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SHORT COMMUNICATION

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Radioenhancement by cisplatin with accelerated fractionated radiotherapy in a human tumour xenograft

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Abstract The aim of the present study was to investigate whether cisplatin would enhance the radioresponse of a human tumour xenograft when given in different schedules combined with accelerated fractionated radiation therapy. A human squamous carcinoma of the hypopharynx, FaDu, was grown in the thigh of athymic nude mice. Tumours were exposed to twice-daily 2-Gy fractions, applied 6 h apart over 2 weeks, 5 days a week, alone or combined with cisplatin given at maximally tolerated doses in three different schedules: (1) i.p. as a single bolus (SB) or (2) i.p. as a daily bolus at 30 min before the first daily radiation fraction or (3) s.c. as a continuous infusion through a mini-osmotic pump over 13 days, commencing 24 h prior to the first daily radiation fraction. The end point for the study was tumour growth delay (TGD), calculated as the difference between the delay in regrowth to 200% of the initial tumour size in treated versus control mice. SB cisplatin plus radiation showed only an additive effect on TGD, whereas daily-bolus and continuous-infusion cisplatin demonstrated a greater than additive effect when combined with accelerated fractionated radiation in this human tumour model. Cisplatin appears to be especially beneficial as a radiation enhancer when given throughout the course of radiation.

Key words Cisplatin · Radiation enhancement · Tumour growth delay · Xenograft · Squamous carcinoma

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Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)] is a highly effective anti-cancer agent with activity against a number of human malignancies, including ovarian, testicular, head and neck, bladder and lung cancer. Cisplatin also potentiates radiation-induced cell killing in a number of experimental systems [1, 4, 8, 10, 16, 18, 19], and clinical trials of this combined-modality treatment are encouraging [5, 6, 12, 13, 17]. Carboplatin is also a potential enhancer of radiation treatment [3] but may be less active as an anti-cancer drug in head and neck cancer [2]. Large single doses of radiation applied in combination with chemotherapeutic agents, including cisplatin, have shown little evidence of a therapeutic advantage in most preclinical studies [18]. However, when clinically relevant fractionated radiation schedules are combined with cisplatin, radiation enhancement is both timing- and sequence-dependent [8, 16]. Few studies have used human tumours growing in mice to investigate cisplatin-induced radiosensitisation, although human tumour xenografts have advantages over murine tumours in that they closely reflect the chemosensitivity [15] and radiosensitivity [14] of the original patient tumours. In addition, the serum-tumour pharmacokinetics of many drugs, including cisplatin, are similar in nude mice and humans [9]. Thus, human tumour xenografts in athymic

nude mice represent a clinically relevant model for investigation of drug-induced radiation enhancement [7].

In the present study we used an accelerated (twice daily for 10 days) fractionated radiation therapy protocol with a clinically relevant dose (2 Gy/fraction). This was given concurrently with three schedules of cisplatin to nude mice bearing the human FaDu tumour, a squamous-cell carcinoma of the hypopharynx. The results of such studies may be useful in the design of further clinical studies to define the most effective scheduling of cisplatin, as well as other potential radiosensitisers, to achieve radioenhancement.

Materials and methods

Animals

Female BALB/c (nu/nu) athymic nude mice aged 5–6 weeks were housed in filter-covered cages under controlled atmospheric conditions and a 12-h light/dark cycle and were given free access to sterile water and mouse chow. All cages, covers, bedding, food and water were changed and sterilised weekly. All animal procedures were approved by the institutional Animal Ethics Committee.

Tumour source and inoculation

The FaDu cell line, derived from a squamous-cell carcinoma of the hypopharynx (American Type Culture Collection, Rockville, Md.), was used as the tumour source after thaw and proliferation in culture until sufficient cells were available for inoculation. Tumours removed from nude mice were confirmed by DNA extraction to be of human origin and were characterised histologically as a poor to moderately differentiated squamous carcinoma. Confluent FaDu cells were disaggregated with 0.025% pronase in phosphate-buffered saline/ethylenediaminetetraacetic acid (PBS-EDTA), and 4×10^6 cells in 50 µl PBS were injected s.c. into the right lateral thighs of the mice. At 24 h before inoculation, mice were further immunosuppressed with 5 Gy whole-body irradiation in a laboratory ^{137}Cs irradiator (1.19 Gy/min). At 10 days after inoculation, mice with tumours ranging in volume from 250 to 400 mm^3 were randomly allocated to treatment or vehicle-control groups of five to ten animals. Treatments consisted of cisplatin alone in the designated schedule and dose, and of radiation alone, cisplatin and radiation combined, and vehicle alone, in the schedules and volumes corresponding to the cisplatin-alone groups.

Irradiation

A 250-kV orthovoltage X-ray unit (HVT 0.5 mmCu, dose rate 1 Gy/min) was used to irradiate unanesthetised mice restrained in a specially designed apparatus that exposed only the tumour-bearing limb to the X-ray beam. A total dose of 40 Gy was given in 20 fractions of 2 Gy twice daily over 2 weeks, 5 days a week, as previous studies in our laboratory have shown this to be a subcurative dose for FaDu tumours [7]. No animal was cured in any of the radiation-alone experiments and no local toxicity resulted from this irradiation schedule.

Cisplatin doses and schedules

Pure cisplatin powder (Institute of Drug Technology, Melbourne, Australia) was dissolved in 0.9% saline, sterilised, and stored at 4 °C protected from light until use. Cisplatin was given in three different schedules at the maximum tolerated dose (MTD), either alone or combined with radiation. The MTD, defined as the maximal dose producing less than a 10% reduction in body weight within the 1st week of treatment, was determined in non-tumour-bearing nude mice for each drug schedule. In the single-bolus schedule a cisplatin dose of 8 mg/kg (0.8 mg/ml) was injected i.p. 45 min prior to the first irradiation on the 1st treatment day. In the daily-bolus schedule, cisplatin at 1 mg/kg per day (0.1 mg/ml) was given i.p. at the same time on each day of irradiation. For the continuous infusion, cisplatin was delivered by a mini-osmotic pump (Alzet model 2002, 0.5 µl/h; Alzet Co., Calif., USA) at 1 mg/kg per day (1.25 mg/ml) over 13 days, commencing 24 h prior to the first irradiation and ceasing 18 h after the final irradiation. The pump was implanted s.c. after mice had been anesthetised with 20% Hypnorm (Janssen Pharmaceuticals, Belgium) injected at 5 ml/kg close to the incision site high on the left side of the flank, and the wound was sealed with metal clips. At the end of treatment, mice were again anesthetised and the pump was removed through an incision at the lower end of the flank.

Tumour growth delay

Tumour size was measured twice weekly with digital calipers (Maxcal). Two perpendicular diameters were used to compute volumes (V) determined from the formula $V = 0.5\ (L \times W^2)$, where L and W represent the longest and shortest diameters, respectively. Calculated volumes were expressed as a percentage of the mean of the pretreatment volume measured for each group on day 1, which was designated as 100%. The number of days required for each tumour to reach 200% of its pretreatment volume was determined for each mouse. The observed tumour growth delay (TGD) was calculated from the difference between the median times required for tumours in the treated groups versus untreated control groups to reach twice their pretreatment volumes. The expected TGDs under an additive model [8] for the drug-plus-radiation schedules were determined by addition of the observed TGDs in the cisplatin-alone and radiation-alone groups for each drug schedule. The normalised TGD [8, 19] was calculated as the difference between the observed TGDs in the cisplatin-alone and the corresponding cisplatin-plus-radiation groups. The normalised TGD gives an indication of the efficacy of the radiation component in the combined drug-plus-radiation treatment groups. The ratio of this value to the observed TGD in the radiation-alone group provides the enhancement factor, which is a measure of the degree of sensitisation provided by cisplatin [19].

Toxicity

To monitor the whole-body toxicity of the treatments, body weight was measured twice weekly for the duration of the experiment and the haematological status was determined weekly for the first 5 weeks, excluding week 2, during which mice in the cisplatin-treated groups were sampled for blood platinum levels. Blood samples (20 µl) were collected from the retro-orbital sinus of each mouse and diluted 1:10 with PBS, and leucocytes, erythrocytes and platelets were counted with a Sysmex TMK-1000 Haematology Analyser.

Drug level evaluation

Blood samples for platinum determination were collected from each mouse at 60–70 min following cisplatin injection, after the first radiation exposure on day 1 for the single-bolus schedules, and at the same time for the daily-bolus and continuous-infusion schedules on the last day of treatment in week 2. In all, 20 µl of blood was sampled from the retro-orbital sinus and diluted with 200 µl heparinized water in 0.1% Triton for platinum determination by flameless atomic absorption spectrophotometry, performed routinely in our laboratory.

Statistical methods

The day on which the tumour volume reached 200% of its initial size was determined by interpolation on the growth curve for each mouse. The Kaplan-Meier product-limit method was used to estimate the median time required to reach 200% of the initial tumour size for each group. The PIL module of the BMDP statistical package was used for the analysis. Haematological data were analysed using one-way repeated-measures analysis of variance (ANOVA), except for the treatment groups that failed a normality test, where a Friedman repeated-measures ANOVA on ranks was performed. All pairwise multiple comparisons were made using the Student-Newman-Keuls method. Blood platinum levels were analysed using a Kruskal-Wallis one-way ANOVA on ranks and Dunn's method for pairwise multiple comparisons between groups. All statistical tests performed were two-sided, and results with $P < 0.05$ were considered significant.

Results

Tumour growth delay

The effects of accelerated fractionated radiation combined with the various treatment schedules for cisplatin on the tumour response are shown in Fig. 1. The tumour response to cisplatin given alone in all three schedules was not significantly different from that seen in controls. Tumours in the radiation-alone group began to regress during the 2nd week of treatment, reaching 90% of their initial size by day 19 and regrowing to 200% of their pretreatment size by day 47. In the single-bolus-plus-radiation group there was an almost immediate and rapid decline in tumour volume, with a nadir of approximately 30% of the initial volume being observed on days 22 to 25. Tumours in the daily-bolus-plus-radiation group increased in volume during the 1st week of treatment before decreasing to 20% of their initial size by day 29. The continuous-infusion-plus-radiation group responded in a manner similar to that shown by the daily-bolus-plus-radiation group, although the tumours regressed to only 50% of their initial volume.

The median times required to reach 200% of the initial tumour size and the observed and expected TGDs for each drug schedule are shown in Table 1. There was no significant difference between the observed and expected TGDs ($P = 0.14$) for the single-bolus-plus-radiation schedule, demonstrating that the effect of combining the two modalities was only additive. The enhancement factor was 1.2 ± 0.1 for this group. The observed TGDs in the daily-bolus- and continuous-infusion-plus-radiation schedules were significantly greater than the expected TGDs ($P = 0.002$ and $P < 0.001$, respectively), thus demonstrating a greater than additive effect of combining the two modalities. The enhancement factors were greatest for the daily-bolus (1.6 ± 0.2) and continuous-infusion schedules (1.5 ± 0.1). There was no difference in the degree of enhancement between these two drug-radiation schedules ($P = 0.65$). These observations suggest a schedule-dependent radiosensitising role for cisplatin.

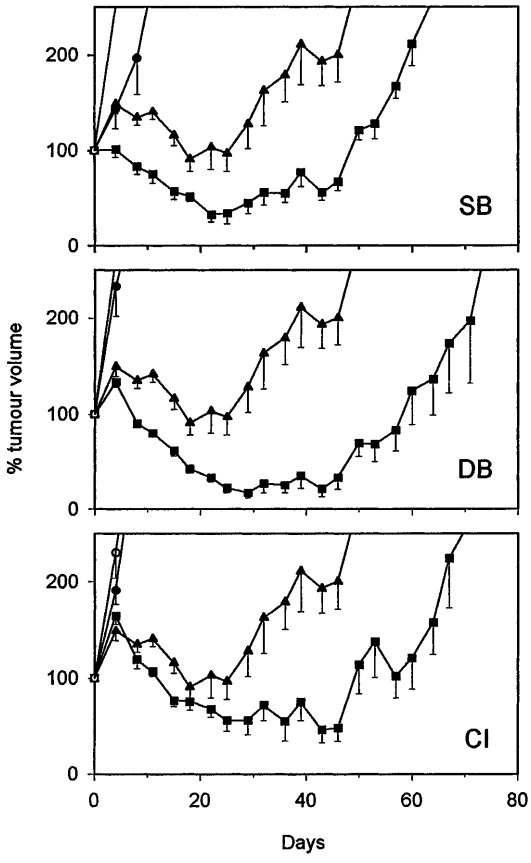

Fig. 1 Effect of cisplatin and/or irradiation on the growth of FaDu tumours. Mice with tumour volumes of 250–400 mm^3 (100% on day 1) were given vehicle alone (○) or cisplatin alone (●) i.p. as a single bolus of 8 mg/kg (*SB*) or a daily bolus of 1 mg/kg per day on each day of irradiation (*DB*), or s.c. as a continuous infusion of 1 mg/kg per day (*CI*), or local irradiation (▲) to the tumour-bearing thigh, or irradiation plus cisplatin (■). *Points* represent mean values ± SEM for 5–10 mice

Toxicity

No reduction in body weight was seen in any of the three vehicle-treated control groups or in the radiation-alone group. There was a mean (±SEM) weight loss of 1.3 ± 0.3 g (8%) in the single-bolus group on day 4, and the mice recovered by day 8. This was similar to the weight loss observed in the single-bolus-plus-radiation schedule (2.2 ± 0.6 g, 15%), although the mice did not recover until day 16. In the daily-bolus cisplatin-alone group the mean weight loss was 3.6 ± 0.3 g (23%) by day 11, with recovery occurring by day 21. The addition of radiation treatment to this schedule of cisplatin did not increase the weight loss, which was 2.6 ± 0.2 g (17%) by day 11, with recovery taking place by day 22. No weight change occurred in mice treated with continuous-infusion cisplatin alone, and there was only a transient weight loss of 4% on day 15 in mice treated with continuous-infusion cisplatin plus radiation. This suggests that the mice may have been capable of tolerating a higher dose in the continuous-infusion schedule,

Table 1 Effect of cisplatin with and without accelerated fractionated irradiation on the growth of FaDu tumours in mice

Schedule and Dose	Time to end point (days)[b]	TGD (days)[a]			Enhancement factor
		Observed	Expected	Normalized	
Single bolus (8 mg/kg):					
Control	4.2 ± 2.3	–	–	–	–
Drug alone	10.0 ± 5.0	5.9 ± 5.5	–	–	–
Radiation alone	46.7 ± 1.0	42.5 ± 2.5	–	–	–
Drug + radiation	61.1 ± 1.7	57.0 ± 2.9	48.4 ± 6.9	51.1 ± 5.3	1.2 ± 0.1
Daily bolus (1 mg/kg per day):					
Control	2.4 ± 0.6	–	–	–	–
Drug alone	2.9 ± 0.1	0.5 ± 0.6	–	–	–
Radiation alone	46.7 ± 1.0	44.3 ± 1.2	–	–	–
Drug + radiation	75.2 ± 9.2	72.8 ± 9.2	44.7 ± 3.3	72.3 ± 9.2	1.6 ± 0.2
Continuous infusion (1 mg/kg per day):					
Control	3.5 ± 0.9	–	–	–	–
Drug alone	4.9 ± 0.6	1.4 ± 1.1	–	–	–
Radiation alone	46.7 ± 1.0	43.2 ± 1.4	–	–	–
Drug + radiation	67.7 ± 2.8	64.3 ± 2.9	44.6 ± 2.2	62.9 ± 2.8	1.5 ± 0.1

[a]Observed TGD, difference between the absolute times required to reach 200% of the pretreatment volume in the treatment versus control groups; expected TGD, sum of the observed TGDs for the cisplatin-alone and radiation-alone groups; normalised TGD, difference between the observed TGDs in the cisplatin-alone versus cisplatin-plus-radiation-groups; enhancement factor, ratio of the normalised TGD to the observed TGD in the radiation-alone group
[b]Time required to reach 200% of the pretreatment tumour volume (median ± SE M)

despite preliminary studies having suggested that 1 mg/kg per day was the MTD.

Whole-body irradiation applied 10 days prior to treatment with cisplatin and radiation led to a 50% decrease in leucocyte counts at the start of treatment. Mice in the vehicle-control groups had elevated leucocyte counts by day 7, which increased 7-fold above normal values by day 21, after which mice were killed due to the presence of very large tumours. The radiation-alone group showed a similar 5.8-fold increase by day 35. In all three cisplatin-alone groups, leucocyte levels increased significantly by day 7, rising 6.2-fold for the single bolus and 4.7-fold for the daily bolus and the continuous infusion. The levels remained elevated until the mice were killed 1 week later. In the combined cisplatin-plus-radiation groups, leucocyte counts were also elevated by day 7, ranging from 2.9-fold for the single bolus to 2.3- and 1.5-fold in the daily-bolus and continuous-infusion schedules, respectively. The levels fell in all three groups by day 21 and then increased again by day 35, rising 5.5-fold in the single-bolus group and 3- and 3.5-fold in the daily-bolus and continuous-infusion groups, respectively.

There was no effect on erythrocytes in mice in the vehicle-control or the daily-bolus groups. Cell numbers increased significantly by 1.4-fold by day 28 in the radiation-alone and single-bolus groups and by 1.2-fold by day 7 for the continuous infusion. In the cisplatin-plus-radiation schedules, erythrocyte levels were significantly elevated from day 7 (1.2-fold) through day 35 (1.5-fold) after the single bolus, from day 28 (1.2-fold) to day 35 (1.4-fold) for the daily bolus, and from day 21 (1.1-fold) to day 35 (1.3-fold) in the continuous-infusion group. Due to considerable variability in platelet numbers the only significant change occurred in the daily-bolus group (3.4-fold increase by day 21).

Blood platinum concentrations

In a preliminary study, peak blood platinum levels of approximately 3.3 μg/ml occurred at 20–30 min following a single i.p. dose of 6 mg/kg cisplatin. In the present study, blood samples for platinum determination following single or daily doses of cisplatin were collected at approximately 60–70 min after injection, on the completion of irradiation. Blood platinum concentrations were pooled in the cisplatin-alone and cisplatin-plus-radiation groups as irradiation had no effect on these levels. The highest median blood levels of platinum occurred in the single-bolus (0.9 μg/ml, range 0.5–1.4 μg/ml) and daily-bolus schedules (0.7 μg/ml, range 0.3–1.0 μg/ml). The levels detected in the continuous-infusion schedule were significantly lower (0.4 μg/ml, range 0.3–0.6 μg/ml; $P < 0.05$).

Discussion

In the present study, cisplatin combined with accelerated fractionated radiation caused a greater than additive effect in delaying the growth of a human tumour xenograft as compared with the two treatment modalities alone. Although the FaDu tumour was unresponsive to cisplatin alone, when the drug was given either daily over two 5-day periods just prior to the first radiation

fraction each day or as a continuous infusion over the entire period of accelerated fractionated radiation, the effect on tumour regrowth for the two modalities combined was greater than that seen when the drug was given as a single bolus just prior to the first radiation fraction.

Previous studies using single-bolus cisplatin in murine tumours resulted in TGDs and therapeutic gain factors that demonstrated radioenhancement by cisplatin when given 24 h before the first of five daily 4-Gy radiation fractions [8]. A greater than additive effect on growth delay also occurred for a human transitional bladder carcinoma in nude mice when a single radiation dose preceded a single dose of cisplatin by 3 or 6 days [10], although, as in the present study, there was no response to cisplatin alone. In the present study a single dose of cisplatin followed by twice-daily irradiation caused an early anti-tumour effect, suggesting the involvement of mechanisms such as drug-induced apoptosis with subsequent tumour shrinkage, which could have led to reoxygenation of previously hypoxic or radioresistant tumour cells, making these cells more sensitive to radiation. This mechanism was proposed by Milas et al. [11] to explain similar observations in a murine tumour treated with single large doses of paclitaxel prior to radiation. More recent work by this group [19] suggests that cisplatin does not sensitise a murine tumour to radiation-induced apoptosis. Since the initial sensitisation in our experiments was short-lived in the single-bolus-plus-radiation schedule, repeated or continuous exposure of the tumour to cisplatin throughout the course of radiation treatment may be necessary for a greater than additive effect.

Radiosensitisation by cisplatin has previously been demonstrated in murine tumours exposed to a daily-bolus schedule of the drug, and supra-additivity was observed, regardless of the size of the radiation fractions or of whether they were delivered daily or twice daily [16]. Hence, further investigations would elucidate whether multiple daily fractions and fractions of less than 2 Gy might be of therapeutic advantage in combined-modality therapy. Clinical trials have demonstrated that fractions of greater than 2 Gy lead to undesirable late normal-tissue toxicity [20]. The delay in tumour response obtained with the daily-bolus-plus-radiation schedule suggests that cisplatin had little effect on the tumour during the initial stages of treatment. However, as the tumour was frequently exposed to cisplatin over the radiation period, an interaction between the two therapeutic modalities may have inhibited tumour regrowth.

Since radioenhancement occurs when cisplatin is given close to each radiation fraction, it could be expected that continuous release of the drug during the entire period of irradiation might be even more effective. Indeed, Fu and co-workers [4] showed that for the combination of cisplatin used with continuous low-dose-rate irradiation over 48 h in a murine squamous-cell carcinoma the anti-tumour effect was optimal when the drug was given as a continuous infusion during the irradiation. Begg et al. [1] found that continuous intratumoural release of cisplatin combined with a fractionated radiation protocol carried out over 5 days did lead to an improved therapeutic ratio. In the present study the level of radioenhancement achieved was similar for both the daily-bolus and the continuous-infusion schedules, although the dose delivered in the continuous-infusion schedule may have been less than that maximally tolerated as there was little loss in body weight. A recent clinical study has also shown good survival in patients with advanced carcinoma of the head and neck [5] after continuous infusions of cisplatin for two periods of 5 consecutive days during the course of daily radiation therapy. Additional studies using higher cisplatin doses are needed to confirm whether radiation enhancement can be improved with the continuous-infusion schedule.

The effect of cisplatin combined with radiotherapy has been studied clinically in a number of tumour types and the results of several randomised trials are now available. A meta-analysis of randomised trials in which radiotherapy alone was compared with the combination of chemotherapy and radiotherapy in non-small-cell lung cancer has shown a small survival benefit for patients treated with the combination when the chemotherapy regimen contained cisplatin [12]. The influence of drug dose, scheduling and mode of administration were not examined in the meta-analysis. In some studies in which concurrent cisplatin-containing chemotherapy given with irradiation has proved superior the schedule of drug administration appears to have been chosen empirically. Cisplatin was given as a single dose on day 1 of radiotherapy in patients with esophageal cancer [6] and head and neck cancer [17]. Further therapeutic gain may be achieved by longer-term exposure to the drug during a course of radiotherapy, as suggested by the results of an EORTC randomised trial in non-small-cell lung cancer in which daily administration of cisplatin during radiotherapy resulted in a survival advantage as compared with weekly administration [13].

Although the present study used both a clinically relevant radiation protocol and a human tumour induced in nude mice, the results cannot be directly extrapolated to clinical practice. However, the observation that more prolonged exposure to cisplatin resulted in greater radioenhancement is consistent with clinical results. Thus, it would appear that the human tumour xenograft/mouse system in combination with accelerated fractionated radiotherapy can be used to predict effectively the outcome of radiation- and drug-based therapies.

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