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Schedule-dependent pharmacodynamic effects of gemcitabine and cisplatin in mice bearing Lewis lung murine non-small cell lung tumours

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Abstract

The combination of 2′,2′-difluorodeoxycytidine (gemcitabine, dFdC) and *cis*-diammine-dichloroplatinum(II) (cisplatin, CDDP) is increasingly applied in clinical oncology. We studied the underlying mechanisms of the *in vivo* schedule dependency and supra-additive interaction between dFdC and CDDP in C57/Bl6 mice bearing Lewis lung (LL) tumours. Mice were treated with CDDP (6 mg/kg) and dFdC (60 mg/kg) either simultaneously or in a 4 or 24 h interval with dFdC preceding CDDP or vice versa. Four, 8 (in some cases 12) and 24 h after treatment mice were sacrificed and tumours, kidneys, blood and bone marrow (BM) were collected. Since CDDP acts by formation of Platinum (Pt)–DNA adducts and dFdC by incorporation of its triphosphate (dFdCTP) into DNA, we measured total Pt levels, dFdCTP accumulation and Pt–DNA adducts by atomic absorption spectrometry (AAS), high performance liquid chromatography (HPLC) and ^{32}P-postlabelling, respectively. These levels were related to the previously determined antitumour efficacy and toxicity of the dFdC/CDDP combination. Peak dFdCTP accumulation in tumours (11 pmol/mg) was found 4 h after dFdC treatment, while CDDP tended to reduce this in a time-dependent way. Peak levels of total Pt in tumours were found 4 h after CDDP treatment (581 fmol/mg) and dropped 1.8-fold after simultaneous treatment with dFdC ($P=0.04$). Treatment with dFdC 4 h after or simultaneously with CDDP increased Pt retention (level 24 h after CDDP treatment) 1.4- and 1.6-fold ($P=0.04$ and $P=0.03$, respectively). Peak Pt–DNA adduct levels in tumours were also found 4 h after CDDP treatment (7 fmol/μg DNA) and were decreased 3-fold by dFdC treatment 24 h prior to CDDP ($P=0.04$). Pt–DNA adduct retention was only decreased when dFdC was given 4 h before CDDP (8-fold ($P<0.01$)). The retention and the area-under the concentration–time curve of Pt–DNA adducts were related to decreased tumour doubling time (linear regression coefficient (R) = 0.95; $P<0.05$, 0.96 $P=0.04$ and 0.90; $P=0.04$. Pt–DNA adduct levels in the BM cells reached a plateau level 4–24 h after CDDP treatment (approximately 10 fmol/μg DNA), which was increased by dFdC when given either simultaneously with, 4 h before or 4 h after CDDP (6-, 3- and 5-fold at 28 h, 8 h and 28 h, respectively ($P\leqslant0.04$)). Peak Pt–DNA adduct formation (24 h: 8 fmol/μg DNA) in kidneys was enhanced by dFdC when given simultaneously with or 4 h before CDDP (4 h timepoint) ($P<0.01$). However, retention was 4- and 6-fold decreased when dFdC was given 4 or 24 h after CDDP, respectively ($P\leqslant0.01$). dFdC given 24 h before CDDP decreased all Pt–DNA adduct levels in kidneys 3-fold or more ($P\leqslant0.03$). Pt–DNA adduct levels were inversely related to kidney toxicity when the most toxic schedule was excluded from the analysis. Peak levels of total Pt in kidneys were reached 24 h after CDDP treatment (4.3 fmol/mg) and the 8 h levels were increased 2-fold by dFdC when given 4 h after CDDP ($P=0.07$). © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Cisplatin; Gemcitabine; Murine; Non-small cell lung cancer; Schedule; Pharmacokinetics; Pharmacodynamics

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1. Introduction

2′,2′-Difluorodeoxycytidine (gemcitabine, dFdC, Gemzar®) is a deoxycytidine analogue [1] with clinical activity against several solid tumours, including ovarian cancer, non-small cell lung cancer (NSCLC), head and neck squamous cell carcinoma (HNSCC), pancreatic cancer and bladder cancer [2]. After entering the cell, dFdC is phosphorylated by deoxycytidine kinase (dCK) to its triphosphate (dFdCTP), which can be incorporated into DNA, followed by one more deoxynucleotide after which DNA polymerisation stops [3]. This process probably determines its cytotoxic effect. dFdCDP is also capable of inhibiting ribonucleotide reductase (RR) [4], an enzyme which plays a key role in DNA repair. Furthermore, dFdCTP can be incorporated into RNA, leading to inhibition of RNA synthesis [5,6].

cis-Diammine-dichloroplatinum(II) (cisplatin, CDDP) is an established anticancer drug with a similar spectrum of antitumour activity as dFdC: ovarian, NSCL, HNSCC, pancreatic and bladder cancer. CDDP is generally considered to exert its cytotoxic effect by binding to DNA, resulting in a number of different adducts [7]. A relationship between platinum (Pt)–DNA adduct levels and antitumour response in cultured cells [8] and in patients has been postulated [9,10].

In *in vitro* studies with colon, ovarian, HNSCC and NSCL cancer cell lines a synergistic effect of these drugs was found [6,11] which was time- and sequence-dependent. The most effective schedule was a 4 h dFdC preincubation followed by the addition of CDDP [11]. The mechanism of this synergy appeared to be an increase in the formation of Pt–DNA adducts, possibly due to the incorporation of dFdC into DNA [6].

In a schedule-finding *in vivo* study of the combination of both drugs in the murine NSCLC Lewis lung (LL) model bearing C57/Bl6 mice, a schedule dependency was observed between the two compounds: dFdC (60 mg/kg intraperitoneal (i.p.) bolus, q3 days×4) preceding CDDP (6 mg/kg i.p. bolus, day 0) by 4 h was shown to be the most active schedule. The reverse schedule also led to an increased antitumour effect [12]. Toxicity was also found to be schedule dependent. dFdC preceding CDDP by 4 h showed acceptable toxicity, whereas increasing the interval to 24 h resulted in unacceptable toxicity (>30% weight loss). The reverse schedule did not lead to increased toxicity.

The aim of this *in vivo* study was to determine the relationship between the schedule-dependent pharmacokinetics and pharmacodynamics of dFdC and CDDP in LL tumours, bone marrow (BM) and kidneys and the antitumour response and toxicity of the combination in C57/Bl6 mice. For this purpose, dFdCTP formation, total Pt accumulation and Pt–DNA adduct formation were studied in LL tumours, BM and kidneys of C57/Bl6 mice treated with several schedules of dFdC and CDDP.

2. Materials and methods

2.1. Materials

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) was kindly provided by Lilly Research Center Ltd (IN, USA). Each ampoule contained gemcitabine HCl 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. All other chemicals were of analytical grade and were commercially available.

2.2. Tumour and treatment schedules

The source and characteristics of the murine LL tumour (NSCLC) have been described elsewhere [13]. 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.

2.3. Treatment

When tumours reached a volume of 200–250 mm^3, treatment was started. Prior to treatment, mice were randomised into several groups, one group serving as a control group. Each group consisted of at least three mice, corresponding to six tumours. Mice were treated by i.p. bolus injection of 60 mg/kg dFdC or 6 mg/kg CDDP, alone or in combination. When the combination was studied, mice were either treated with both drugs simultaneously, or with dFdC preceding CDDP by 4 or 24 h, or with the reverse treatment. All protocols were approved by the ethics committee for animal experiments of the Vrije Universiteit in Amsterdam and comply with the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia [14].

2.4. Sample collection

At several time points after treatment (4, 8, 12, 24 and 28 h) mice were anaesthetised and one tumour was removed for dFdCTP analysis and immediately put in liquid nitrogen. After removing one tumour, mice were sacrificed by cervical dislocation and the other tumour, kidneys, BM and blood were removed. Tumours and kidneys were immediately frozen in liquid nitrogen and stored at −80°C. Frozen tumours were pulverised using

a micro-dismembrator as previously described [15]. BM cells were isolated and immediately stored on ice until washing in ice-cold phosphate buffered saline (PBS) and the determination of the cell amount with a Bürker counter. After centrifugation (1 min, 10 000 *g*, 4°C) the BM cell pellet was frozen in liquid nitrogen and stored at −80°C until analysis. Blood was collected in heparin containing Eppendorf vials and kept on ice until centrifugation (5 min, 3000 *g*, 4°C). After centrifugation, the upper plasma layer was removed, transferred into a new Eppendorf vial and stored at −80°C until analysis. Urea and creatinine plasma levels were analysed with a Technicon RA-1000 auto-analyser (Technicon, Gorinchem, The Netherlands) and aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) levels were analysed with a Hitachi 737 Automatic Analyser (Boehringer Mannheim B.V., Almere, The Netherlands).

2.5. dFdCTP accumulation in LL tumours

Frozen tumour powder was weighed and suspended in ice-cold PBS (1 g frozen tumour material/1 ml PBS). dFdCTP in tumour powder extract was extracted and analysed by high performance liquid chromatography (HPLC) as previously described [11,16]. Briefly, 1/3 volume of 40% trichloro-acetic acid (TCA; 10% final concentration) was added to the PBS-suspended tumour material and the test-tube was left on ice for 20 min to precipitate the proteins and nucleic acids. Subsequently, the mixture was centrifuged for 10 min at 10 000 *g* at 4°C. A 2-fold excess of a freshly prepared mixture of trioctylamine and 1,1,2-trichlorotrifluoroethane (1:4) was added to neutralise the supernatant. After centrifugation (1 min, 10 000*g*, 4°C), the nucleotide extract (upper aqueous layer) was carefully removed and stored at −20°C until analysis. Separation and quantification of dFdCTP was achieved with a gradient HPLC system (Partisphere SAX anion-exchange column) connected to a photo-diode array detector, regularly set at 254 and 280 nm as previously described [16]. Peaks were quantified by Chromeleon Version 3.02 (Chromeleon Chromatography Data Systems, Gynkotek HPLC, Germering, Germany).

2.6. Measurement of total Pt concentration in tumours and kidneys

100 mg of frozen tumour or kidney was destructed overnight with 0.5 ml benzethoniumhydroxide (Sigma, Zwijndrecht, The Netherlands) and diluted with 4.25 ml 0.2 M hydrochloric acid, as previously described for the destruction of red blood cells [17]. Calibration standards were prepared by spiking control tumour and kidney samples with known amounts of CDDP and were handled in the same way as the treated samples. Total Pt concentration was analysed by flameless atomic absorption spectrophotometry using a Spectra AA-300 Zeeman AAS (Varian, Houten, The Netherlands).

2.7. Platinum–DNA adduct determination in BM cells, tumours and kidneys

Pt–DNA adduct levels in tumours, BM cells and kidneys were analysed by ^{32}P-postlabelling as previously described [18–20]. Briefly, DNA was isolated and completely digested to mononucleosides and the main Pt–DNA dinucleotides: Pt–GG and Pt–AG. The platinated dinucleotides were purified on a strong cation-exchange column coupled to a fast performance liquid chromatography (FPLC) system (Mono-S, Pharmacia Biotech Benelux, Roosendaal, The Netherlands). The adducts were collected in Eppendorf vials, containing NaCN, and after addition of thymidylyl 3′,5′ thymidine (TpT) as an internal standard, they were dried under a vacuum. After drying, the mixture was dissolved in NaCN and incubated in order to remove the Pt from the adducts. Each sample was labelled with [γ-^{32}P]ATP using T4-polynucleotide kinase. The separation of the radioactively labelled products was performed by thin layer chromatography (Polygram Cel 300 PEI TLC sheets) with NH_4-formate buffer and each spot was quantified using a Phosphor Imager 425 with Image Quant software (Molecular Dynamics, Sunnyvale, CA, USA). The amounts of Pt–GG and Pt–AG thus measured account for over 80% of all adducts formed by CDDP [21]. Therefore, the total of these adducts was taken as the total amount of Pt–DNA adducts formed by CDDP.

2.8. Statistical evaluation

Changes in the pharmacokinetics and pharmacodynamics compared with dFdC or CDDP alone were evaluated using the parametric two-tailed Students *t*-test and the non-parametric Mann–Whitney *U*-test (*P*-values are only given in the text when lower than 0.15).

The area under the concentration versus time curve (AUC) from $t=0$ (start of treatment) to infinity of all pharmacokinetic and pharmacodynamic data were calculated by the linear trapezoidal rule using the pharmacokinetic computer program Topfit, version 2.0. Relationships between the pharmacokinetic and pharmacodynamic data and AUCs with toxicity and antitumour response of the different schedules (as previously published [12]) were studied by multiple linear regression. All statistics were performed with the Statistical Package for the Social Sciences (SPSS) computer program, version 7.0.

3. Results

3.1. dFdCTP accumulation in tumours

dFdCTP is the active metabolite of dFdC that can be incorporated into DNA and is generally thought to be responsible for the cytotoxic effect. CDDP decreased dFdCTP accumulation in LL tumours in a time-dependent manner: tumours of mice treated with CDDP 4 or 24 h before dFdC or dFdC and CDDP simultaneously accumulated 132-, 1.3- and 8-fold less dFdCTP than after treatment with dFdC alone (Fig. 1). Furthermore, mice treated with CDDP after dFdC tended to retain dFdCTP longer in their tumours than mice treated with dFdC alone. However, none of these effects were significant, probably due to the high variation of dFdCTP measurements in this study, even though tumours were removed from mice under anaesthesia and were immediately frozen.

3.2. Total platinum levels in tumours

Peak levels of total Pt of 581 fmol/mg tumour were measured at 4 h after treatment with CDDP alone. dFdC influenced both the time and height of the total Pt peak depending on the schedule and the interval between the drugs (Fig. 2). Simultaneous treatment with dFdC decreased the peak Pt levels at 4 h 1.8-fold ($P=0.04$) and shifted the total Pt peak to 8 h with maximal levels of 698 fmol Pt/mg ($P=0.12$). However, dFdC given 4 h before CDDP decreased peak Pt levels 1.5-fold ($P=0.11$). Furthermore, dFdC given 4 h after CDDP or simultaneously with CDDP increased the 28 h levels of total Pt 1.4- and 1.6-fold ($P=0.04$ and 0.03) respectively, compared with CDDP alone.

3.3. Platinum–DNA adduct formation in tumours

Since Pt–DNA adduct formation is generally considered to be responsible for the cytotoxic action of

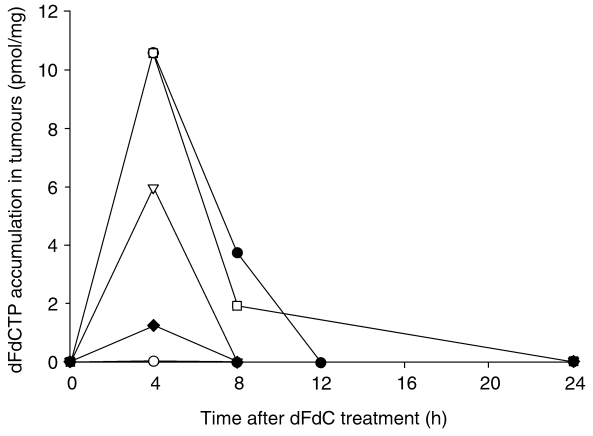

Fig. 1. 2′,2′-difluorodeoxycytidine triphosphate (dFdCTP) accumulation in Lewis lung (LL) tumours after treatment of mice with 2′-2′-difluorodeoxycytidine (dFdC) alone and different schedules of combinations with cisplatin (CDDP): dFdC alone (□); simultaneous treatment (◆), dFdC 4 h before CDDP (●); CDDP 4 h before dFdC (○); and CDDP 24 h before dFdC (▽). Values are means of measurements in at least three tumours of separate mice. The standard error of the mean (SEM) ranged from 53 to 96% of the mean, but are not shown so that the clarity of the figure is not affected.

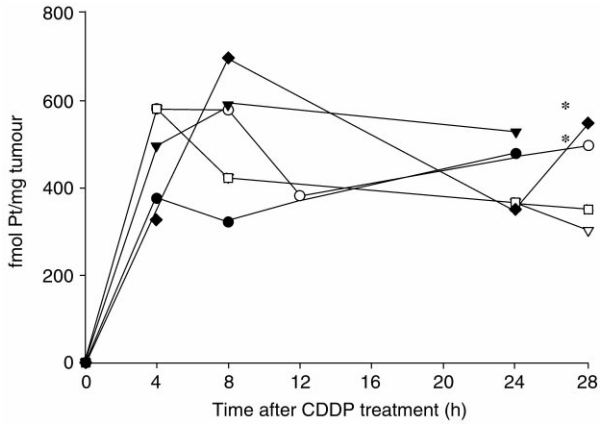

Fig. 2. Total platinum (Pt) levels in Lewis lung (LL) tumours after treatment of mice with cisplatin (CDDP) alone and different schedules of combinations with 2′,2′-difluorodeoxycytidine (dFdC): CDDP alone (□); simultaneous treatment (◆); dFdC 4 h before CDDP (●); CDDP 4 h before dFdC (○); dFdC 24 h before CDDP (▼); and CDDP 24 h before dFdC (▽). Values are the means of measurements in at least three tumours of separate mice. The standard error of the mean (SEM) ranged from 4 to 94% of the mean, but are not shown to maintain the clarity of the figure. *Significantly different from CDDP alone ($P<0.05$).

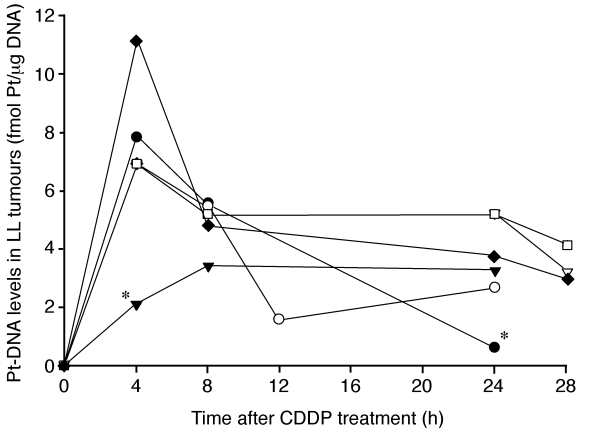

Fig. 3. Platinum (Pt)–DNA adduct levels in Lewis lung (LL) tumours after treatment of mice with cisplatin (CDDP) alone and different schedules of combinations with 2′,2′-difluorodeoxycytidine (dFdC): CDDP alone (□); simultaneous treatment (◆); dFdC 4 h before CDDP (●); CDDP 4 h before dFdC (◇); dFdC 24 h before CDDP (▼); and CDDP 24 h before dFdC (▽). Values are means of measurements in at least three tumours of separate mice. The standard error of the mean (SEM) ranged from 12 to 53% of the mean, but are not shown on the figure for clarity. *Significantly different from CDDP alone ($P<0.05$).

Table 1
Schedule dependency of the platinum (Pt)–GG/Pt–AG ratio in Lewis lung (LL) tumours, bone marrow (BM) and kidneys of C57/Bl6 mice treated with 2′,2′-difluorodeoxycytidine (dFdC) and cisplatin (CDDP)

	Pt–GG/Pt–AG ratio					
	Tumours		BM		Kidneys	
Exposure	T_{max}	24 h	T_{max}	28 h	8 h	T_{max}
CDDP alone	6.2±1.6	9.0±3.6	6.1±2.2	2.5±0.4	6.0±0.3	7.2±2.4
dFdC and CDDP	4.2±0.8	2.0±0.4	1.5±0.1	1.9±0.2	6.0±0.5	5.5±0.1
dFdC 4 h before CDDP	8.2±2.5	3.5±1.8	1.6±0.1	2.6±0.3[b]	4.3±0.3*	3.3±0.5
CDDP 4 h before dFdC	6.2±1.6	2.8±0.5[a]	2.6±0.2	1.5±0.0	4.3±0.9	2.9±0.5*,[a]
dFdC 24 h before CDDP	7.2±3.1	10.8±7.1	1.9±0.3	6.1±2.6[b]	3.8±0.4*	8.5±1.2
CDDP 24 h before dFdC	6.2±1.6	9.0±3.6	6.1±2.2	1.6±0.2	6.0±0.3	7.2±2.4

T_{max} is the time at which maximal Pt–DNA adduct levels were achieved after treatment with CDDP alone (T_{max} tumours: 4 h, T_{max} kidneys: 24 h, and for BM T_{max} represents the ratio for the 8 h levels). Values are the means of 3–9 tumours, BM samples or kidneys of separate mice ±standard error of the mean (SEM).

[a] Pt–GG/Pt–AG ratio for the 28 h levels.

[b] Pt–GG/Pt–AG ratio for the 24 h levels.

*Significantly different from CDDP alone ($P < 0.05$).

CDDP, we measured Pt–DNA adducts in LL tumours. Peak levels of Pt–DNA adducts in LL tumours were found 4 h after treatment with CDDP alone (Fig. 3) and increased by simultaneous treatment, but were unchanged when dFdC was given 4 h before CDDP. Pt–DNA adduct peak levels decreased 3-fold by treatment with dFdC 24 h before CDDP ($P = 0.04$), which tended to be accompanied by a 1.2-fold increase in the Pt–GG over Pt–AG ratio (Table 1). Furthermore, the 24 h level of Pt–DNA adducts was 8-fold lower when dFdC was given 4 h before CDDP, compared with treatment with CDDP alone ($P < 0.01$), and was accompanied by an approximately 3-fold decrease in the tumour Pt–GG/Pt–AG ratio. In addition, the 24 h ratio between Pt–GG and Pt–AG levels in the tumour was reduced approximately 4-fold by simultaneous treatment with dFdC and CDDP, compared with CDDP alone ($P = 0.12$). The Pt–DNA adduct levels did not correlate with the total Pt levels in tumours, clearly indicating that dFdC interferes with the normal pharmacokinetics of CDDP.

3.4. dFdC and CDDP pharmacokinetics and antitumour effect of treatment

All pharmacokinetic and pharmacodynamic parameters determined in this study and the area under the concentration–time curves of these parameters (Table 2) were related to the antitumour effect of these combinations of dFdC and CDDP [12]. No relationships between the tumour dFdCTP accumulation and antitumour effect were found. However, Pt–DNA adduct levels at 28 h, especially Pt–GG, and Pt–DNA AUC in the tumours were related to the decreased tumour doubling time of tumours treated with the combination ($R = 0.95$ ($\pm < 0.05$), 0.96 ($P = 0.04$) and 0.90 ($P = 0.04$) (Fig. 4), respectively).

Table 2
Schedule dependency of the area under the concentration–time curves (AUCs) of 2′,2′-difluorodeoxycytidine triphosphate (dFdCTP), total platinum (Pt) and Pt–DNA adducts in Lewis lung (LL) tumours, bone marrow (BM) and kidneys of C57/Bl6 mice treated with dFdC and cisplatin (CDDP)

	dFdCTP in tumours (pmol×h/mg)	Pt in tumours (nmol×h/g)	Pt–DNA in tumours (pmol×h/μg DNA)[a]	Pt–DNA in BM (pmol×h/μg DNA)	Pt in kidneys (nmol×h/g)	Pt–DNA in kidneys (pmol×h/μg DNA)
dFdC alone	132.33	–	–	–	–	–
CDDP alone	–	10.67	0.47	0.23	91.09	0.11
dFdC and CDDP	5.04	11.22	0.25	0.38	100.97	0.17
dFdC 4 h before CDDP	120.58	8.58	0.11	0.33	83.60	0.12
CDDP 4 h before dFdC	0.32	11.56	0.16	0.29	83.87	0.09
dFdC 24 h before CDDP	132.33	12.82	1.58	0.12	81.22	0.03
CDDP 24 h before dFdC	23.84	10.67	0.50	0.23	91.09	0.11

The AUCs were calculated from the start of exposure (injection) to 24 h after exposure from the mean values at each time point with the computer program Topfit.

[a] $AUC_{0-\infty}$: AUC calculated from drug injection to infinity.

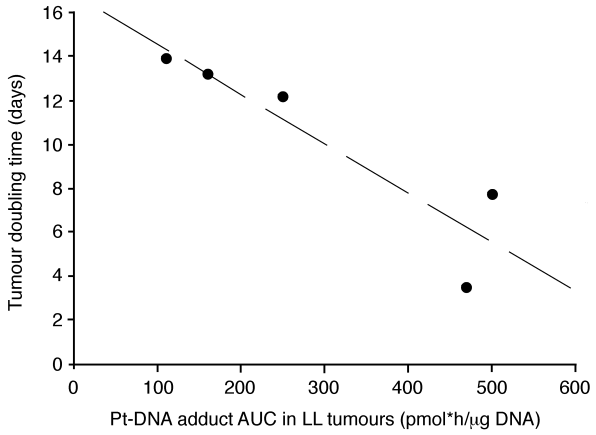

Fig. 4. Relationship between the area under the concentration–time curve (AUC) of platinum (Pt)–DNA adducts and tumour doubling time in Lewis lung (LL) tumours treated with different combination schedules of 2′,2′-difluorodeoxycytidine (dFdC) and cisplatin (CDDP). Multiple linear regression coefficient $R = -0.95$ ($P = 0.04$).

3.5. Platinum–DNA adduct formation in BM

We studied Pt–DNA adduct formation in BM cells since they are most likely responsible for the toxicity of the combination of dFdC and CDDP. A plateau of Pt–DNA adduct levels in the BM was found, which lasted from 4 until 24 h after treatment with CDDP alone (Fig. 5). However, when given simultaneously or 4 h before CDDP, dFdC changed the peak of Pt–DNA adducts to 8 h after CDDP treatment and increased adduct levels approximately 2- ($P = 0.06$) and 3-fold ($P = 0.04$), respectively, accompanied by an approximately 4-fold decrease in the Pt–GG/Pt–AG ratio ($P = 0.12$) (Table 1). Of interest, dFdC caused an approximately 3-fold reduction of the ratio between Pt–GG and Pt–AG adducts when given 24 h before CDDP ($P = 0.14$). The latter finding was observed despite the fact that there was no effect on the total Pt–DNA adduct levels (data not shown). Similarly, dFdC affected Pt–DNA adduct retention in BM cells. Both simultaneous treatment and dFdC given 4 h after CDDP significantly increased the 28 h levels 6- ($P < 0.01$) and 5-fold ($P < 0.01$), respectively (Fig. 5), again accompanied by a 1.3- and 1.7-fold ($P = 0.11$) decrease in the Pt–GG/Pt–AG ratio (Table 1).

3.6. Total platinum accumulation in kidneys

The peak level of total Pt in kidneys after treatment with CDDP alone, which measured 4.3 fmol/mg (AUCs shown in Table 2), was reached 24 h after treatment. dFdC affected total Pt in kidneys only when mice were treated with dFdC 4 h after CDDP, this caused a shift in the peak of total Pt to 8 h with a level of 6.8 fmol/mg ($P = 0.07$). None of the other schedules induced significantly different levels of Pt in kidneys compared with CDDP alone.

3.7. Platinum–DNA adduct formation in kidneys

The peak of Pt–DNA adduct formation in kidneys appeared at 24 h after treatment with CDDP (Fig. 6). Simultaneous treatment with dFdC increased the 8 h Pt–DNA adduct levels 1.9-fold ($P < 0.01$), but these

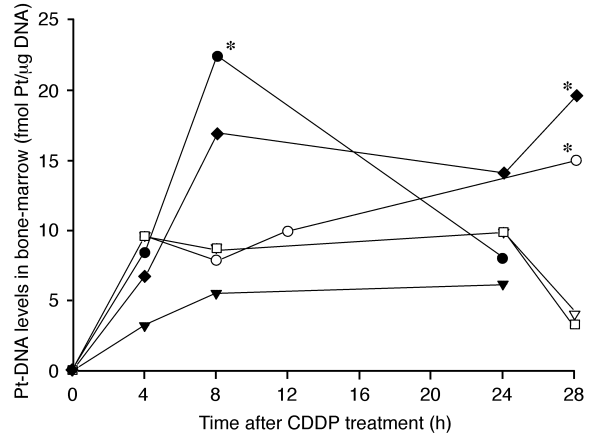

Fig. 5. Platinum (Pt–DNA) adduct levels in bone marrow (BM) of mice after treatment with cisplatin (CDDP) alone and different schedules of combinations with 2′,2′-difluorodeoxycytidine (dFdC): CDDP alone (□); simultaneous treatment (◆); dFdC 4 h before CDDP (●); CDDP 4 h before dFdC (○); dFdC 24 h before CDDP (▼); and CDDP 24 h before dFdC (▽). Values are means of measurements in at least three BM pellets of separate mice. The standard error of the mean (SEM) ranged from 9.8 to 49.3% of the mean, but are not shown on the figure for clarity. *Significantly different from CDDP alone ($P < 0.05$).

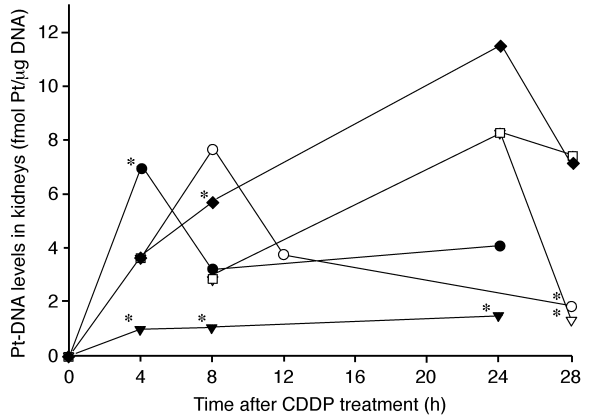

Fig. 6. Platinum (Pt)–DNA adduct levels in kidneys of mice after treatment with cisplatin (CDDP) alone and different schedules of combinations with 2′,2′-difluorodeoxycytidine (dFdC): CDDP alone (□); simultaneous treatment (◆); dFdC 4 h before CDDP (●); CDDP 4 h before dFdC (○); dFdC 24 h before CDDP (▼) and CDDP 24 h before dFdC (▽). Values are means of measurements in at least three kidneys of separate mice. The standard errors of the mean (SEMs) ranged from 6.4 to 68.0% of the mean, but are not shown on the figure for clarity. *Significantly different from CDDP alone ($P < 0.05$).

changes were not accompanied by a change in the Pt–GG/Pt–AG ratio (Table 1). dFdC caused a shift of the Pt–DNA adduct peak to 4 and 8 h, when given 4 h before and 4 h after CDDP ($P=0.01$ and 0.06), respectively. These changes were accompanied by a 1.4-fold decrease of the Pt–GG/Pt–AG ratios ($P<0.10$). Due to the shift in Pt–DNA adduct peak, dFdC decreased the 24 h Pt–DNA adduct levels 2-fold when given 4 h prior to CDDP ($P=0.11$), accompanied by a 2-fold decrease in Pt–GG/Pt–AG ratio ($P=0.08$). Pt–DNA adduct retention was also affected by dFdC. When dFdC was given either 4 or 24 h after CDDP, the 28 h Pt–DNA adduct levels in the kidneys dropped 4- and 6-fold ($P<0.01$ and $P=0.01$), respectively. Of interest, only dFdC given 4 h after CDDP resulted in a significant decrease in the Pt–GG/Pt–AG ratio (2-fold, $P=0.01$). All Pt–DNA adduct levels were significantly decreased when dFdC was given 24 h before CDDP (4, 8 and 24 h levels: 4- ($P<0.01$), 30- ($P=0.03$) and 5-fold ($P=0.02$), respectively), only decreasing the Pt–GG/Pt–AG ratio 8 h after CDDP treatment 1.6-fold ($P<0.01$).

3.8. CDDP pharmacokinetics and BM, kidney and liver toxicity

All schedules of dFdC and CDDP induced a high degree of acute BM, kidney and liver toxicity as shown in Table 3. dFdC and CDDP alone decreased BM cell count to 48.8 and 66.5% of the control levels, respectively, whereas the simultaneous administration of both compounds reduced BM cell counts to 26.6%. Renal toxicity appeared to be somewhat less. dFdC and CDDP alone reduced creatinine levels in plasma to 64 and 92% of control levels, respectively, but the combination caused a further decrease to 82%. dFdC and CDDP alone failed to affect urea levels, whereas the combination of both compounds decreased urea levels to 81% of the control levels. Hepatic toxicity was very severe in these mice and macroscopically observable: CDDP alone decreased ALAT levels to 81%, whereas all combinations with dFdC, where measurable, decreased the ALAT and ASAT levels to 47–91% of control levels with the exception of ASAT levels for the CDDP 4 h before dFdC treatment. Overall, the schedule of CDDP preceding dFdC for 4 h caused the least BM, kidney and liver toxicity, whereas dFdC 24 h before CDDP was the most toxic schedule.

Surprisingly the maximal weight loss (MWL) [12], BM and renal toxicity due to the dFdC and CDDP treatment were not directly related to any of the measured CDDP parameters. However, since this appeared to be mainly due to the most toxic schedule, i.e. dFdC 24 h before CDDP, we excluded this schedule from the analysis. As a consequence, the amount of Pt–DNA adducts in the kidneys 4 h after treatment, mainly Pt–GG adducts, showed a relationship with decreased urea levels in plasma ($R=-1.00$ ($P<0.01$)). However, this relationship was based on only three data points.

4. Discussion

This is the first extensive study of the intracellular pharmacology and pharmacodynamics in tumour-bearing animals of one of the most used combinations of cytotoxic agents. We found that the mechanistic interactions between dFdC and CDDP in mice depended on the sequence and time interval between the administration of the two drugs. CDDP affected dFdCTP accumulation and retention in tumours, whereas dFdC affected the accumulation and retention of both total Pt and Pt–DNA adducts in kidneys, tumours and BM. However, there was no clear correlation between these parameters and the more than additive antitumour effects and toxicity of the combination of dFdC and CDDP in LL tumour-bearing mice.

Depending on length of the interval between the drugs, CDDP decreased dFdCTP accumulation in

Table 3
Schedule dependency of the toxicity of the different 2′,2′-difluorodeoxycytidine (dFdC) and cisplatin (CDDP) schedules in C57/Bl6 mice

	BM cell count (%)	Creatinine (%)	Urea (%)	ASAT (%)	ALAT (%)
dFdC alone	48.8	64	105	nd	105
CDDP alone	66.5	92	102	nd	81
dFdC and CDDP	26.6	82	101	nd	67
dFdC 4 h before CDDP	42.4	87	81	52	65
CDDP 4 h before dFdC	34.6	90	96	110	91
dFdC 24 h before CDDP	25.6	79	91	49	47
CDDP 24 h before dFdC	29.6	85	98	nd	60

BM, bone marrow; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase. All parameters were measured 4 h after the last treatment (BM cell count 24 h). For creatinine, urea, ASAT and ALAT detection, blood from at least three mice were pooled, due to the low volumes, and the levels in the pooled blood samples from treated C57/Bl6 mice are given as a percentage of the levels in the non-treated tumour-bearing C57/Bl6 mice (control levels: 9.5×10^6 BM cells, 39 μM creatinine, 10.1 mM urea, 489 units/l ASAT and 43 units/l ALAT). nd, value not measurable due to haemolysis.

tumours, which was in agreement with previous studies in the LL and several other cell lines *in vitro* [6,11]. A change in the balance between the UTP, CTP, and ATP pools might be responsible for these effects, since these nucleotides regulate the activity of dCK [22]. However, in this study no increase in the CTP and UTP pools was found (data not shown). Possibly, CDDP inhibits dFdC uptake in LL tumours directly; since CDDP also inhibited the *in vitro* uptake of 2′-deoxy-5-azacytidine (DAC), another deoxycytidine analogue [23]. Furthermore, *in vitro* studies have shown that CDDP increased the dFdCTP incorporation into DNA [6], which may have led to the decreased dFdCTP levels in the tumour.

The peak of total Pt accumulation in LL tumours, 581 fmol/mg (174 ng/g tissue) was approximately 10-fold lower than that at 4 or 3 h in squamous-cell carcinoma xenografts after treatment with 7.5 or 7 mg CDDP/kg i.p., respectively [24,25], but only 4-fold lower than that at 24 h in ovarian cancer xenografts after treatment with 5 mg/kg CDDP i.v. [26]. dFdC decreased the total Pt levels when given simultaneously or 4 h before CDDP, but not when given later or 24 h before CDDP. Previous *in vitro* studies in the LL cell line have shown that dFdC, when given simultaneously with CDDP for 24 h, did not affect the accumulation of Pt [6], indicating that pharmacokinetic interactions might be important for the inhibition of total Pt accumulation. Indeed, dFdC given before CDDP decreased total Pt levels in the plasma of patients [20]. Increased CDDP binding to glutathione (GSH), due to inhibition of the CDDP detoxifying glutathione-S-transferase, might decrease the amount of CDDP actively transported out of the cell [27,28]. However, preliminary studies in the human ovarian cancer cell line A2780 did not reveal an effect of dFdC on the amount of cellular GSH (A.M. Bergman, G.J. Peters, University Hospital Vrije Universiteit, Amsterdam, The Netherlands). However, *in vivo* these interactions might be different.

Pt–DNA adduct peak levels in tumours (6.9 fmol/μg DNA, 2.3 adducts/10^6 nucleotides) were approximately 3-fold lower than those measured in HNSCC xenografts [25], whereas *in vitro* exposure to 20 μM CDDP led to 50-fold higher Pt–DNA adduct levels in LL cells [6]. dFdC decreased Pt–DNA adduct formation when given 24 or 4 h before CDDP, while total Pt and Pt–DNA adduct levels in the tumour were not correlated. This indicates that dFdC interfered with the normal pharmacokinetics of CDDP. Indeed, in patients dFdC given before CDDP tended to decrease Pt–DNA adduct formation in white blood cells (WBC) [20]. These results were in line with our previous *in vitro* studies with the human ovarian cancer cell line A2780, in which simultaneous treatment with dFdC increased Pt–DNA adduct repair [29]. Induction of nucleotide excision repair (NER) processes after incorporation of dFdC into DNA [30,31], might result in the removal of Pt–DNA adducts from the DNA [32] leading to either decreased Pt–DNA adduct formation, or to increased Pt–DNA adduct retention, depending on the interval between dFdC and CDDP treatment.

Both Pt–DNA adduct retention and the Pt–DNA AUC in the tumour were inversely related to the antitumour activity of the combination. This is in contrast to that described for Pt–DNA adduct AUC and the antitumour effects of CDDP alone [10,25,33–37], implicating that for the combination of dFdC and CDDP the level of repair of Pt–DNA adducts is a more important parameter for antitumour activity than the height of Pt–DNA adduct levels in the tumour.

In BM cells the peak Pt–DNA adduct levels were 1.4-fold higher than in tumours, whereas in the kidneys the peak was later and somewhat higher than in tumours. In contrast, dFdC increased peak Pt–DNA adduct formation in both BM cells and kidneys, indicating a direct interaction between dFdC incorporation into DNA and Pt–DNA adduct formation. *In vitro* for both the combination of dFdC and CDDP [6], and that of CDDP with DAC, a relationship between Pt-adducts and DNA incorporation was found [23]. Incorporation of dFdC into DNA could lead to structural changes altering the binding of Pt to the guanine nucleotide opposite to the cytosine nucleotide. Since dFdC decreased the Pt–GG/Pt–AG ratio in the BM cells and kidneys for most treatments, the Pt–AG adduct was more easily formed than the Pt–GG adduct. Thus, the conformational changes induced by dFdC might hamper the binding of Pt to the guanine nucleotide opposite the cytosine nucleotide, favouring the binding to the adenine nucleotide. Since the Pt–AG nucleotide has been described to be the most important adduct for the cytotoxic action of CDDP [38], this might be an important underlying mechanism for the more than additive toxicity of both compounds.

Surprisingly, only in BM cells did dFdC increase Pt–DNA adduct retention when given simultaneously with or 4 h after CDDP. This may be the result of the inhibition of ribonucleotide reductase (RR) by dFdC [4], causing depletion of deoxyribonucleotide pools in cells and inhibition of repair processes. Thus, BM cells may be more sensitive to the RR inhibition than tumour and kidney cells, which might indicate an additional reason for the more than additive toxicity. The increased Pt–DNA adduct formation and decreased repair in normal cells might underlie clinical toxicity, such as dose-limiting thrombocytopenia in the once weekly gemcitabine times three schedule with cisplatin in the first week followed by a one week rest [39–44]. The omission of the last scheduled gemcitabine administration in over 25% of all cases [45], led many researchers to use a once weekly times two week schedule with a 1 week rest, allowing for platelet recovery.

In conclusion, we have shown that the combination of dFdC and CDDP has multiple mechanistic interactions *in vivo*. CDDP decreased dFdCTP accumulation in tumours, possibly via increased incorporation of dFdCTP into DNA. CDDP may have other effects on the metabolism of dFdC contributing to or even responsible for the supra-additive effect of the combination. The decreased Pt–DNA adduct formation and retention in tumours could be due to the induction of repair by dFdC, causing an inverse relationship with antitumour activity, while the increase in BM cells and kidneys may result from conformational changes of DNA after incorporation of dFdC and lead to the more than additive toxicity. Furthermore, dFdC may affect deoxyribonucleotide levels, resulting in inhibition of DNA repair and altering the metabolism of dFdC itself.

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