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Schedule-dependent Therapeutic Efficacy of the Combination of Gemcitabine and Cisplatin in Head and Neck Cancer Xenografts

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Gemcitabine and cisplatin are both drugs with proven clinical activity in various tumour types, have no overlapping toxic side-effects and are different with respect to cellular metabolism. We, therefore, performed an *in vivo* study to determine the efficacy of the combination of these two drugs using two human head and neck squamous cell carcinoma xenograft lines, subcutaneously growing in athymic nude mice. 100 mg/kg gemcitabine was given intraperitoneally on days 0, 3, 6 and 9 and 4 mg/kg cisplatin intravenously on days 0 and 6. In one tumour line, the combination treatment resulted in better effects than those observed when the drugs were administered individually. In the other cell line, addition of cisplatin did not increase the moderate effect of gemcitabine. Experiments with single dose injections of both drugs showed adverse effects when the interval was extended to 24 h. These data are of potential interest for clinical application, and suggest that the drugs should be administered either simultaneously or with a short time interval in which cisplatin should precede gemcitabine.

Key words: athymic mice, cisplatin, combination therapy, gemcitabine, head and neck cancer, nude mice, preclinical, xenografts

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INTRODUCTION

CISPLATIN IS an established anticancer drug with activity in a variety of solid tumours, including head and neck squamous cell carcinoma (HNSCC). Thirty per cent of patients with local and regionally advanced HNSCC show a response to monochemotherapy with cisplatin, but unfortunately an enhancement of survival has not been observed [1]. The action of cisplatin is generally considered to be based on its interaction with DNA [2]. Although it has been established which cisplatin–DNA adducts are formed, the nature of the "key lesion" is still unknown [3].

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is a recently synthesised deoxycytidine analogue [4], which, unlike cytosine arabinoside, shows broad spectrum activity against panels of murine tumours and human tumour xenografts [5]. As for the xenografts, activity has been observed in lung, breast, colon [5], ovarian [6], and HNSCC [7]. Promising activity has also been observed in phase I and II trials of lung, breast and ovarian cancer [8]. The mechanism of action of gemcitabine is characterised by a complex metabolic pathway, in which an array of enzymes and metabolites are involved [9, 10]. Heinemann and associates [11] has described unique mechanisms of self potentiation, including, among others, the blockade of ribonucleotide reductase. The main mechanism of action is assumed to be the incorporation of the triphosphate form of gemcitabine, dFdCTP, into DNA. The first rate limiting step in the phosphorylation of dFdCTP is catalysed by deoxycytidine kinase. This enzyme plays a crucial role in the mechanism of action of gemcitabine, since deficiency of this enzyme results in resistance to gemcitabine [12]. Furthermore, the extent of dFdCTP formation and retention has been related to the *in vitro* sensitivity to gemcitabine [13]. Incorporation into RNA may play a role in the cytotoxic action of this drug [14].

Up to now, no studies have been reported on the combination of gemcitabine with cisplatin. However, a number of studies have described a synergistic effect between two other cytidine analogues, cytosine arabinoside (ara-C) or 5-aza-2′-deoxycytidine (5-aza-CdR) and cisplatin. *In vitro*, these synergistic effects

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of the combination of ara-C with cisplatin have been reported for murine [15] and human leukaemia [16], and a variety of human cancer cell lines [17, 18], including colon, ovarian and breast cancer. *In vivo*, it has been shown that ara-C and cisplatin have synergistic activity in murine ovarian cancer [19]. Furthermore, a synergistic effect between cisplatin and 5-aza-CdR has been observed for a panel of six human cell lines [20]. With respect to the mechanism of action underlying the combination of cisplatin and 5-aza-CdR, it has been postulated that the degree of DNA hypomethylation did not influence this synergistic effect [20]. At the clinical level, the addition of ara-C to cisplatin did not increase the therapeutic index when tested in a phase II HNSCC trial [21].

As listed above, cisplatin and gemcitabine display differences with respect to cellular metabolism and interaction with DNA. Moreover, both drugs cause different side-effects in patients. Cisplatin causes renal [22], gastrointestinal [23] and neurological toxicity [24], whereas for gemcitabine, myelosuppression is dose limiting [8].

HNSCC xenografts are tumours which seem to be the best reflection of human HNSCC, in terms of the sensitivity/activity profile for the drugs that are used to treat patients [25]. Therefore, for the present study, we chose two HNSCC xenograft lines with different sensitivities to both drugs [7] as being representative of the clinical situation. Since gemcitabine was the least toxic drug with considerable activity, we aimed to improve the activity of this drug by combining it with cisplatin at a non-toxic dose.

The aim of this study was to determine the efficacy of the combination of gemcitabine with cisplatin in HNSCC xenograft lines, and in addition, the effect of sequence and time interval in this combination. The results will be of relevance for the design of the optimal combination treatment schedule in future clinical trials.

MATERIALS AND METHODS

Animals and tumours

Female nude mice (Hsd: athymic nude-nu, 8–10 weeks old) were obtained from Harlan Olac, CPB, Zeist, The Netherlands. The conditions under which the mice were kept have been reported elsewhere [26]. HNX-14C was established from a poorly differentiated human squamous cell carcinoma of the oral cavity; the HNX-22B line originated from a well differentiated tumour of the hypopharynx. The cell lines (UM-SCC-14C and UM-SCC-22B) used to establish tumour lines were kindly provided by Dr T.E. Carey, Ann Arbor, U.S.A., and have been characterised previously [27]. Serial transplantation of tumour lines was performed by inserting slices measuring about $3\times3\times1$ mm subcutaneously in the lateral thoracic region on both sides of the animal. Tumour volume in mm^3 was measured biweekly using vernier callipers and calculated according to the formula: length × width × height × 0.5. This way of determining the volume has proven to be the most accurate [28].

The animal experiments were performed according to Dutch law. Approval was obtained from the University authorities.

Drugs, doses and schedules

Gemcitabine (LY 188011) was donated by Lilly Research, Windlesham, Surrey, U.K. Cisplatin was purchased from Bristol-Myers Squibb, Woerden, The Netherlands. The various schedules of the combination of gemcitabine and cisplatin are listed in Table 1. The study was designed so that the dose of gemcitabine was the maximum tolerated one, corresponding to a weight loss between 5 and 15% [29] and that adding cisplatin would not increase toxicity.

Multiple dose schedules. Four schedules (1–4, Table 1) were based on the optimal schedule of gemcitabine as found in previous studies [7]. Gemcitabine was given intraperitoneally (i.p) at a dose of 100 mg/kg on days 0, 3, 6 and 9. On days 0 and 6, cisplatin (4 mg/kg) was injected intravenously (i.v.), either simultaneously, 4 h before or 4 h after the injection with gemcitabine. An experimental group without cisplatin was included.

Single dose schedules. Single doses of both drugs were administered applying schedules 5–10 (Table 1). This decision was based on the concept that to see a "clean" effect multiple dosing of the same drug should be avoided. Gemcitabine (240 mg/kg) was given either simultaneously, 4 or 24 h before or following an i.v. injection with cisplatin (7 mg/kg). As a control, an experimental group was included in which cisplatin as a single agent was given, 7 mg/kg (i.v.) on days 0 and 7.

Evaluation of chemotherapy

Treatment was started when the tumours reached a volume between 50 and 250 mm^3, according to the guidelines described by Boven and associates [29]. The animals were randomly divided into treatment and control groups (5–10 tumours/group). An antitumour effect was expressed in terms of the growth delay factor, defined as the difference between the median values of the time required by tumours of treated and control animals to double their volume, divided by the median value of the time required by the tumours of control mice to double their volume [30]. For the second method, tumour volumes were calculated as values relative to the tumour volume at the time treatment was started. The mean of these values of the treatment group is expressed as a percentage of that obtained for the control group and is described as the treated/control value; the optimal treated/control value is considered to be the lowest value at a certain time point during the post-treatment observation period. Cures were scored when a tumour regressed and did not show regrowth for a 3 month period. A growth delay factor between 1 and 2 or a treated/control value between 25 and 50% reflects a moderate, and a growth delay factor > 2 or a treated/control value $< 25\%$ a real response [29].

RESULTS

Therapy with multiple dose schedules

The HNX-22B line was sensitive to single agent treatment with gemcitabine and moderately sensitive to cisplatin (see Table 1), confirming a previous report [7]. The antitumour effect of the combination was better than that seen with single agent therapy; the growth delay factor for the combination when administered simultaneously was 6.3, whereas this was 3.8 and 1.8 for gemcitabine and cisplatin single agent therapy, respectively. The tumour volume doubling times of the combination groups were significantly ($P < 0.05$, Mann–Whitney U-test) smaller than that of cisplatin but not of gemcitabine. Beyond 10 days after the start of treatment, the mean values of the tumour volumes were for the combination groups significantly ($P < 0.05$, Student's t-test) smaller than for both single agent groups. When both drugs were given, simultaneous administration had a better therapeutic index than gemcitabine given 4 h before or after the cisplatin injection (Table 1, Figure 1). Although the responses were more or less similar in all three combination schedules, the toxicity was significantly lower

Table 1. Cisplatin/gemcitabine combinations in the tumour line HNX-22B

Treatment schedule			Toxicity (% weight loss)*	Antitumour effect		
Number	Dose frequency	Sequence and interval		Growth delay factor	Optimal treated/control (%)	No. cures/total
1	Multiple	Cisplatin 0 h gemcitabine	9	6.3†	4	1/6
2	Multiple	Cisplatin 4 h gemcitabine	13	5.5†	4	0/5
3	Multiple	Gemcitabine 4 h cisplatin	16	6.3†	4	0/6
4	Multiple	Gemcitabine	8	3.8†	12	1/6
5	Single	Gemcitabine 0 h cisplatin	14	1.8†	27	0/6
6	Single	Gemcitabine 4 h cisplatin	17	2.7†	13	0/7
7	Single	Cisplatin 4 h gemcitabine	17	3.4†	25	1/6
8	Single	Cisplatin 24 h gemcitabine	8	0.3	57	0/5
9	Single	Gemcitabine 24 h cisplatin	7	0.4	48	0/8
10	Single	Gemcitabine	5	0.9†	46	0/8
11	Multiple	Cisplatin	11	1.8†	24	0/7

For an explanation of the calculation of the antitumour effects see Materials and Methods. With schedules 1–4, 100 mg/kg gemcitabine on days 0, 3, 6 and 9 and 4 mg/kg cisplatin on days 0 and 6 was given. With schedules 5–10, single injections of drugs were given, 240 mg/kg gemcitabine and 7 mg/kg cisplatin. With schedule 11, 7 mg/kg cisplatin was given on days 0 and 7.

*The highest percentage weight loss in the post-treatment period is expressed as the means of weights of the treated mice relative to that of the control mice; †$P < 0.05$, significant growth delay: a difference in growth rate between tumours from treated and control animals was analysed for a statistical difference with the two-tailed Mann–Whitney U-test.

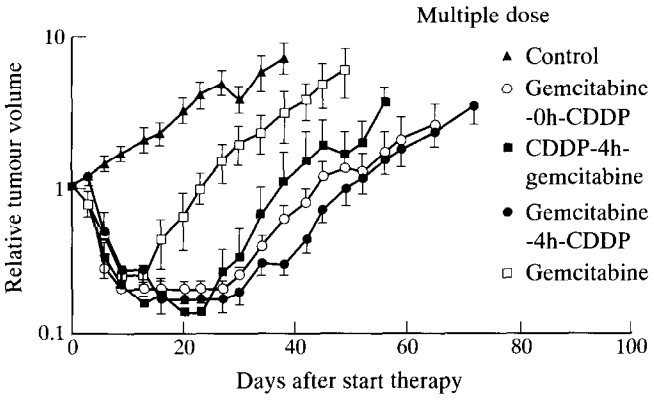

Figure 1. Antitumour effect of the combination of gemcitabine with cisplatin (CDDP) in the tumour line HNX-22B. The schedules with multiple dosing are shown. In addition to the growth curve of the control group, those of the treatment schedules 1–4 (Table 1) are shown. Volumes are expressed relative to that observed at the start of treatment and are presented as means ± standard error of the mean.

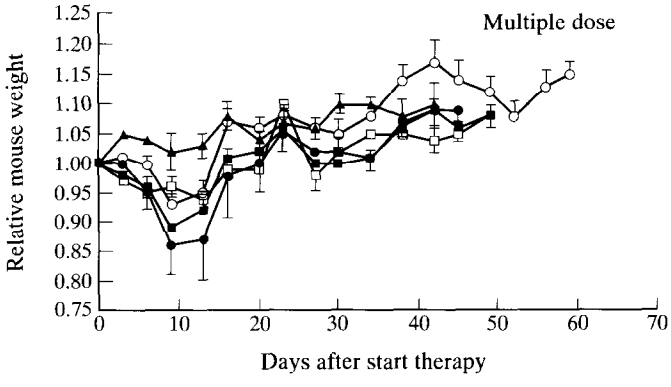

Figure 2. Weight curves of the animals bearing HNX-22B tumours. The corresponding tumour growth curves are shown in Figure 1. Results are expressed relative to those observed at the start of treatment and are presented as means ± standard error of the mean. For symbols see legend to Figure 1.

in the experimental group with simultaneous injections. The maximal weight loss differed (Table 1) and there was a significant difference ($P < 0.05$) in animal weights on days 10 and 14 post-treatment as determined with the Student's t-test (Figure 2).

The HNX-14C line is not sensitive to cisplatin (growth delay factor = 0.2) and only moderately sensitive to gemcitabine (growth delay factor = 1.1, see Table 2). Addition of cisplatin did not lead to an enhancement of the antitumour effect of gemcitabine. A significant finding was that, irrespective of the sequence, an interval of 4 h was much more toxic to the animals than when the drugs were given at the same time. In both experimental groups with the 4 h interval, the treatment frequency of gemcitabine had to be limited to three times instead of four times to prevent the animals from dying. The mice bearing the HNX-14C line were much more sensitive to the drug combination induced side-effects than the ones bearing HNX-22B tumours.

Therapy with single dose schedules

Generally, antitumour activity using single doses was less for both tumour cell lines, than when applying multiple doses. As for HNX-22B, the combination was significantly ($P < 0.05$, Mann–Whitney U-test) better than gemcitabine alone, but not better than cisplatin (Table 1). It should be realised that cisplatin was given twice for a therapeutic purpose. The single dose of cisplatin was expected to induce a lower effect. When gemcitabine was given 4 h before cisplatin, the results were worse than when either cisplatin was given first or simultaneously with gemcitabine ($P < 0.05$, Student's t-test, Figure 3). When cisplatin preceded gemcitabine administration, a cure was observed. An interval of 24 h between injections was minimally active, even antagonistic effects could be observed compared to single agent gemcitabine therapy (Figure 4).

Table 2. Cisplatin/gemcitabine combinations in HNX-14C

Treatment schedule			Toxicity (% weight loss)*	Antitumour effect		
Number	Dose frequency	Sequence and interval		Growth delay factor	Optimal treated/control	No. cures/total
1	Multiple	Cisplatin 0 h gemcitabine	11‡	1	52	0/7
2	Multiple	Cisplatin 4 h gemcitabine	25	0.9	42	0/7
3	Multiple	Gemcitabine 4 h cisplatin	24‡	0.9	36	0/5
4	Multiple	Gemcitabine	7	1.1	44	0/5
5	Single	Gemcitabine 0 h cisplatin	7	0.7†	66	0/6
6	Single	Gemcitabine 4 h cisplatin	12	0.7	65	0/7
7	Single	Cisplatin 4 h gemcitabine	11	1.2†	47	0/6
8	Single	Cisplatin 24 h gemcitabine	20	0.3	73	0/6
9	Single	Gemcitabine 24 h cisplatin	14§	0.3§	59§	0/2§
10	Single	Gemcitabine	9	0.2	81	0/6
11	Multiple	Cisplatin	11	0.2	69	0/7

*The highest percentage weight loss in the post-treatment period is expressed as the means of weights of the treated mice relative to that of the control mice; †$P < 0.05$, significant growth delay: a difference in growth rate beween tumour from treated and control animals was analysed for a statistical difference with the two-tailed Mann-Whitney U-test; ‡Gemcitabine was given three times instead of four; §Three out of five mice died of toxicity.

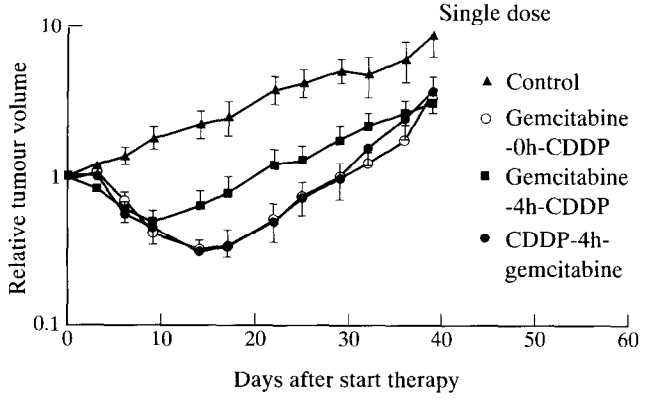

Figure 3. Antitumour effect of the combination of gemcitabine with cisplatin (CDDP) in the tumour line HNX-22B. The schedules with single dosing of both drugs are shown. In addition to the growth curve of the control group, those for treatment schedules 5–7 (Table 1) are shown. Volumes are expressed relative to that observed at the start of treatment and are presented as means ± standard error of the mean.

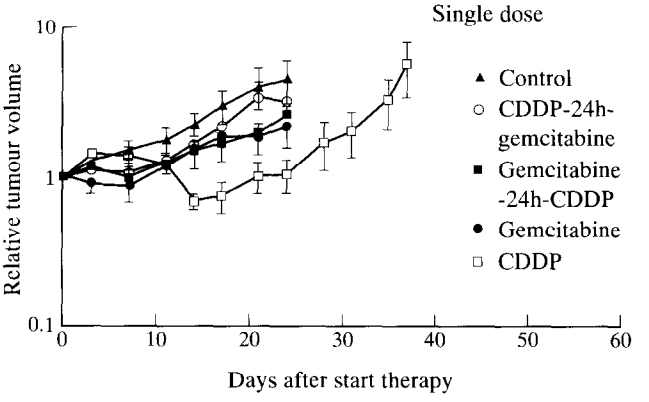

Figure 4. Antitumour effect of the combination of gemcitabine with cisplatin (CDDP) in the tumour line HNX-22B. The schedules with single dosing of both drugs are shown. In addition to the growth curve of the control group, those of the treatment schedules 8–11 (Table 1) are shown. Volumes are expressed relative to those observed at the start of treatment and are presented as means ± standard error of the mean.

The responses with HNX-14C were less than those observed for HNX-22B, but essentially the implication of the results was similar (Table 2). With the first tumour line, the results were best when cisplatin was injected 4 h before gemcitabine. Judged upon the volume doubling times, the differences were not statistically different. However, for mean values of tumour volumes, the group with cisplatin as first drug had a better response at day 10 post-treatment than all the other groups. The schedule with a 24 h interval between the drugs did result in a similar response as seen with the administration of each drug alone. However, the combination dramatically increased toxicity.

DISCUSSION

This study shows that the combination of the standard drug cisplatin with the relatively new antimetabolite gemcitabine can produce a better antitumour effect than when the drugs are given alone.

The sequence and time interval appear to be important factors in the antitumour activity of the combination of gemcitabine and cisplatin. When multiple injections of both drugs are given to mice bearing human HNSCC xenografts, the drugs can best be given at the same time. A 4 h interval leads to a similar, at least, additive antitumour effect, but also to an increased toxicity. Apparently at a 4 h time interval, the normal tissues are more seriously affected than the tumours. This effect was less pronounced when only a single injection of both drugs was given, indicating that accumulation of damage plays a role in this increased severity of the side-effects. With single injections, the best results were observed when cisplatin was given first, followed by gemcitabine after 4 h. This finding is also supported by *in vitro* experiments, showing that the best results are obtained when human ovarian cancer cells are first exposed to cisplatin [31]. Both the single and multiple dose schedules demonstrate that a 24 h interval between administration of gemcitabine and cisplatin is the least advantageous. Side-effects

became very severe and the antitumour effect was even less than when observed with single agent therapy.

The data of the present study do not permit definitive conclusions to be drawn on additivity and synergism, since no extensive study on the dose–response relationship of each drug (and the combination) was performed in this setting. However, such experiments would provide only limited new data on single drug treatment for each compound. Firstly, it is already known that gemcitabine activity is very dose-dependent in HNSCC xenografts, and secondly, cisplatin is only minimally active at the low dose studied here. The approach was chosen so that addition of cisplatin to gemcitabine would not increase the toxicity observed when gemcitabine was given alone. This better effect of the combination was observed in the tumour line HNX-22B, which is sensitive to gemcitabine treatment alone. The effect of the combination was only minimal in the HNX-14C line, which is less sensitive to gemcitabine, suggesting that sensitivity may be a precondition for an additive effect of the combination. The approach of using cisplatin as the initial drug and gemcitabine as the modulating drug was not studied here, but is still an interesting option.

The studies reported here indicate that the drugs may act additively or even synergistically. Mechanisms to explain synergism are theoretically at hand. The incorporation of gemcitabine into DNA might facilitate the formation of cisplatin–DNA adducts, as has been postulated for the combination of cisplatin and 5-aza-CdR [32, 33]. Another possibility is that DNA repair is inhibited by gemcitabine when cisplatin is given. This hypothesis is supported by the fact that gemcitabine is known to disturb the intracellular ribonucleotide and deoxyribonucleotide pools, an essential aspect to repair DNA damage [9, 10].

Some preclinical studies have been performed on the combination of cisplatin and two other pyrimidine analogues, ara-C and 5-aza-CdR. *In vitro* synergism is described for a large number of murine and human cancer cell lines [15–21]. These studies all concerned simultaneous exposure for 2 to 4 days. Sequence and short term exposures, however, have not been studied. Synergism was also observed in murine *in vivo* models [19]. Although the sequence of drug administration was not studied, the main conclusion in that study was that ara-C and cisplatin, when given simultaneously daily for 3 days, was much better than when a simultaneous once only injection was given.

The reason for the observed tumour-dependent severity of the toxic side-effects can only be speculated upon. It might be related to tumour-dependent metabolic effects exerted by the drug, related to signal transduction, nucleotide pools and energy status of the tumour. This effect has also been observed in euthymic mice (Peters GJ, unpublished observation).

At the clinical level, the combination of ara-C and cisplatin showed limited activity in a phase II HNSCC trial [21]. A continuous infusion of ara-C was given for 3 days and cisplatin was injected at 12, 36 and 60 h. This negative result might be attributed to the choice of the sequence of drugs, the antimetabolite being given first. In patients with ovarian cancer, treatment with cisplatin combined with ara-C proved to be effective [34]. Recently, good antitumour activity of the combination gemcitabine with cisplatin was observed in a phase II lung cancer trial [35]. These preliminary results indicate that the combination produces higher response rates than each drug alone. The results of the present study suggest that gemcitabine and cisplatin should be administered either simultaneously or with a short time interval in which cisplatin should precede gemcitabine. These findings are of potential interest for the clinical trials that are planned with this combination in the near future. Interpretation of the results presented here should be linked to the already known clinical experience, namely that the administration of gemcitabine with a weekly interval is currently most frequently used [8].

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