Time Dependence of the Selective Modulation of Cisplatin-induced Nephrotoxicity by WR2721 in the Mouse[1]

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ABSTRACT

2-(3-Aminopropylamino)ethylphosphorothioic acid (WR2721; ethiofos) was shown to selectively protect nontumor tissues from *cis*-diamminedichloroplatinum(II) (cisplatin)-induced toxicity, when administered 30 min prior to the platinum drug. Selectivity of protection by WR2721 is probably due to the preferential formation and uptake of the thiol metabolite 2-(3-aminopropylamino)ethanethiol (WR1065), which can inactivate toxic platinum-species inside the cell. We investigated the protective potential of WR2721, when administered at different time points relative to cisplatin. BALB/c mice treated with WR2721 (200 mg/kg i.p.) either 30 min or 5 min prior to cisplatin (i.p.) allowed a 2.2-fold increase in cisplatin dose to 19 mg/kg before the occurrence of nephrotoxicity as expressed by an increase in plasma urea. A small part of the protection could be ascribed to the mannitol (200 mg/kg), present in the formulated WR2721. WR2721 (200 mg/kg) 30 min after 14.5-16-mg/kg cisplatin did not offer any protection against the rise in plasma urea. WR2721 (200 mg/kg) 5 min before 19-mg/kg cisplatin did not cause liver toxicity (increase in serum glutamic pyruvic transaminase or serum glutamic oxaloacetic transaminase). Furthermore, WR2721 (200 mg/kg) 5 min prior to cisplatin did not reduce antitumor activity in nude mice bearing well-established human ovarian cancer xenografts. Under protection of WR2721, the dose of cisplatin could be increased by a factor of 1.6 to 8 mg/kg (administered twice weekly), resulting in an increased antitumor activity.

INTRODUCTION

WR2721[3] was originally developed as a radioprotective agent. It was shown to selectively protect nontumor tissues from radiation-induced damage due to radical scavaging, oxygen depletion, and hydrogen donation to damaged DNA by the thiol metabolite WR1065 (1). Selectivity of protection may be explained by a preferential formation and uptake of the thiolmetabolite WR1065 by nontumor tissues (2-4). WR2721 was also shown to protect against the dose-limiting nephrotoxicity of the cytostatic agent cisplatin without reduction of antitumor efficacy (5-8). In addition, myelosuppression and neurotoxicity, which become a problem at high-dose cisplatin (9), appear to be modulated selectively by WR2721 (8, 10). WR2721 is administered 30 min prior to cisplatin, and the timing of WR2721 administration relative to cisplatin has not yet been studied. Recent results from *in vitro* studies suggest that the mechanism of protection by WR2721 is most likely the prevention of cisplatin-induced cellular damage by WR1065 rather than the reversal of this damage by WR1065 (11, 12). Therefore, maximal protection by WR2721 is to be expected at the time

intracellular WR1065 has reached the highest concentration. Considering the rapid uptake and subsequent fast clearance of WR1065 in the kidney [maximum levels at 5-15 min (3, 4)], protection from cisplatin-induced nephrotoxicity might be improved when WR2721 is administered only 5 min before cisplatin.

WR1065 was shown to reverse part of the cisplatin-DNA adducts and platinum-protein interactions *in vitro* (11, 12). Furthermore, an increase in enzymatic repair of damage to the DNA was hypothesized as a result of WR1065-induced DNA unwinding (13). Thus, WR2721 may also offer protection when administered after cisplatin (rescue).

We have shown that cisplatin is hardly inactivated by WR2721 or its main metabolites (14). Therefore, a significant inactivation of cisplatin in the circulation, causing a decrease in the antitumor activity of cisplatin, is not expected when WR2721 is administered close to or together with cisplatin.

To verify the indications obtained from our *in vitro* studies, we investigated in mice the possibility of increasing the selective protection from cisplatin-induced nephrotoxicity by WR2721, by comparing the effects of WR2721 given 5 min prior to and 30 min after cisplatin with the drug given 30 min prior to cisplatin. Special attention was paid to the occurrence of liver toxicity in mice treated with the MTD of cisplatin and WR2721, because of potential hepatic damage of the combination as suggested in clinical studies (15). Finally, we investigated whether WR2721 5 min prior to cisplatin affected the antitumor activity in nude mice bearing an OVCAR-3 xenograft.

MATERIALS AND METHODS

Chemicals. Platinol (0.5 mg/ml cisplatin in 0.9% NaCl) was obtained from Bristol Myers-Squibb, Woerden, The Netherlands. Ethiofos (500 mg WR2721 and 500 mg mannitol) and analytically pure WR2721 (obtained from U.S. Bioscience, West Coshohocken, PA) were reconstituted immediately prior to injection with sterile water and phosphate buffered saline (pH 7.4), respectively.

Toxicity Studies. Seven- to 9-week-old male BALB/c mice, obtained from Harlan/Cpb (Zeist, The Netherlands), were housed and fed *ad libitum* according to standard conditions. Mice (8 mice/group) were given i.p. injections of cisplatin and/or WR2721 1-2 h after light onset. WR2721 was used at its MTD of 200 mg/kg (16). Nephrotoxicity was determined by measurement of urea and creatinine plasma levels at the expected zenith on day 4 (17), using a Technicon RA-1000 autoanalyzer (Technicon, Gorinchem, The Netherlands). Liver toxicity was assayed by plasma sGPT and sGOT activities 1 and 3 days after treatment, using a Hitachi 737 Automatic Analyzer (Boehringer Mannheim B.V., Almere, The Netherlands). Heparinized plasma was isolated from 200-μl blood samples obtained from the retro-orbital plexus under light ether anesthesia.

Antitumor Activity Studies. Female athymic nude mice (Harlan/Cpb, Zeist, Netherlands) were housed and fed as published previously (18). Eight-week-old animals were inoculated s.c. with fragments (2-3 mm in diameter) of OVCAR-3 xenografts. These xenografts were previously grown from the *in vitro* cell line NIH:OVCAR-3, kindly provided by Dr. T. C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA) and originally established from the malignant ascites of a patient with poorly differentiated papillary adenocarcinoma of the ovary. Treatment was

started at the time tumors had reached a size of approximately 150 mm³. Mice (6 mice/group, 2 tumors/mouse) were given injections at days 0 and 7 with cisplatin i.v. at a standard dose of 5 mg/kg (18) with and without 200 mg/kg WR2721 i.p. and with the higher MTD of cisplatin and WR2721 200 mg/kg i.p.. This MTD, allowing 10% weight loss, was determined in non-tumor-bearing nude mice (3 mice/group). Tumor volume (length × width × height × 0.5, expressed in mm³) was measured weekly with slide calipers by the same observer. For evaluation of treatment efficacy, tumor volumes were calculated relative to the volume at the start of treatment. The T/C% and the gain in tumor doubling time (t_D) of the treated group:

$$\text{Specific growth delay} = \frac{(t_{D,\text{ treated}} - t_{D,\text{ control}})}{(t_{D,\text{ control}})}$$

were used to express antitumor effects.

Statistics. Results were validated with both Student's t test ($P \leq 0.05$) and Wilcoxon's rank test (double sided, $2\alpha \leq 0.05$).

RESULTS

Toxicity Studies. The protective potential of 200-mg/kg WR2721 30 min prior to cisplatin was compared to that of a similar dose of WR2721 5 min prior to or 30 min after cisplatin. As depicted in Table 1, cisplatin-induced nephrotoxicity was greatly reduced by both WR2721 given 30 min prior to and 5 min prior to cisplatin. The dose of cisplatin could be increased from 8 mg/kg to 19–22 mg/kg before nephrotoxicity occurred; 200-mg/kg WR2721 administered 30 min after cisplatin did not show any protection at dose levels of 14.5–16 mg/kg. Cisplatin-induced nephrotoxicity became clear by an increase in mean plasma urea levels at day 4 (Table 1), while mean plasma creatinine levels were hardly changed at that time (results not shown). The number of mice with a greater than 2-fold increase in plasma urea level correlated well with lethality at day 7, while such a correlation was poor for plasma creatinine levels (Table 1). Two deaths occurred in both the WR2721 30 min prior to and the WR2721 5 min prior to 16-mg/kg cisplatin groups that were probably stress-related, inasmuch as these deaths occurred within 24 h after treatment and an increase in plasma urea levels was not observed in these groups. There were no deaths in the WR2721 30 min prior to the 19-mg/kg cisplatin-treated group and only one animal, with a >2-fold increase in plasma urea, died in the WR2721 5 min prior to the 19-mg/kg cisplatin-treated group.

Formulated ethiofos contains an equivalent amount of man-

Table 2 *sGPT and sGOT activities (IU/liter) in plasma of BALB/c mice (n = 8) treated with 200-mg/kg WR2721 5 min prior to 19-mg/kg cisplatin as compared to untreated mice*

	Day 1		Day 3	
	sGPT	sGOT	sGPT	sGOT
Control	39 ± 33	91 ± 63	44 ± 55	69 ± 35
WR2721/cisplatin	55 ± 17	105 ± 52	43 ± 14	70 ± 21

nitol which, together with the injected saline (0.9 ml at a high dose of cisplatin), may offer some protection from cisplatin-induced nephrotoxicity. Therefore, another group of mice was treated with 200 mg/kg of analytically pure WR2721 5 min prior to cisplatin. Although the mean plasma urea level of the WR2721 (analytically pure) prior to the 19-mg/kg cisplatin-treated group did not significantly differ from the WR2721 (ethiofos) prior to the 19-mg/kg cisplatin-treated group, 3 mice suffered from severe nephrotoxicity (of which 2 died) in the first compared to one (which died) in the latter. With WR2721 (analytically pure) 5 min prior to 16-mg/kg cisplatin, no nephrotoxicity was observed comparable to the mannitol-containing formulation. Thus, mannitol may have slightly added to the protective effect of WR2721 as formulated ethiofos.

In mice treated with 200-mg/kg WR2721 (ethiofos) 5 min prior to 19-mg/kg cisplatin, liver toxicity was analyzed. No significant increase of sGPT or sGOT activity in plasma was observed compared to untreated mice either 1 or 3 days after treatment (Table 2).

Antitumor Activity Studies. With 200-mg/kg WR2721 either 30 min or 5 min prior to cisplatin, the weekly (twice) MTD of cisplatin, causing a mean 10% decrease in body weight of non-tumor-bearing nude mice, increased from 5 mg/kg i.v. by a factor of 1.6 to 8 mg/kg i.v. The −5-min interval was as protective as the −30-min interval, which had already been demonstrated not to interfere with the antitumor activity of cisplatin (5–8). Therefore, we determined the antitumor activity for the −5-min interval. WR2721 by itself did not affect tumor growth (Fig. 1). At the standard 5-mg/kg dose of cisplatin, WR2721 5 min prior to cisplatin did not affect the antitumor activity. T/C% at the time of minimal tumor size did not differ significantly among the groups receiving cisplatin either with or without WR2721 (Table 3). Although the specific growth delay was slightly lower for the cisplatin 5 mg/kg + WR2721 group (8.0) compared to the cisplatin 5-mg/kg group (9.2), the difference in mean tumor volume between the groups at day 49

Table 1 *Modulation of cisplatin-induced nephrotoxicity in BALB/c mice (n = 8) by WR2721 (200 mg/kg i.p.) at 30 min before, 5 min before, or 30 min after cisplatin as measured by plasma urea levels (mM) at day 4*

	Cisplatin dose (mg/kg)								
	0	8.5	10	11.5	13	14.5	16	19	22
No WR2721	9.0 ± 1.8 0/0/0[d]	6.3 ± 0.6 0/0/0	20.4 ± 22.4 2/0/2	19.4 ± 9.0[a,b] 4/1/1	35.8 ± 26.6[a,b] 4/1/4	21.4 ± 14.0[a,b] 4/2/5	42.7 ± 27.8[a,b] 6/2/7	NM[c]	NM
WR2721 30 min before cisplatin + mannitol	9.0 ± 0.4 0/0/0	NM	NM	8.1 ± 1.5 0/0/0	7.4 ± 5.3 1/0/0	6.2 ± 1.0 0/0/0	6.0 ± 1.6 0/0/2	5.8 ± 1.6 0/0/0	14.7 ± 6.6[a] 4/0/5
WR2721 5 min before cisplatin + mannitol	9.0 ± 0.4 0/0/0	NM	NM	NM	NM	8.0 ± 1.9 0/0/0	7.5 ± 1.0 0/0/2	10.6 ± 7.6 1/0/1	13.9 ± 9.5 2/2/1
WR2721 5 min before cisplatin − mannitol	9.0 ± 2.0 0/0/0	NM	NM	NM	NM	NM	7.2 ± 1.2 0/0/0	17.5 ± 8.1[a,b] 3/0/2	NM
WR2721 30 min after cisplatin + mannitol	9.0 ± 0.4 0/0/0	NM	NM	NM	NM	45.7 ± 34.5[a,b] 5/3/3	55.9 ± 22.9[a,b] 7/2/5	NM	NM

[a] Significantly different from control (Student's t test, $p \leq 0.05$).
[b] Significantly different from control (Wilcoxon's rank test, double sided, $2\alpha \leq 0.05$).
[c] NM, not measured.
[d] Number of mice: with plasma urea > 2 × control/with plasma creatinine > 2 × control/dead at day 7.
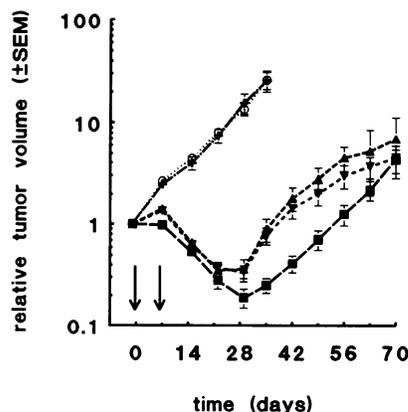
Fig. 1. Relative volumes of well established s.c. OVCAR-3 xenografts grown in female nude mice after 5-mg/kg (twice weekly) cisplatin alone (▼) and in combination with 200-mg/kg WR2721 5 min prior to the platinum drug (△) or 200-mg/kg WR2721 5 min prior to an equitoxic dose of 8-mg/kg cisplatin (twice weekly) (■) in comparison to untreated (○) and WR2721-treated (+) mice.

was not statistically significant. At the higher weekly (twice) dose of 8-mg/kg cisplatin with WR2721 protection (5 min prior to cisplatin), a significant decrease in T/C% (day 29) was achieved compared to the 5-mg/kg cisplatin treatment without protection (Table 3; Fig. 1). Specific growth delay also increased, but the difference in mean tumor size between these groups at day 49 was statistically not significant.

DISCUSSION

These studies present data on the protective potential of WR2721 when administered 5 min before or 30 min after cisplatin as compared to the usual administration of 30 min before cisplatin. The dependence of time for the protection of cisplatin-induced toxicities by WR2721 has not been questioned before. Recent *in vitro* results (11, 12, 14) from our group together with (tissue) kinetics of WR2721 and its metabolites (3, 4) prompted us to perform such a study.

Cisplatin caused nephrotoxicity in BALB/c mice at doses of 10 mg/kg or higher. WR2721 was equally active in protecting mice from cisplatin-induced nephrotoxicity, when administered either 30 min or 5 min before cisplatin. Due to the large interindividual variation in plasma urea levels, statistical differences are not readily achieved in these relatively small groups, when nephrotoxicity becomes evident. Therefore, precise DMF, especially with the rather large increases in dose, are hard to establish. On basis of a "no-toxicity criterium," cisplatin dose could be increased from 8.5 to 19 mg/kg (DMF = 2.2) with WR2721 administered 30 min prior to cisplatin and from 8.5 to 16 mg/kg (DMF = 1.9) with WR2721 administered 5 min prior to cisplatin. However, the conclusion that protection is higher for the −30-min interval may not be drawn from these DMFs, which is demonstrated by the lower toxicity at 22-mg/kg cisplatin for the −5-min interval compared to the −30-min interval. Our hypothesis, based on *in vitro* results (11, 12, 14) and tissue kinetics of WR1065 (3, 4), that WR2721 5 min prior to cisplatin might offer better protection as compared to a time interval of −30 min, was not confirmed. This shows that *in vitro* test systems can be of great help in understanding the molecular actions of drugs, but care should be taken when extrapolating to the complicated *in vivo* situation. In our mouse studies, cisplatin was administered as a bolus injection, causing excessive damage to the kidney over a short period of time as was shown by the lack of protection by WR2721 administered

30 min after the cisplatin injection. In a clinical setting, where cisplatin is usually administered as a continuous infusion over several h, damage will be inflicted over a longer period of time. Therefore, the administration of WR2721 just before a short-term cisplatin infusion (of a few h) may still improve its protective potential compared to a delay between the WR2721 and cisplatin infusion. At least, protection is not expected to decrease.

The DMF of 2.2 for the −30-min interval is slightly larger than found in previous studies in mice with WR2721 30 min prior to cisplatin (5–7). An explanation might be the differences in the strain of mice used. Also, the formulated WR2721 contains an equal amount of mannitol, which is known, at a high dose (3 g/kg), to offer some protection from cisplatin-induced nephrotoxicity in rats (19). In a phase I study, an enhanced protection from cisplatin-induced nephrotoxicity was observed with WR2721 + mannitol as compared to WR2721 alone (20). Indeed, mice appeared to be somewhat less protected from cisplatin-induced nephrotoxicity if WR2721 was administered without mannitol.

Protection from cisplatin-induced nephrotoxicity was totally absent, when WR2721 was administered 30 min after 14.5–16-mg/kg cisplatin. Although care must be taken when extrapolating *in vitro* results to the *in vivo* level, these results support our hypothesis (11, 12) that prevention of the platination of macromolecules by WR1065 (and mixed disulfides?) is the main mechanism of protection. Reversal of damage by WR1065 (11, 12) and increased enzymatic DNA repair (13) do not appear to be major mechanisms of protection from cisplatin-induced nephrotoxicity. The lack of protection by WR2721 30 min after 14.5–16-mg/kg cisplatin suggests that most of the damage to the kidney is exerted within 30 min after the administration of cisplatin.

Mice treated with 200-mg/kg WR2721 5 min prior to 19-mg/kg cisplatin did not show an increase in sGOT and sGPT levels at 1 or 3 days after treatment. Thus, toxicity to the liver of this combination, as suggested by clinical findings (15), was not demonstrated in this study.

WR2721 (200 mg/kg) 5 min prior to a standard weekly dose of 5 mg/kg did not compromise the reduction of OVCAR-3 tumor growth in nude mice. This is in excellent agreement with our expectations based on the relatively low reaction rates of cisplatin with WR2721 and its main metabolites (14), and we do not expect the administration of WR2721 immediately preceding the cisplatin infusion to reduce antitumor activity in the clinic. With WR2721 (either 30 min or 5 min before cisplatin), the MTD of cisplatin could be increased by a factor of 1.6 to 8 mg/kg. Again the −5-min interval was equal to the −30-min interval in its protective capacity. The DMF (1.6) is slightly smaller than those obtained in the nephrotoxicity studies (1.9–2.2). This again may be explained by differences between mouse strains, but also by the criteria defined for MTD, *i.e.*, increase in plasma urea *versus* weight loss. With the 1.6-

Table 3 *Antitumor activity of cisplatin with or without WR2721 (5 min prior to cisplatin)*

Cisplatin dose (mg/kg)	WR2721 dose (mg/kg)	T/C^a (%)	t_D (days)	SGD
0	0	100	4.8	0
5	0	2.5	49.0	9.2
5	200	2.7	43.4	8.0
8	200	1.4	62.6	12

^{a} Mean tumor volume of the treated group *versus* control at day 29; t_D, tumor doubling time; SGD, specific growth delay.

fold increased dose of cisplatin in combination with WR2721, a statistically significant increase in the extent of tumor regression was obtained, although tumors could not be cured.

In summary, mice were well protected from cisplatin-induced nephrotoxicity by WR2721 either 5 min or 30 min before the platinum drug, while WR2721 30 min after cisplatin offered no protection. WR2721 5 min prior to cisplatin did not interfere with antitumor activity in tumor-bearing nude mice. The appreciable increase in therapeutic efficacy obtained by a higher MTD of cisplatin preceded by WR2721 with an interval of 5 min should be explored in future trials in cancer patients.

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