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Original Paper

Scheduling of Gemcitabine and Cisplatin in Lewis Lung Tumour Bearing Mice

C.J.A. van Moorsel,[1] H.M. Pinedo,[1] G. Veerman,[1] J.B. Vermorken,* P.E. Postmus[2] and G.J. Peters[1]

[1]Department of Medical Oncology, University Hospital VU, Amsterdam; and [2]Department of Pulmonology, University Hospital VU, P.O. Box 7057, 1007 MB, The Netherlands

We used the gemcitabine (dFdC) and cisplatin (cis-diamine dichloroplatinum CDDP) resistant murine NSCLC tumour Lewis Lung (LL) in C57/Bl6 mice to optimise scheduling of both drugs, since in previous *in vivo* studies no effective combination schedule of both compounds was found to overcome resistance to either drug. dFdC could not be combined at the previously determined maximum tolerated dose (MTD) (120 mg/kg, q3dx4) with CDDP at its MTD (9 mg/kg, q6dx2) (mean weight loss < 15% and < 15% toxic deaths), because of additive toxicity. Therefore, we lowered the dose of dFdC to 60 mg/kg (q3dx4) and of CDDP to 3 mg/kg (q6dx2), which caused an increase in antitumour effect compared with the activity of each compound alone at its MTD (growth delay factor (GDF) = 0.55, 0.13 and 2.56 for dFdC and CDDP alone and the combination, respectively). Changing the CDDP treatment schedule giving the total dose (6 mg/kg) only at day 0 caused unacceptable toxicity. This effect was not seen when mice were treated with the total dose of CDDP on day 9, but, the anti-tumour effect was not enhanced. To decrease toxicity, the dosage of dFdC was lowered to 50 mg/kg and combined with the total dose of CDDP on day 0, which caused a better antitumour effect than the combination of 60 mg/kg dFdC and 3 mg/kg CDDP (q6dx2) with acceptable toxicity. Schedule dependency was found for the combination: dFdC preceding CDDP by 4 h was the best treatment schedule in the LL tumours (GDF: 2.1) with acceptable toxicity. However, when the interval was increased to 24 h, toxicity became unacceptable (> 30% weight loss). The reverse schedule, in which CDDP preceded dFdC, did not lead to an increased antitumour effect or to increased toxicity. Adding amifostine, a selective chemoprotector, to the treatment decreased toxicity of the combination without affecting the antitumour effect. Increasing the CDDP dose to 9 mg/kg (day 0) under amifostine protection led to an improved therapeutic index. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: cisplatin, gemcitabine, amifostine, non-small cell lung cancer, schedule dependence
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INTRODUCTION

2′,2′-DIFLUORODEOXYCYTIDINE (GEMCITABINE, dFdC) is a deoxycytidine analogue [1] with clinical activity against several solid tumours, such as ovarian cancer, non-small cell lung cancer (NSCLC), head and neck cancer (HNC), pancreatic cancer and bladder cancer [2]. After entering the cell, dFdC is phosphorylated to its triphosphate (dFdCTP) which can be incorporated into DNA, followed by one more deoxynucleotide after which DNA-polymerisation stops [3], which probably determines its cytotoxic effect. dFdC is also capable of inhibiting ribonucleotide reductase (RR) [4], an enzyme with a key role in DNA-repair mechanisms, and can be incorporated into RNA probably leading to inhibition of RNA synthesis [5].

Correspondence to G.J. Peters, e-mail: gj.peters@azvu.nl
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*Present address: Department of Oncology, University Hospital Antwerp, Belgium.

cis-Diamminedichloroplatinum (cisplatin, CDDP) is an established anticancer drug with a similar spectrum of antitumour activity as dFdC. CDDP is generally considered to exert its cytotoxic effect by binding to DNA, resulting in a number of different adducts [6]. A relationship between platinum–DNA adduct (Pt–DNA adduct) levels and antitumour response in cultured cells [7] and in patients has been postulated [8, 9].

Because of the different, possibly complementary mechanisms of action, a similar spectrum of antitumour activity and different side-effects, CDDP being nephrotoxic and dFdC being mildly myelotoxic, combination of these drugs has been investigated. In *in vitro* studies with colon-, ovarian-, HNC and NSCLC cancer cell lines a synergistic effect of both drugs was found [10, 11] which was time- and sequence-dependent. The most effective schedule was 4 h dFdC preincubation followed by the combination of dFdC and CDDP [10]. The mechanism of synergy appeared to be an increase in the formation of platinum (Pt)-DNA adducts [12] possibly due to the incorporation of dFdC into the DNA.

In vivo studies in the dFdC and CDDP sensitive human HNC xenograft HNX-22B in nude mice also showed that the combination of both drugs resulted in higher response rates than for each single agent and that dFdC (100 mg/kg, intraperitoneally (i.p.), q3dx4) and CDDP (4 mg/kg, intravenously (i.v.) d0 and 6) should be administered either simultaneously or with a short time interval. However, addition of dFdC to CDDP did not lead to an enhancement of activity in the CDDP resistant HNX-14C [13]. In a study with the murine Colon 26-10 tumour in Balb/C mice dFdC at 120 mg/kg i.p. (q3dx4) [14] was effective as a single agent, but the combination with 4 mg/kg CDDP i.p. showed enhanced toxicity, which was not expected from results with non-tumour-bearing mice [15]. At a lower dose of CDDP (3 mg/kg), schedules with a 24 h interval after or before dFdC were much more toxic than dFdC alone. The combination of dFdC and CDDP was also effective in the dFdC sensitive human NSCLC xenograft Calu-6 in nude mice when dFdC was given as a bolus injection every 3 days (150 mg/kg, q3dx4) and CDDP was given the day after the last dFdC injection (10 mg/kg, day 10) [16]. However, the efficacy was not greater than expected from single agent activity of the two compounds. In the less dFdC sensitive human NSCLC xenografts LX-1 and NCI-H460 the combination of both drugs was not as effective as in the Calu-6 xenograft.

Based on preclinical studies several clinical studies have been initiated in NSCLC patients [17–21]. Response rates of up to 54% have been found in these phase II studies with both compounds. In these studies again a schedule dependency was observed; the highest response rates were found when dFdC preceded CDDP for 24 h [18]. However, major responses were also observed with other schedules and confidence intervals were overlapping.

The aim of this study was to optimise scheduling of both drugs in a dFdC-resistant tumour, since in previous *in vivo* studies with several other tumour types no effective combination schedule of both compounds was found to overcome resistance to either drug. For this purpose we chose the murine NSCLC tumour LL. The cell line derived from this tumour showed *in vitro* synergism [12]. Furthermore, we tried to decrease the toxicity of the combination with pretreatment with amifostine, a protector against chemotherapy-induced toxicity to the bone marrow and kidneys [22].

MATERIALS AND METHODS

Materials

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) was kindly provided by Lilly Research Center Ltd (Indianapolis, Indiana, U.S.A.). Each ampoule contained gemcitabine HCI equivalent to 500 mg gemcitabine, 500 mg mannitol, and 80 mg sodium acetate. The gemcitabine powder was dissolved in 0.9% NaCl to reach a final concentration of 12 mg/ml. Cisplatin (CDDP, Platinol®) was obtained from Bristol-Myers Squibb B.V. (Woerden, The Netherlands) at a concentration of 0.5 mg/ml. Amifostine (S-2-(3-aminopropylamino)-ethylphosphorothioic acid, WR2721, Ethyol®) was obtained from USB Pharma (Nijmegen, The Netherlands) and dissolved in 0.9% NaCl to reach a final concentration of 24 mg/ml. All other chemicals were of analytical grade and were commercially available.

Tumour

The sources and characteristics of the murine LL tumour (non-small cell lung cancer (NSCLC)) have been described elsewhere [23]. LL tumours were grown in female C57/Bl6 mice. The mice were kept in an area maintained on a standardised light/dark cycle and had access to food (RMH-B 10 mm code 2100, Hope Farms, Woerden, The Netherlands) and water *ad libitum*. Tumours were transplanted subcutaneously (s.c.) in both flanks in the thoracic region in small fragments of 1–5 mm^3. When tumours reached a volume of 50–150 mm^3, treatment was started. Tumour size was determined using electronic calliper measurement ($0.5 \times length \times width \times height$) twice a week, which was shown to be the most reliable method [24]. The calliper was connected to a computer enabling a direct evaluation of the data. The tumour volume was expressed relative to that determined on the first day of treatment (day 0). Prior to treatment, mice were randomised into several groups, one group serving as a control group. Each group consisted of at least 6 mice, corresponding to 12 tumours. Antitumour activity was evaluated using two different methods; by calculation of the T/C values, i.e. by dividing the relative tumour volume (RTV) of treated mice by that of control mice; and with the growth delay factor (GDF) which was defined as the mean number of tumour-doubling times gained by the treatment and was calculated with the formula: $GDF = (TD_{tr} - TD_{con})/TD_{con}$. In this formula TD_{tr} is the tumour-doubling time of treated tumours and TD_{con} that of untreated tumours. Partial responses were determined as a reduction of more than 50% of initial tumour volume after treatment. As a general policy mice were sacrificed when the tumour volume reached approximately 2000 mm^3. Body weights of the animals were recorded immediately before treatment and once daily during and after treatment. Deaths were seen as being drug related when the tumour volume on the day of death did not exceed 500 mm^3, when death occurred within a week of treatment or when death was preceded by a sharp weight loss of > 10%. Differences in significance between the antitumour effects of a particular treatment were determined by means of a Student's *t*-test.

Doses and schedules

Mice were treated by i.p. bolus injection. An initial dose finding study in non-tumour-bearing C57/Bl6 mice found the dose that caused a maximal weight loss of 15% was defined as the maximum tolerated dose (MTD). Treatment

Table 1. *Summary of the antitumour activity of gemcitabine and cisplatin in Lewis Lung tumours*

Dose	Antitumour effect					Toxicity	
	Mean doubling time of tumours (days)	Growth delay factor (GDF)	Max T/C	(day)†	PR‡	MWL§ (%)	Deaths‖ *n*/total
Gemcitabine*							
Control	3.2 ± 0.9						
50 mg/kg	7.15	0.59	0.51	10	0/11	4.7	0/6
60 mg/kg	5.1	0.88	0.50	11	0/11	6.5	0/6
90 mg/kg	4.86	0.55	0.42	13	0/10	9.3	1/6
120 mg/kg	¶	¶	0.4 ± 0.3	9	0/26	11.6	5/17
Cisplatin							
Control	3.2 ± 0.9						
3 mg/kg (d0, d6) CDDP	3.7 ± 1.0	0.3 ± 0.5	0.8 ± 0.3	6	0/28	0.5	0/18
6 mg/kg (d0, d6) CDDP	5.07	0.13	0.91	9	0/11	4.1	0/6
6 mg/kg (d0) CDDP	3.53	0.12	0.44	3	0/10	4	0/6

*dFdC was dosed as q3dx4. †The day at which this was observed. ‡Partial response (greater than 50% reduction in volume) recorded per total number of tumours. §Maximal weight loss (%) due to drug treatment as compared with the first day of treatment (day 0). ‖Total of deaths due to drug treatment. ¶Tdt, too toxic to determine, although the dose was tolerable in previous studies with dFdC [14]. *GDF*, $(TD_{tr} - TD_{con})/TD_{con}$. Values of > 1 indicate that the tumour is sensitive; T/C, RTV_{tr}/RTV_{con}, tumour:control ratio.

was initiated at day 11 after transplantation. All protocols were approved by the ethics committee for animal experiments of the Free University of Amsterdam and comply with the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia [25].

RESULTS

Therapy with single drugs

A summary of the single agent antitumour activity and toxicity of each compound given in a number of schedules and doses is shown in Table 1. The LL tumour was resistant to single agent treatment with CDDP (MTD = 6 mg/kg (q6dx2, d0, d6); GDF = 0.13) and only moderately sensitive to dFdC (MTD = 90 mg/kg (q3dx4); GDF = 0.55) (Figure 1). No significant differences between the tumour-doubling times (TDt) of treatments with both compounds alone were seen. Toxicity was schedule dependent, the lowest toxicity was seen after treating mice with only one injection of 6 mg/kg CDDP on the first day.

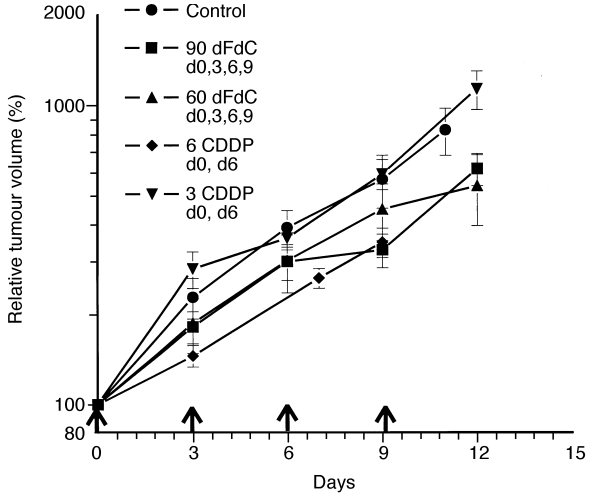

Figure 1. Antitumour effect of dFdC or CDDP in LL tumours. Growth curves of untreated tumours (●) and tumours treated with dFdC at its MTD (90 mg/kg; ■) and at 60 mg/kg alone (▲) and CDDP at its MTD (6 mg/kg; ◆) and at 3 mg/kg alone (▼) are shown. Volumes are expressed relative to those observed at the start of the treatment and are presented as means ± SEM (standard error of the mean). The arrows indicate the days of treatment. The average of the maximal tumour volume of control mice was 1975 ± 234 mm^3.

Maximum tolerated dose of the combination of dFdC and CDDP

Based on previous combination studies in which almost full doses of both dFdC and CDDP could be given [13, 15] and based on the observation that the MTD of Colon-38 tumour bearing C57/Bl6 mice of dFdC alone was 300 mg/kg, q3dx4, i.p. [14], we started the combination studies with both compounds at 90 mg/kg dFdC (q3dx4, i.p.) and 9 mg/kg CDDP (q6dx2, i.p.). Unexpectedly, this initial schedule was excessively toxic for C57/Bl6 mice (29% weight loss, with all toxic deaths occurring at day 10); therefore, we decreased the dFdC dose to 60 and 50 mg/kg. This was, however, not sufficient to decrease toxicity to an acceptable degree (still 29% weight loss with 3/6 toxic deaths). Since the toxicity profile appeared to be typical for CDDP we decreased the CDDP dose to 3 mg/kg once every 6 days (q6dx2) in combination with 120 mg/kg dFdC (q3dx4). With this schedule toxicity varied between the experiments and was overall not acceptable; lowering the dFdC dose to 60 mg/kg resulted in an antitumour activity better than that observed with single agent CDDP or dFdC at their MTD (Figure 2). The TDts of the combination were significantly ($P < 0.01$) lower than those for CDDP or dFdC alone (GDF = 2.56; 0.13 and 0.55, respectively). In tumour-bearing mice the toxicity pattern was different from that in non-tumour-bearing mice. Therefore, subsequent scheduling studies were also performed in tumour-bearing mice.

Schedule dependency of the combination of dFdC and CDDP

Since CDDP is given at different schedules clinically and since CDDP has a steep dose–response relationship, we investigated several schedules of CDDP. However, administration of the total dose of CDDP (6 mg/kg) on day 0 combined with 60 mg/kg dFdC (q3dx4) was more toxic than the combination of 3 mg/kg CDDP twice (d0, d6) combined with 60 mg/kg dFdC (q3dx4) (Table 2). Since the tumour volumes of the mice who survived treatment were much

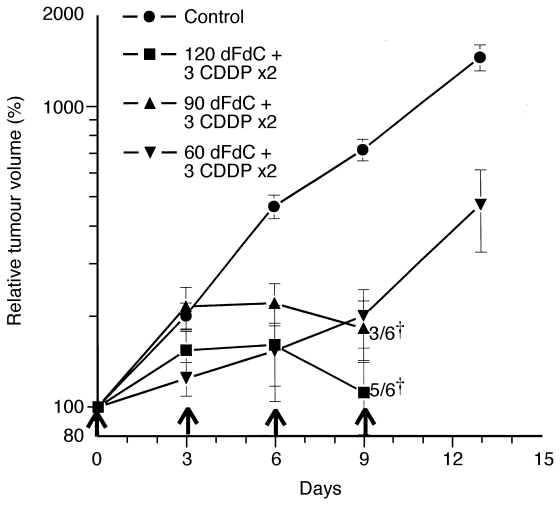

Figure 2. Antitumour effect of the combination of dFdC and CDDP in LL tumours. Growth curves of untreated tumours (●) and tumours treated with several combinations are shown: 120 mg/kg dFdC (q3dx4) combined with 3 mg/kg CDDP (d0, d6) (■), 90 mg/kg dFdC (q3dx4) combined with 3 mg/kg CDDP (d0, d6) (▲) and 60 mg/kg dFdC (q3dx4) combined with 3 mg/kg CDDP (d0, d6) (▼). Volumes are expressed relative to those observed at the start of the treatment and are presented as means ± SEM (standard error of the mean). The inset indicates the number of mice which died during the experiment. The arrows indicate the days of treatment. The average of the maximal tumour volume of control mice was 1655±471 mm³.

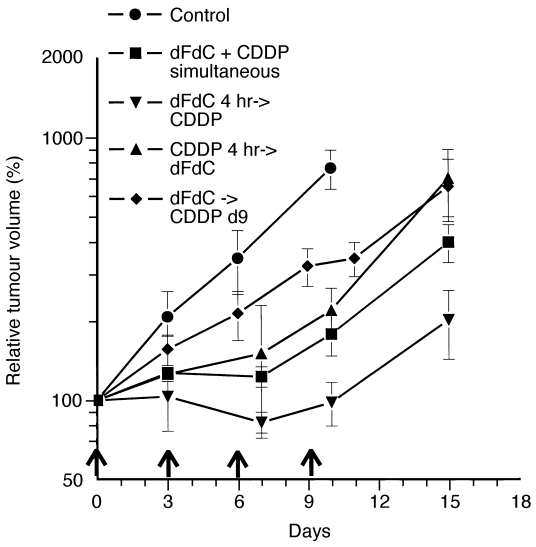

Figure 3. Schedule dependency of the antitumour effect of the combination of CDDP and dFdC in LL tumours. Growth curves of untreated tumours (●) and tumours treated with several combinations of 50 mg/kg dFdC (q3dx4) and 6 mg/kg CDDP (d0) are shown: simultaneous (■), dFdC 4 h before CDDP (▼), CDDP 4 h before dFdC (▲) and CDDP on day 9 combined with 60 mg/kg dFdC (◆). Volumes are expressed relative to those observed at the start of the treatment and are presented as means ± SEM (standard error of the mean). The arrows indicate the days of treatment. The average of the maximal tumour volume of control mice was 1348±205 mm³.

lower for the combination of 60 mg/kg dFdC (q3dx4) with 6 mg/kg CDDP on day 0 than for the combination of 60 mg/kg dFdC (q3dx4) with 3 mg/kg CDDP (d0, d6), we treated mice with a reduced dose of dFdC (50 mg/kg (q3dx4)) combined with 6 mg/kg CDDP on day 0. This treatment had a significantly improved antitumour efficacy ($P = 0.02$) compared with the combination of 60 mg/kg dFdC (q3dx4) combined with 3 mg/kg CDDP (d0, d6). Previous *in vitro* studies had shown that sequential treatment with dFdC or CDDP was more synergistic than simultaneous treatment. Therefore, dFdC and CDDP were not only administered simultaneously but also sequentially at similar doses. These *in vitro* scheduling studies showed different degrees of efficacy, therefore, we used intervals of 4 and 24 h. Thus, mice were treated with 50 mg/kg dFdC 4 h or 24 h before CDDP (6 mg/kg) on day 0, or with 6 mg/kg CDDP 4 h or 24 h before 50 mg/kg dFdC on day 0, followed by three treatments with dFdC alone (days 3, 6, 9). In the same experiment the interval with dFdC 4 h before CDDP was significantly more effective than the simultaneous exposure of both drugs ($P = 0.03$)

Table 2. Schedule dependency of the efficacy of the gemcitabine–cisplatin combination in LL tumours

	Antitumour effect					Toxicity	
Dose/schedule★	TDt† (days)	GDF	Max T/C	(day)‡	PR§	MWL‖ (%)	Deaths¶ *n*/total
Control	2.58					0	0/6
60 mg/kg dFdC + 3 mg/kg (d0, d6) CDDP	9.18	2.56	0.21	9	0/9	7.9	0/6
Control	2.72					0	0/5
60 mg/kg dFdC + 6 mg/kg (d0) CDDP	**	**	0.15	11	0/11	18.6	3/6
60 mg/kg dFdC + 6 mg/kg (d9) CDDP	6.13	1.25	0.42	11	0/11	19.5	1/6
Control	4.5					0.3	0/4
50 mg/kg dFdC + 6 mg/kg (d0) CDDP	11.5	1.56	0.23	10	0/11	13.5	0/6
6 mg/kg (d0) CDDP 4 h→50 mg/kg dFdC	13.2	1.93	0.29	10	1/12	15.5	0/6
50 mg/kg dFdC 4 h→6 mg/kg (d0) CDDP	13.9††	2.08	0.13	10	1/11	18.4	0/6
Control	2.5					0	0/6
50 mg/kg dFdC + 6 mg/kg (d0) CDDP	12.8‡‡	4.12	0.11	10	0/11	19.4	2/6
50 mg/kg dFdC 24 h→6 mg/kg (d0) CDDP	**	**	0.16	7	0/11	27.6	2/6
6 mg/kg (d0) CDDP 24 h→50 mg/kg dFdC	7.81	2.12	0.29	10	0/10	14.7	1/6

★dFdC was dosed as q3dx4. †Mean doubling time of tumours. ‡The day at which this was observed. §Partial response (greater than 50% reduction in volume) recorded per total number of tumours. ‖Maximal weight loss (%) due to drug treatment as compared with the first day of treatment (day 0). ¶Total of deaths due to drug treatment. **TDt, too toxic to determine. ††Significantly higher than TDt of mice treated with 50 mg/kg dFdC + 6 mg/kg CDDP d0 ($P = 0.03$). ‡‡Significantly higher than TDt of mice treated with CDDP 24 h before dFdC ($P < 0.01$). *GDF*, $(TD_{tr} - TD_{con})/TD_{con}$. Values of > 1 indicate that the tumour is sensitive; $T/C = RTV_{tr}/RTV_{con}$, tumour:control ratio.

Table 3. Effect of amifostine on the toxicity and antitumour efficacy of the combination of gemcitabine and cisplatin

Dose*	Antitumour effect					Toxicity	
	TDt† (days)	GDF	Max T/C	(day)‡	PR§	MWL‖ (%)	Deaths¶ *n*/total
Control	2.58					0	0/6
60 mg/kg dFdC + 6 mg/kg (d0) CDDP + 120 mg/kg amifostine	9.99	2.87	0.16	9	3/10	9.9	0/6
60 mg/kg dFdC + 3 mg/kg (d0, d6) CDDP + 120 mg/kg amifostine	7.83	2.03	0.32	9	0/11	14.9	0/6
Control	2.69					0	0/6
50 mg/kg dFdC + 6 mg/kg (d0) CDDP + 120 mg/kg amifostine	9.59	2.57	0.23	10	1/12	18.1	2/6
50 mg/kg dFdC + 9 mg/kg (d0) CDDP	**	**	0.11	7	1/11	29.6	6/6
50 mg/kg dFdC + 9 mg/kg (d0) CDDP + 120 mg/kg amifostine	13.48††	4.01	0.12	10	0/11	23.3	0/6

*dFdC was dosed as q3dx4. †Mean doubling time of tumours. ‡The day at which this was observed. §Partial response (greater than 50% reduction in volume) recorded per total number of tumours. ‖Maximal weight loss (%) due to drug treatment as compared with the first day of treatment (day 0), including the day after first treatment at which this was observed. ¶Total of deaths due to drug treatment. **TDt, too toxic to determine. ††Significantly higher than TDt of mice treated with 50 mg/kg dFdC + 6 mg/kg CDDP (d0) alone or in combination with amifostine ($P = 0.04$ and 0.05, respectively). *GDF*, $(TD_{tr} - TD_{con})/TD_{con}$. Values of > 1 indicate that the tumour is sensitive; T/C RTV_{tr}/RTV_{con}, tumour:control ratio.

(Figure 3, Table 2). However, when dFdC preceded CDDP by 24 h, the combination was lethal to all mice. Similarly, when CDDP preceded dFdC by 4 h the combination was more effective than the simultaneous exposure, although not significant. This combination was effective with a 24-h interval, although less effective than the simultaneous combination ($P < 0.01$). Previous studies reported by Tanzer and colleagues [16] indicated that treatment of mice with CDDP after the last dFdC dose increased the antitumour activity of treatment with dFdC alone and was better than with CDDP alone, when administered at a similar tumour volume. Therefore, we administered the total dose of CDDP (6 mg/kg CDDP) on day 9. However, this was significantly less effective ($P < 0.01$) than the treatment with 6 mg/kg CDDP at day 0 combined with 60 mg/kg dFdC (q3dx4).

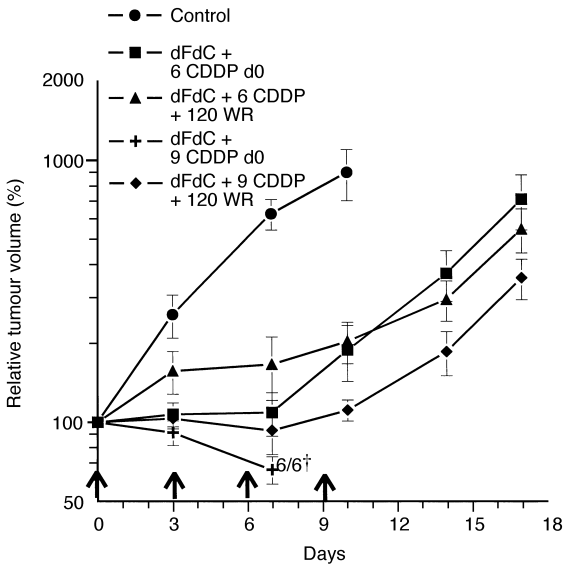

Figure 4. The antitumour effect of increasing doses of CDDP in combination with dFdC under amifostine (WR) protection. Growth curves of untreated tumours (●) and tumours treated with the combination of 50 mg/kg dFdC (q3dx4) and CDDP (d0) with and without amifostine are shown: dFdC combined with 6 mg/kg CDDP with (▲) or without amifostine (■) and dFdC combined with 9 mg/kg CDDP with (◆) or without amifostine (+). Volumes are expressed relative to those observed at the start of the treatment and are presented as means ± SEM (standard error of the mean). The inset indicates the number of mice which died during the experiment. The arrows indicate the days of treatment. The average of the maximal tumour volume of control mice was 2101±284 mm³.

Decreasing the toxicity of the combination

Since control of CDDP toxicity appeared to be a major hurdle in the combination studies of CDDP with dFdC we attempted to control toxicity with amifostine. Since amifostine decreased toxicity of the combination of 5-fluorouracil (5-FU) plus CDDP [26], we used a schedule similar to that given previously. Thus, amifostine was given 5 min before CDDP at 120 mg/kg in the simultaneous combination of 60 mg/kg dFdC (q3dx4) and 6 mg/kg CDDP (d0) or 3 mg/kg CDDP (d0, d6) (Table 3, Figure 4). Toxicity was not decreased in the combination of 3 mg/kg CDDP (d0, d6) and 60 mg/kg dFdC (q3dx4), but was significantly decreased ($P = 0.03$; day 6) for the 6 mg/kg CDDP combination. Amifostine did not affect the antitumour activity of the combination. However, its addition resulted in a clearly improved therapeutic index. Thereafter, the dose of CDDP was escalated in combination with amifostine, resulting in a tolerable dose of CDDP of 9 mg/kg, which is lethal on its own. Toxicity of the combination of 50 mg/kg dFdC (q3dx4) with 9 mg/kg CDDP (d0) was significantly decreased ($P = 0.03$). Much to our surprise, the toxicity of the combination of 50 mg/kg dFdC with 6 mg/kg CDDP (d0) was not decreased. Altogether, amifostine increased the antitumour effect of both combinations, resulting in the best therapeutic index for the combination of 9 mg/kg CDDP (d0) and 50 mg/kg dFdC (q3dx4) (GDF = 4.01; $P = 0.04$).

DISCUSSION

This study shows that the efficacy of the combination of dFdC with CDDP is very schedule dependent, but is more effective than each drug alone at its optimal schedule. The sequence and time interval between the two drugs appear to be important determinants of antitumour activity, similar to what was previously shown in HNC xenografts and a murine colon tumour [13, 15]. Moreover, the efficacy of the combination could be even further increased by the addition of amifostine, which allowed an increase in the dose of CDDP.

In a previous study we failed to increase the antitumour activity of the dFdC insensitive HNC xenograft HNX-14C [13], whereas the sensitive HNX-22B xenograft could be

made much more sensitive. Therefore, it was postulated that a tumour should have a certain sensitivity to each drug in order to achieve an additive effect from the combination. The LL tumour is less sensitive to either dFdC and CDDP alone than the HNX-14C; however, from this study it appears that the resistance to the combination according to the HNC xenograft schedule, could be overcome in the LL tumour by adjusting the CDDP schedule.

The optimal schedule in C57/Bl6 mice bearing the LL tumour was based on a high-dose of CDDP treatment only on day 0. This schedule was not only different from the schedule used in the HNC xenografts, but also from the effective schedule used in Calu-6 human lung xenografts, either CDDP on day 0 and day 6 or a high-dose of CDDP at day 9, respectively [16]. The schedule with delayed high-dose CDDP was ineffective for the LL tumour, possibly because the schedule used in the human lung xenografts was designed to treat tumours with a similar size by CDDP, i.e. tumours which were treated with CDDP alone had a similar size as tumours treated with dFdC, for that purpose tumours have to be sensitive to dFdC alone, which LL tumours are not, resulting in tumours too large to be treated at day 9. This might also account for the inactivity of the schedule used in the HNC xenograft studies. Apparently, alternative schedules such as repeated low-dose CDDP and delayed CDDP, are only effective in relatively sensitive tumours.

The schedule dependency studies revealed that LL tumours responded best to a treatment with a sequential administration of dFdC and CDDP with a 4-h interval. These data agree with previous studies in the HNX-22B xenograft in which dFdC preceding CDDP with a 4 h interval, showed the highest antitumour activity [13]. However, in both studies treatment of mice with dFdC 24 h before CDDP increased toxicity to an unacceptable level, while CDDP 24 h before dFdC failed to show an increase in the efficacy compared with simultaneous combination. Partial responses were observed in both 4 h interval studies in LL tumours, this in contrast to the simultaneous exposure where no partial responses were observed. However, in contrast to the study in the HNX-22B xenograft where three cures were found, no cures were seen in this study. However, the HNX-22B xenograft already showed sensitivity to each drug alone.

The reason for the difference in toxicity between non-tumour bearing and tumour-bearing mice remains unknown. However, these more severe side-effects were also found in previous studies with HNC xenografts and a murine colon tumour [15]. It was proposed that this toxicity might be related to tumour-dependent metabolic effects exerted by the drug related to signal transduction, nucleotide pools and energy status of the tumour, as was found in euthymic mice [27]. However, the increased toxicity is found in almost all studies combining both compounds, even though three different mouse strains and four different tumour types were used. Therefore, it appears to be related to the interaction of both compounds, which might be, as was postulated for 5-FU, related to an increased RNA synthesis inhibition by dFdC and CDDP [12].

In this study we managed to reduce toxicity by pretreating mice with amifostine without affecting the antitumour effect of the combination of dFdC and CDDP. In previous studies with combined 5-FU/CDDP and carboplatin treatment an increase in gastrointestinal and haematological toxicity was found compared with each compound alone [26, 28]. Amifostine could protect the myelotoxicity, gastrointestinal toxicity and nephrotoxicity typical for CDDP and carboplatin. Since amifostine also reduced dFdC-CDDP induced toxicity but not dFdC toxicity, dFdC apparently increased CDDP-related toxicities. This finding might be very important for future use of the combination in the clinic. Since the clinical activity of these compounds is strongly dose-related, pre-treatment with amifostine might improve the response rate of the combination of dFdC and CDDP in NSCLC [17–19].

The results presented here can give further insight in the *in vivo* interaction between dFdC and CDDP. This study shows that the schedule dependency between both compounds plays an important role in both antitumour response and toxicity patterns, which is of major interest for the treatment of tumours in the clinic: more schedules may be active. Furthermore, this study shows that resistance to the combination can probably be overcome by adjusting the CDDP schedule and that the total dose of the combination of both compounds can be increased by co-treatment with the toxicity protector amifostine, which might be an indication for a role for this compound in patient treatment.

1. Hertel LW, Kroin JS, Misner JW, Tustin JM. Synthesis of 2′-deoxy-2′,2′-difluoro-D-ribose and 2-Deoxy-2,2-difluoro-D-ribofuranosyl nucleosides. *J Org Chem* 1988, **53**, 2406–2409.
2. Van Moorsel CJA, Peters GJ, Pinedo HM. Gemcitabine: future prospects of single-agents and combination studies. *The Oncologist* 1997, **2**, 127–134.
3. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. *Cancer Res* 1991, **51**, 6110–6117.
4. Heinemann V, Hertel LW, Grindey GB, Plunkett W. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. *Cancer Res* 1988, **48**, 4024–4031.
5. Ruiz van Haperen VWT, Veerman G, Vermorken JB, Peters GJ. 2′,2′-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. *Biochem Pharmacol* 1993, **46**, 762–766.
6. Sundquist WI, Lippard SJ. The coordination chemistry of platinum anticancer drugs and related compounds with DNA. *Coord Chem Rev* 1990, **100**, 293–322.
7. Terheggen PMAB, Emondt JY, Floot BGJ, *et al.* Correlation between cell killing by cisdiamminedichloroplatinum(II) in six mammalian cell lines and binding of a cisdiamminedichloroplatinum(II)–DNA antiserum. *Cancer Res* 1990, **50**, 3556–3561.
8. Parker RJ, Gill I, Tarone R, *et al.* Platinum DNA-damage in leucocyte DNA of patients receiving cisplatin and carboplatin chemotherapy, measured by atomic absorption spectrometry. *Carcinogenesis* 1991, **12**, 1253–1258.
9. Schellens JHM, Ma J, Planting ASTh, *et al.* Relationship between the exposure to cisplatin, DNA–adduct formation in leucocytes and tumour response in patients with solid tumours. *Br J Cancer* 1996, **73**, 1569–1575.
10. Bergman AM, Ruiz van Haperen VWT, Veerman G, Kuiper CM, Peters GJ. Synergistic interaction between cisplatin and gemcitabine *in vitro*. *Clin Cancer Res* 1996, **2**, 521–530.
11. Van Moorsel CJA, Veerman G, Bergman AM, *et al.* Combination chemotherapy studies with gemcitabine. *Semin Oncol* 1997, **24** (Suppl. 7), 17–23.
12. Van Moorsel CJA, Pinedo HM, Veerman G, *et al.* Mechanisms of synergism between cisplatin and gemcitabine in ovarian and non-small cell lung cancer cell lines. *Br J Cancer* 1999, in press.
13. Braakhuis BJM, Ruiz van Haperen VWT, Welters MJP, Peters GJ. Schedule-dependent therapeutic efficacy of the combination of gemcitabine and cisplatin in head and neck cancer xenografts. *Eur J Cancer* 1995, **31A**, 2335–2340.
14. Veerman G, Ruiz van Haperen VWT, Vermorken JB, *et al.* Antitumor activity of prolonged as compared with bolus administration

of 2′,2′-difluorodeoxycytidine *in vivo* against murine colon tumors. *Cancer Chemother Pharmacol* 1996, **38**, 335–342.
15. Peters GJ, Bergman AM, Ruiz van Haperen VWT, Veerman G, Kuiper CM, Braakhuis BJM. Interaction between cisplatin and gemcitabine *in vitro* and *in vivo*. *Semin Oncol* 1995, **22** (Suppl.), 72–79.
16. Tanzer LR, Rutherford PG, Self TD, Seymour PD, Theobald KS, Merriman RL. Antitumor activity of gemcitabine in combination with cisplatin against the human NSCLC xenograft Calu-6. *Proc AACR* 1995, **36**, abstract 1761.
17. Abratt RP, Bezwoda WR, Goedhals L, Hacking DJ. Weekly gemcitabine with monthly cisplatin: effective chemotherapy for advanced non-small cell lung cancer. *J Clin Oncol* 1997, **15**, 744–749.
18. Crino L, Scagliotto G, Marangolo M, *et al*. Cisplatin–gemcitabine combination in advanced non-small cell lung cancer: a phase II study. *J Clin Oncol* 1997, **15**, 297–303.
19. Steward WP, Dunlop DJ, Dabouis G, Lacroix H, Talbot D. Phase I/II study of gemcitabine and cisplatin in non-small cell lung cancer: preliminary results. *Semin Oncol* 1996, **5**(Suppl. 10), 143–147.
20. Shepherd FA, Anglin G, Abratt R, *et al.* Influence of gemcitabine (gem) and cisplatin (cp) schedule on response and survival in advanced non-small cell lung cancer (NSCLC). *Proc ASCO* 1998, **17**, abstract 1817.
21. Abratt RP, Sandler A, Crino L, *et al*. Combined cisplatin and gemcitabine for non-small cell lung cancer: influence of scheduling on toxicity and drug delivery. *Semin Oncol* 1998, **25**(Suppl. 9), 35–43.
22. Van der Vijgh WJF, Peters GJ. Protection of normal tissues from the cytotoxic effect of chemotherapy and radiation by amifostine (Ethyol): preclinical aspects. *Semin Oncol* 1994, **21**, 2–7.
23. Mayo JG. Biologic characterization of the subcutaneously implanted Lewis Lung tumor. *Cancer Chemother Rep* 1972, part 2 (3) **1**, 325–330.
24. Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* 1989, **24**, 148–154.
25. UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia, 2nd Ed. *Br J Cancer* 1998, **77**(1).
26. Van der Wilt CL, Van Laar JAM, Gyergyay F, Smid K, Peters GJ. Biochemical modification of the toxicity and antitumour effect of 5-fluorouracil and cisplatinum by WR-2721 in mice. *Eur J Cancer* 1992, **28A**, 2017–2024.
27. Peters GJ. Therapy related disturbances in nucleotides in cancer cells. *Adv Exp Med Biol* 1995, **370**, 95–107.
28. Van Laar JAM, Van der Wilt CL, Treskes M, Van der Vijgh WJF, Peters GJ. Effect of WR-2721 on the toxicity and antitumour activity of the combination of carboplatin and 5-fluorouracil. *Cancer Chemother Pharmacol* 1992, **31**, 97–102.

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