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Adriamycin/Cyclophosphamide Combination Chemotherapy: The Importance of Drug Scheduling*

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Abstract—*The effect of adriamycin/cyclophosphamide combination chemotherapy was evaluated against a spectrum of transplantable tumours in mice (L1210 leukaemia, Lewis lung carcinoma and C22LR osteosarcoma) and critical normal tissue. Treatment was given either simultaneously or sequentially with a time interval of* 24 hr. *Drug synergism was observed in the treatment of L1210 when the two agents were administered simultaneously. In the solid tumour experiments, neither synergism nor schedule dependency could be demonstrated. From the bone marrow stem cell assays, it is concluded that simultaneous treatment is the least effective and therefore the least toxic schedule. Based on these data, it may be recommended that, in clinical cancer treatment, adriamycin and cyclophosphamide be given simultaneously and not sequentially.*

INTRODUCTION

FOR OPTIMAL chemotherapy of tumours with combinations of different types of drugs, it is of great importance to have available detailed data on dose, time and sequence of drug applications that result in maximum tumour response and minimal side effects. Such procedures of drug application are designated as "drug scheduling".

The combination of the DNA-intercalating antibiotic adriamycin (Adria) and the alkylating agent cyclophosphamide (Cyclo) has been shown to be very effective in treating several animal and human tumours. Because of the increasing interest in the use of the Adria–Cyclo combination in adjuvant therapy for breast cancer patients, it is worthwhile to investigate whether it is possible to increase the therapeutic ratio by employing different treatment schedules with Adria–Cyclo.

The work reported here is concerned with the question of selective potentiation of effects on the tumour relative to the normal tissue. The method of evaluation was based on a differential effect against a spectrum of transplantable tumours in mice and on bone marrow stem cells. Thus, two major considerations in this study were (a) the sequence in which the two drugs should be administered to obtain the optimal antitumour effect; (b) the least toxic schedule of Adria–Cyclo combination chemotherapy. The present experiments were designed as part of a study on drug schedule design of which results have been reported [1–4].

MATERIALS AND METHODS

Adriamycin (NSC 123127) was a gift from Farmitalia, Milan, Italy, and cyclophosphamide (NSC 26271) was provided gratuitously by Asta-Werke, Brackwede, Germany. The L1210 leukaemia survival experiments were carried out in CD2 F_1 mice. The Lewis lung carcinoma was maintained and transplanted in the C57 Bl/Ka mouse and the C22LR osteosarcoma studies were perfor-

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med in BCBA F_1 mice. The solid tumour cell lines were of one passage from frozen samples [1,2]. Resting and rapidly proliferating haemopoietic bone marrow stem cells of BCBA F_1 mice were employed for studying responses of the normal tissue.

From an exponentially growing L1210 *in vitro* culture, 10^5 cells were injected i.p. into mice. Treatment i.p. with Adria and Cyclo was either simultaneously or sequentially: Adria 24 hr before (Adria→Cyclo) or 24 hr after Cyclo injection (Cyclo→Adria). The median survival time (MdST) excluding long term survivors was used as an index of antitumour activity. Mice surviving for 60 days after inoculation and showing no evidence of leukaemia at autopsy were recorded as long term survivors. Data are expressed in terms of the percent increase in life-span (% ILS) as determined by the equation:

$$\frac{\text{MdST of treated} - \text{MdST of control}}{\text{MdST of control}} \times 100 = \%\ \text{ILS}.$$

Synergism in L1210 is suggestive when the % ILS corresponding to the effect observed after combined treatment is greater than the algebraic sum of the values of % ILS determined for the two agents separately. Drug induced toxic death is defined as death within 10 days of the end of treatment unless clearly caused by tumour growth. The technique of preparing a single cell suspension of the Lewis lung carcinoma and the C22LR osteosarcoma have been described in detail [1]. The method of evaluation of the effect on solid tumours is presented here only in outline: tumour growth delay in days is the displacement in time between the growth curve of the control group and the growth curve of the tumours recurring after treatment. If no original tumour volume was known (e.g., for tumour inocula exposed to treatment before a tumour was palpable), the delay in comparison with the control group to reach an arbitrary volume between 400–800 mm^3 was estimated.

To assess the effect of drug scheduling against normal cells, the survival of resting haemopoietic stem cells was determined by the spleen colony assay technique. After cytostatic drug treatment, the normally resting stem cells will be recruited into cycle and this may result in increased toxicity. Therefore, the effect of treatment on recruited, rapidly proliferating normal bone marrow stem cells was also investigated [1]. The survival of resting and proliferating CFU was calculated as the ratio of the mean number of CFU per donor femur and per spleen, respectively, of treated to that of simultaneously assayed control mice. Synergistic effect on bone marrow cells is suggestive when the observed percentage is smaller than the expected percentage of surviving cells calculated by multiplying the values of percentage surviving cells determined for the two agents separately.

For the statistical evaluation of growth delay data, the Student's *t*-test was used. Comparison of survival for the different groups of leukaemic mice was accomplished by means of the Wilcoxon test.

RESULTS

The therapeutic effect of Adria + Cyclo and the influence of drug scheduling (Adria⇄Cyclo) on the percentage increase in life-span (% ILS) for L1210 leukaemic mice is shown in Table 1. The expected % ILS calculated from the data of the single drug treatments by assuming an additive effect was smaller than the observed % ILS after simultaneous application of the drugs, indicating drug synergism. Furthermore, the data in Table 1 show that sequential treatment with an interval of 24 hr was less effective than simultaneous treatment, but not significantly different as compared to the calculated % ILS; this indicates that the effect was additive in these treatments. The results of treatment in the two solid tumour lines are shown in Table 2. Simultaneous treatment was additive and no effect of drug scheduling with an interval of 24 hr could be demonstrated except in the Lewis lung tumour experiment No. 240 LL. To obtain a growth delay after Adria administration comparable to the growth delay measured after Cyclo treatment, the dosage of Adria was increased from 12.5 to 15 mg/kg i.p. As shown in Table 3, this resulted in a high proportion of toxic deaths in BCBA F_1 and C57Bl/Ka mice. The incidence of toxic death was significantly lower when the two drugs were given simultaneously. Schedule dependent host toxicity could also be demonstrated in a survival experiment using L1210 leukaemic mice. A slight increase in Adria dosage from 2.5 to 3 mg/kg and, most notably, when the route of administration was changed from i.p. to i.v. resulted in a dramatic increase in the incidence of toxic deaths. In Table 4, the effects of Adria and Cyclo on normal bone marrow

Table 1. *The influence of drug scheduling with adriamycin–cyclophosphamide combination chemotherapy on L1210 leukaemic mice*

Drugs and treatment schedules	C136 Exp. No.	Per cent increase in life span (% ILS) C142	C146	C156	C160	C178
Adria	84	81	45	12	23	71
Cyclo	37	41	47	20	32	71
Adria $\xrightarrow{24\ hr}$ Cyclo	105	141	105	40	65	186
Adria + Cyclo	238*	205	95	96	135	285
Cyclo $\xrightarrow{24\ hr}$ Adria	94	164	120	81	125	171
Calculated effect of combination	121	122	92	42	55	142

On days 2 and 3 or 3 and 4 after the i.p. inoculation of 10^5 L1210 cells, groups of 10 CD2 mice were treated i.p. with Adria (2.5 mg/kg) and Cyclo (100 mg/kg). Synergism is evident when the % ILS corresponding to the effect observed after combined treatment is greater than the algebraic sum of the values of % ILS determined for the two agents separately. Except for experiment C146, the differences between the observed % ILS of simultaneous Adria + Cyclo were always significantly higher than the calculated effects of the combination. The asterisk indicates % ILS excluding 6/10 mice with no evidence of disease at day 49.

Table 2. *The effect of adriamycin–cyclophosphamide combination chemotherapy on Lewis lung carcinoma and C22LR osteosarcoma*

Drugs and treatment schedules	Growth delay (days) Lewis lung carcinoma 233 LL	240 LL	Osteosarcoma 6053	6099	6124
Adria	2.2	1.3	1.6	2.0	2.6
Cyclo	5.8	4.0	6.9	6.8	7.1
Adria $\xrightarrow{24\ hr}$ Cyclo	6.2	6.2	10.0	7.5	8.2
Adria + Cyclo	8.7	9.3	10.0	9.2	7.3
Cyclo $\xrightarrow{24\ hr}$ Adria	8.4	10.4	9.7	7.9	8.6
Calculated effect of combination	8.0	5.3	8.5	8.8	9.7

Groups of 10 mice were inoculated s.c. with tumour cells on day 0. Treatment was given on two subsequent days during the early course of the tumour growth. A dose of 10–12.5 mg/kg Adria was given i.p. in combination with a dose of Cyclo which, in the Lewis lung experiments, was 100 and, in the osteosarcoma experiments, 50 mg/kg i.p. Drug induced early toxic death was not observed. The mean growth delay is given. The maximum standard error was 1.6 days. The differences between expected and observed effects of the various treatment schedules are not statistically significant except in experiment 240 LL ($P<0.01$).

Table 3. *Drug induced early toxic death in tumour bearing mice: the influence of drug scheduling*

Treatment schedule (mg/kg, i.p.)	Exp. No.	No. of toxic deaths/total No. of mice per treatment group Osteosarcoma in BCBA (F_1) 6082	Lewis lung in C57Bl/Ka 308 LL	L1210 in CD2 C201
Dose of Adria		15	15	3*
Dose of Cyclo		50	75	100
Adria $\xrightarrow{24\ hr}$ Cyclo		3/4	3/5	8/15
Adria + Cyclo		0/5	2/5	1/14
Cyclo $\xrightarrow{24\ hr}$ Adria		2/5	5/5	1/15

Drug induced early toxic death is defined as death of a mouse within 10 days after the last drug administration and presumably not caused by tumour disease.
*Intravenously.

Table 4. The influence of drug scheduling with adriamycin and cyclophosphamide on the percentage of surviving normal haemopoietic bone marrow stem cells

Drugs and treatment schedules	Surviving cells (%)						
	Resting stem cells				Proliferating stem cells		
Exp. No.	I	II	III	IV	V	VI	VII
Dosages Adria	30	30	20	20	5	5	5
(mg/kg i.p.) Cyclo	100	100	75	75	50	50	50
Adria	18.7	20.3	35.5	N.D.	21.9	25.3	27.1
Cyclo	27.5	14.5	40.9	N.D.	14.7	21.4	16.1
Adria $\xrightarrow{24\text{ hr}}$ Cyclo	0.1	2.1	3.0	3.5	0.6	3.0	5.9
Adria + Cyclo	8.3	2.3	14.5	8.9	2.9	3.0	6.1
Cyclo $\xrightarrow{24\text{ hr}}$ Adria	0.6	0.1	2.1	2.1	2.0	3.6	6.8
Calculated effect of combination	5.1	2.9	14.5	—	3.2	5.4	4.4

Synergism is evident when the observed percentage is smaller than the expected percentage of surviving cells calculated by multiplying the individual percentages. N.D. indicates the investigation was not done.

stem cells are demonstrated. Experiments on resting haemopoietic stem cells showed an additive effect for the simultaneous treatment and a synergistic effect when the two drugs were given in sequence at a 24 hr interval.

Data obtained with the rapidly proliferating haemopoietic systems show an additive effect for the various combinations and no schedule dependency is observed.

DISCUSSION

The following three questions will be discussed: What is the effect of simultaneous administration of Adria and Cyclo? What is the influence of drug scheduling (Adria $\rightleftarrows$ Cyclo) on the effect of Adria/Cyclo combination chemotherapy? How should the experimental data be interpreted to be relevant to clinical management of malignant diseases?

Synergism is suggestive when the observed effect of the combination is greater than the expected effect calculated from the single drug data assuming a linear dose–response curve for the individual drugs.

A dose–response curve for each drug was determined in L1210 survival experiments. From the data (not shown) it was concluded that, in the dose range used in our L1210 experiments, both Adria and Cyclo show a linear dose–response relationship. Therefore, according to data presented in Table 1, Adria + Cyclo was a synergistic combination in all but one of the L1210 survival experiments. In the solid tumour experiments shown in Table 2, however, the differences between calculated and observed growth delay for the simultaneously given treatment were usually not significant. Although the results of experiment No. 240 LL in Table 2 seems to indicate drug synergism, this conclusion may be too optimistic. As Adria in contrast to Cyclo treatment was marginally effective in the treatment of Lewis lung carcinoma, it was not possible to obtain a meaningful dose response curve for Adria. Therefore, it seems preferable to interpret the data of experiment 240 LL with caution. Simultaneous treatment of resting and proliferating bone marrow stem cells with Adria + Cyclo was always additive: the observed percentages were as expected as calculated by multiplying the individual percentages. Whether the effect of simultaneous administration of Adria and Cyclo is synergistic depends on the type of cell line used in an experiment: the effect of Adria and Cyclo was synergistic in L1210 and, in contrast, it was additive in Lewis lung carcinoma, osteosarcoma and in resting and proliferating haemopoietic stem cells.

Our second question concerns the influence of drug scheduling. The effect of sequential treatment is best demonstrated in data of the L1210 experiments (Table 1) and the haemopoietic stem cell assays (Table 4). An additive effect was observed when L1210 leukaemic mice were treated either with Adria $\xrightarrow{24\text{ hr}}$ Cyclo or with Cyclo $\xrightarrow{24\text{ hr}}$ Adria, which is in contrast to the synergistic effect after simultaneously given treatment. In the resting stem cell assay, sequential treatment was significantly more effective than the additive effect observed after simultaneous administration of Adria and Cyclo. No effect of drug scheduling was observed in either the recruited bone marrow stem cell system or the Lewis lung carcinoma and osteosarcoma investigations.

In conclusion, of the three schedules tested, sequential treatment is the least effective in L1210 leukaemia and the most toxic in resting bone marrow cells. In contrast, simultaneous administration of Adria and Cyclo results in a synergistic anti-L1210 effect without an accompanying synergistic effect on resting stem cells. These conclusions are in agreement with the observed schedule dependent toxicity of high dose treatment as shown in Table 3. Sequentially given treatment will lead to a synergistic effect on haemopoietic stem cells and therefore to an increased bone marrow toxicity eventually leading to the death of the animals.

Our results are not totally in agreement with those in the literature. A summary of the relevant experimental data is given in Table 5 [5–13]. Therapeutic synergism has been described in L1210 and P388 leukaemia by Avery and Roberts. Corbett *et al.* showed a synergistic effect of Adria and Cyclo in C3H mammary carcinoma, B16 melanoma and the Ridgway osteosarcoma, which is in marked contrast to the present results obtained for Lewis lung carcinoma and C22LR osteosarcoma. Of all the mentioned investigators, there is only one, Vietti from St. Louis, who studied the effect of Adria and Cyclo combination chemotherapy on tumour and concomitantly on critical normal tissue. Neither drug synergism not schedule dependency was demonstrated in L1210 cells and in marrow stem cells. The discrepancy between Vietti's results and ours is difficult to explain, but differences in sensitivity and repair after exposure to Adria have been described for different lines of L1210 [14].

The final question to be discussed concerns the clinical applicability of the findings. Influenced by experimental results [6, 7] and in an effort to reduce nausea and vomiting, some clinical investigators [15, 16] have opted for sequential treatment with Adria given i.v. on day 1 followed by Cyclo given orally in divided doses on days 3–6. Their clinical results, especially those obtained in patients with mammary carcinoma, are promising. Other clinical investigators have evaluated Adria/Cyclo combination chemotherapy, both drugs administered simultaneously in a bolus injection [17–19]. Their results are also encouraging, most notably those observed in a resistant tumour type such as bladder car-

Table 5. Synergism and schedule dependency of adriamycin and cyclophosphamide in experimental tumour systems; A summary from the literature

Author (ref.)	Tumour type	Author's conclusions
Wodinsky [5]	L1210	Synergism after Cyclo $\xrightarrow{24\ hr}$ Adria
Tobias [6]	L1210	Synergism when given simultaneously Schedule dependent
Corbett [7]	C3H mammary carcinoma B16 melanoma Ridgway osteosarcoma P388 leukaemia	Synergism No schedule dependency
Vietti [8]	L1210 marrow stem cells	No synergism No schedule dependency
Johnson [9]	L1210	Synergism Schedule dependency not investigated
Avery [10, 11]	L1210	Synergism when given simultaneously Schedule dependent
Jesair [12]	P388 leukaemia	Synergism Slight preference for Adria $\xrightarrow{24\ hr}$ Cyclo
Braunschweiger [13]	Rat mammary tumour	Synergism after Cyclo $\xrightarrow{24\ hr}$ Adria
Mulder (this paper)	L1210 Resting bone marrow stem cells	A differential schedule dependent synergistic effect
	Lewis lung carcinoma C22LR osteosarcoma	No synergism No schedule dependency

cinoma. Therefore, there is insufficient data from clinical results to recommend any particular Adria/Cyclo treatment schedule. From experimental data, however, there is evidence to optimize the combination chemotherapy with Adria and Cyclo in rapidly proliferating tumour cell lines, the two drugs should be administered simultaneously. The data obtained with the Lewis lung carcinoma and osteosarcoma seem to suggest that neither synergism nor schedule dependency may be expected with these combinations of drugs in solid tumour treatments. For many data, there seems to be a much greater variation among tumours in both mice and man than among normal tissue response in different species. In this study the effect of drug scheduling on critical normal tissue was investigated in detail. From the bone marrow stem cell data, there is in order to minimize toxicity a preference to administer the two drugs simultaneously. On the basis of the differential effect of Adria/Cyclo combination chemotherapy in tumours and in bone marrow stem cells, it may be recommended that, in clinical cancer treatment, Adria and Cyclo be given simultaneously and not sequentially until more experimental data are available to conclude otherwise.

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