal tissue.

The number of reports on 5-FU/Cyclo combination chemotherapy in experimental tumour systems is surprisingly scarce, this in marked contrast to the large amount of literature on the clinical efficacy of this combination. Mutz *et al.* demonstrated that the cytotoxicity of 5-FU given 24 to 36 hr before Cyclo leads to maximum synergistic killing of L1210 leukaemic colony forming units but not of normal haemopoietic stem cells [8]. Fernandes *et al.* [9] showed a synergistic effect of simultaneously administered 5-FU and Cyclo in L1210 leukaemia. An additive effect of 5-FU and Cyclo administration in sarcoma-180 was shown by Kanzawa *et al.* [10]. The discrepancies among the results of different investigators are disturbing and, apart from differences in tumour cell lines, difficult to explain. To explain the observed synergistic effect of 5-FU and Cyclo and the effect of scheduling, various mechanisms must be considered. An alkylating agent such as Cyclo with the potential to cross-link complementary strands of the DNA double helix may cause synergistic inhibition of tumour growth when employed in combination with antimetabolites. 5-FU, which inhibits thymidilate synthetase after conversion to a deoxyribonucleotide, will limit the availability of deoxyriboncleoside triphosphate needed for both repair and the replication of DNA. This complementary inhibition [11] of 5-FU and Cyclo directed at the precursor level and polymer level, respectively, may be responsible for the synergistic interaction observed in the L1210 leukaemia experiments and for the less convincingly demonstrated synergism in the solid tumour experiments. Synergism based on a pharmacokinetic interaction of 5-FU and Cyclo seems unlikely. The results of the investigations described by Fernandes *et al.* indicate that 5-FU does not alter the rate of conversion of Cyclo nor could an increased intracellular level of 5-fluoro-2′-deoxyuridine-5′-monophosphate be demonstrated after Cyclo administration. Drug synergism in the resting stem cell assays was observed only after Cyclo $\xrightarrow{24\,hr}$ 5-FU treatment. The explanation may be that, after the administration of Cyclo, recruitment of resting cells takes place and, because 5-FU is more effective on proliferating than on resting cells, an enhanced effect of 5-FU should then be anticipated [12].

The design of a chemotherapeutic regimen will generally be based on cell kinetic and pharmacokinetic information and on clinical experiences. Clinical trials comparing different dose intervals of 5-FU and Cyclo will be difficult to initiate logistically, among other reasons, because the number of variables in such a schedule will be too many. In designing treatment protocols, the clinical investigator tends to use common sense in employing data on effectiveness, tolerance and feasibility as principal guidelines. In the CMF regimen, 5-FU and Cyclo have overlapping bone marrow toxicity and clinicians must often reduce dosages to avoid toxicity, and compromise the therapeutic effect. As Broder and Carbone convincingly demonstrated, both drugs appear to have a linear dose-response curve and the clinically observed tumour response rate of 5-FU and Cyclo appears to be dependent on the product of drug concentration and drug exposure time [13]. As a consequence, when one uses a multi-drug regimen including 5-FU and Cyclo, it is desirable to utilize maximally tolerated schedules for effectiveness. From our data, the best tolerated and generally also the most effective antitumour schedule is the simultaneous administration of 5-FU and Cyclo. This conclusion restricts one to single dose administrations of 5-FU and Cyclo and may not be fully applicable to multidose schedules as utilized, for instance, in Bonadonna's CMF regimen. Notwithstanding this limitation, when 5-FU/Cyclo combination chemotherapy is considered in patients with metastatic mammary carcinoma, it seems preferable to give the two drugs simultaneously.

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