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Effects of the Modulating Agent WR2721 on Myelotoxicity and Antitumour Activity in Carboplatin-treated Mice

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The selective modulation of carboplatin [diammine(1,1-cyclo-butanedicarboxylato)platinum(II)]-induced myelotoxicity was investigated in mice, using the protective agent WR2721 [*S*-2-(3-aminopropylamino)ethyl-phosphorothioic acid, ethiofos]. In female BALB/c mice, WR2721 (200 mg/kg intraperitoneally, i.p.) partly prevented the reduction of *in vitro* proliferation of whole bone marrow cells and non-adherent cells when administered at different time points relative to 90 mg/kg carboplatin (i.p.). Protection was highest when WR2721 was administered 5 min prior to carboplatin. *In vitro* proliferation of whole bone marrow cells and non-adherent cells in liquid culture increased from 15% of control for carboplatin alone to 45% when WR2721 was administered 5 min prior to carboplatin. However, WR2721 did not significantly prevent the loss in clonogenic capacity of early hematopoietic progenitors in the bone marrow, as determined by a bilayered soft agar colony forming units assay. In nude mice, bearing well-established subcutaneous human ovarian carcinoma xenografts OVCAR-3, WR2721 (200 mg/kg i.p.) 5 min prior to intravenous carboplatin allowed a 1.5-fold increase in the maximum tolerated dose of carboplatin as determined by overall weight loss. WR2721 alone did not affect tumour growth. However, WR2721 had a potentiating effect on the tumour growth inhibition of a standard dose of carboplatin in this model. Minimal tumour volume compared to control (T/C) decreased from 9.4% with carboplatin alone to 2.2% with WR2721 5 min prior to the same dose of carboplatin. Specific growth delay (SGD) increased from 7.4 to 10.3. With the 1.5-fold increased, equitoxic dose of carboplatin in combination with WR2721, the antitumour activity was only slightly further increased (T/C = 1.4%, SGD = 10.5).
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INTRODUCTION

FROM THE extensive search for cisplatin analogues with reduced nephrotoxicity but equal or higher antitumour activity, carboplatin emerged as one of the most promising second generation platinum compounds [1]. The clinical use of carboplatin is limited by bone marrow suppression [1]. A selective reduction of this side-effect, allowing an increase in the administered dose, could improve the efficacy of the treatment [2]. WR2721, initially developed as a radioprotector [3], was shown to protect mice from cisplatin-induced nephrotoxicity and myelosuppression, without a reduction in antitumour activity [4–6]. Early clinical trials suggested a similar selective protection [7]. The selectivity in protection of non-tumour tissues by WR2721 may be explained by a preferential formation and uptake of the thiol metabolite WR1065 in non-tumour tissues [8, 9]. Preliminary results indicate that WR2721 may also protect mice against carboplatin-induced myelotoxicity without an apparent loss in antitumour activity [10]. However, the nadir of white blood cell counts at day 4, which was used in that study as an indicator of myelotoxicity, may not properly reflect the extent of the bone marrow toxicity [11–13].

In vitro studies from our laboratory suggested prevention of intracellular damage by the thiol metabolite WR1065 as the main mechanism of protection against platinum-induced cytotoxicity [14, 15]. Considering the rapid tissue kinetics of WR1065 [9], optimum protection against platinum-induced toxicity was postulated when WR2721 is administered shortly before the platinum drug [16]. This was confirmed by the efficient protection from cisplatin-induced nephrotoxicity when WR2721 was administered 30 and 5 min prior to cisplatin, while administration 30 min after cisplatin did not offer any protection [17]. As carboplatin is less rapidly cleared from the plasma, and its action is exerted over a longer period of time as compared to cisplatin [18], the time dependence of the selective modulation of carboplatin-induced myelotoxicity may differ from that observed for cisplatin-induced nephrotoxicity. The low reac-

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tivity of cisplatin and carboplatin with WR2721 and its main metabolites is expected to result in a low level of inactivation in the circulation, even when WR2721 is given concomitantly with the platinum drug [16]. Therefore, WR2721 is not expected to interfere with antitumour activity. This was already confirmed in the case of cisplatin [17].

To investigate the protection against carboplatin-induced myelotoxicity by WR2721, mice were treated with carboplatin with or without WR2721 at different time points relative to carboplatin. Bone marrow function was determined after 24 h using three different assays: *in vitro* proliferation of whole bone marrow cells, *in vitro* proliferation of 3-day non-adherent cells from the bone marrow, and clonogenic capacity of the bone marrow. Furthermore, we investigated the effect of WR2721 5 min prior to carboplatin on the antitumour activity obtained with carboplatin in a human ovarian cancer (OVCAR-3) xenograft nude mouse model.

MATERIAL AND METHODS

Chemicals

Paraplatin^R (150 mg lyophilised carboplatin) was obtained from Bristol Myers-Squibb (Woerden, The Netherlands) and reconstituted with 15 ml of sterile water prior to use. Ethiofos (500 mg WR2721 and 500 mg mannitol) was obtained from US Bioscience (West Conshohocken, Pennsylvania, U.S.A.) and reconstituted with 9.3 ml of sterile water prior to use. Interleukin-3 (IL-3) was obtained from the supernatant of 10^6 cells/ml of the myelomonocytic cell line WEHI-3B (Dr G. Wagemaker, Erasmus University, Rotterdam, The Netherlands) after 3 days of culture in Dulbecco's minimal essential medium (Flow Laboratories, Irvine, U.K.) supplemented with 1 mmol/l L-glutamine (Merck, Darmstadt, Germany) and 10% heat-inactivated fetal calf serum (Gibco BRL, Breda, The Netherlands) [19]. Purified pregnant mouse uterus extract (Dr G. Wagemaker, Erasmus University) was used as a source of macrophage colony-stimulating factor (M-CSF) [20].

Myelotoxicity studies

Seven to nine-week-old female BALB/c mice (Harlan/Cpb, Zeist, The Netherlands) were housed and fed according to standard conditions. Mice were injected with carboplatin without or with WR2721, 1–2 h after light onset. Carboplatin was injected intraperitoneally (i.p.) at a non-lethal dose of 90 mg/kg, which was shown to cause severe bone marrow suppression in pilot experiments. WR2721 was used at its maximum tolerated dose (MTD) of 200 mg/kg i.p. [21].

Mice were killed 24 h after treatment by cervical dislocation, and whole bone marrow (WBM) was isolated by flushing both femurs with medium. WBM cells were seeded (1.5×10^6 cells/well) in 6-well plates (Costar, Cambridge, Massachusetts, U.S.A.). Non-adherent cells (NAC), enriched for clonogenic myeloid progenitors [22], were collected by aspirating the medium after 3 days of culture. For the determination of *in vitro* proliferation of WBM cells and NACs, these were cultured in 8-fold, using 96-well flat-bottomed plates (Greiner, Alphen a/d Rijn, The Netherlands). For the determination of the clonogenic capacity of the bone marrow, a bilayered soft agar culture was performed [23]. Briefly, NACs were cultured, in 12-fold, in a 0.3% agar layer (0.5 ml) on top of a 0.5% agar layer (0.3 ml) in 24-well flat-bottomed plates (Costar).

All cultures were performed in α-modified Eagle's medium (MEM) (Gibco BRL) supplemented with 5% heat-inactivated horse serum (Gibco BRL), 10% heat-inactivated fetal calf serum (Gibco BRL), 100 U/ml penicillin, 100 μg/ml streptomycin and IL-3 (10% WEHI-3B supernatant), and M-CSF (5 μl pregnant mouse uterus extract/ml) as growth factors. Cells were cultured at 37°C under humidified air with 5% CO_2.

In vitro proliferation of WBM cells and NACs was determined by quantification of the mitochondrial respiration (cellular activity), using a formazan (MTT) dye [24] and by quantification of protein (biomass), using a sulphorhodamine B (SRB) dye [25]. A small modification was made to the SRB assay: before fixing the cells, cells were pelleted (5 min, $275 \times g$) and the protein-rich medium was replaced by phosphate-buffered saline to reduce the background staining. Clonogenic capacity of the bone marrow was determined by counting the colonies (defined as ≥ 50 cells) using a converting microscope (Zeiss, Weesp, The Netherlands).

Antitumour activity studies

Female athymic nude mice (Harlan/Cpb) were housed and fed as described previously [26]. Eight-week-old animals were inoculated subcutaneously (s.c.) with fragments (2–3 mm in diameter) of human ovarian cancer (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 Centre, Philadelphia, Pennsylvania, U.S.A.), and originally established from the malignant ascites of a patient with a poorly differentiated papillary adenocarcinoma of the ovary. Treatment was started at the time tumours had reached a size of 50–150 mm^3 (day 0). Mice (six per group, two tumours per mouse) were injected twice weekly (at days 0 and 7) with a standard dose of 60 mg/kg carboplatin intravenously (i.v.), inducing 10% weight loss [26] with and without 200 mg/kg WR2721 (i.p.). Another group received the higher MTD of carboplatin obtained by the co-administration of WR2721 (200 mg/kg i.p.). This MTD, allowing 10% weight loss within 2 weeks after the first injection, was first determined in non-tumour-bearing nude mice (three per group). Tumours were measured weekly with slide calipers by the same observer, and the volume calculated as length × width × height × 0.5, expressed in mm^3. For evaluation of treatment efficacy, tumour volumes were calculated relative to the volume (V_0) at the start of treatment. Tumour doubling time (t_D) was the number of days for a tumour V_0 to double its volume. The mean relative tumour volume of the treated group divided by the mean relative tumour volume of the control group × 100% (T/C%) and the gain in mean tumour doubling time (t_D) for the xenografts [specific growth delay, SGD = $(t_{D,treated} - t_{D,control})/t_{D,control}$] were used to express antitumour effects.

Statistics

Results were validated with Wilcoxon's rank test (double sided, $2\alpha \leq 0.05$).

RESULTS

Myelotoxicity studies

Isolated bone marrow from untreated mice did not display any proliferation *in vitro* without the addition of IL-3 and M-CSF as growth factors. IL-3+M-CSF-dependent proliferation of WBM cells, as measured by SRB and MTT staining, reached a plateau after 6 days. Optimal *in vitro* proliferation over 6 days was observed at 2.5×10^4 WBM cells/well, while for NACs optimal proliferation over 6 days occurred at 0.5×10^4 NACs/well. Optimal cloning efficiency (approximately 100 colonies/3×10^3 NACs) was obtained after 14 days at 250 NACs/well.

The number of WBM cells obtained from two femurs (approximately 1.1–1.5 × 10^7) did not vary between groups. NACs (0.35–0.66 × 10^6) were obtained 3 days after seeding 4.5 × 10^6 WBM cells. These numbers also did not vary between groups. Four per cent of the NAC fraction of bone marow from untreated mice formed colonies.

With a dose of 90 mg/kg carboplatin, bone marrow function decreased to approximately 15% of the control as indicated by all three assays (Fig. 1). At this non-lethal dose which caused severe bone marrow suppression, the protective potential of 200 mg/kg WR2721 at several time points relative to carboplatin was tested. WR2721 (200 mg/kg) alone did not affect bone marrow function (data not shown). WR2721 30 min prior to carboplatin appeared to offer some protection against loss in bone marrow function as determined by the *in vitro* proliferation of WBM cells and NACs, using both MTT and SRB staining. However, differences were hardly or non-significant. The clonogenic capacity of the bone marrow was only slightly increased. The difference was not statistically significant. WR2721 5 min prior to carboplatin appeared to offer good protection as determined by the *in vitro* proliferation of WBM cells and NACs, using both MTT and SRB staining. The decrease in bone marrow function was limited to about 45% of control. In contrast, only a slight increase in clonogenic capacity could be observed which did not reach the level of statistical significance. WR2721 30 min after carboplatin appeared to protect bone marrow when assayed by the *in vitro* proliferation of WBM cells, which was comparable to the −5 min interval. However, this protective effect was not observed for the *in vitro* proliferation of the NACs or the clonogenic capacity of the bone marrow.

Antitumour activity studies

Both schedules of 200 mg/kg WR2721 30 min before or 5 min before the injections of carboplatin (at days 0 and 7) allowed a 1.5-fold increase in the MTD (weight loss ≤ 10%) to 90 mg/kg carboplatin. The −30 min interval was already shown not to interfere with the antitumour efficacy of carboplatin [10].

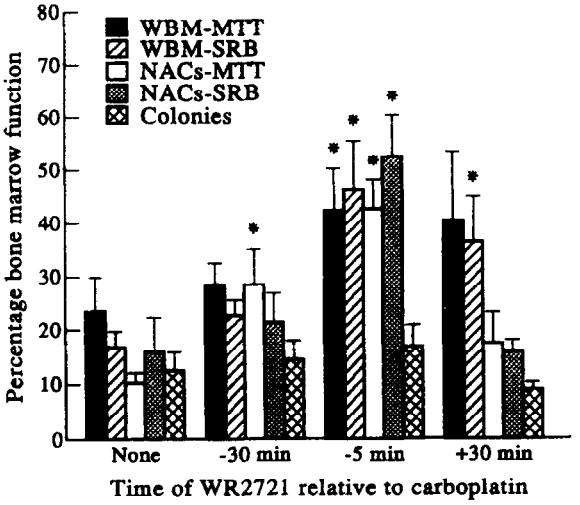

Fig. 1. Bone marrow function (% of control ± S.E.M.) of female BALB/c mice treated with 90 mg/kg of carboplatin i.p. with or without 200 mg/kg WR2721 i.p. at various administration times relative to carboplatin. *In vitro* proliferation of whole bone marrow (WBM) and non-adherent cells (NACs) was determined both by MTT and SRB assay. Clonogenic capacity of the bone marrow was determined by a bilayered soft agar assay. *Significantly different from the group treated with carboplatin alone (double sided, $2\alpha \leq 0.05$).

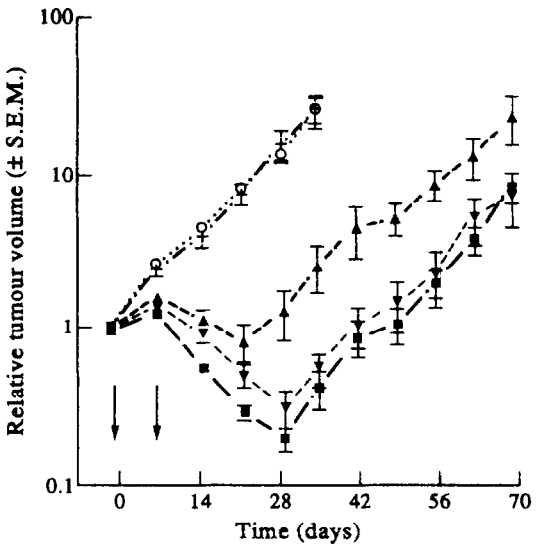

Fig. 2. Relative volumes of s.c. OVCAR-3 xenografts grown in female nude mice after two injections (days 0 and 7) of 60 mg/kg carboplatin (i.v.) alone (▲) and in combination with 200 mg/kg WR2721 (i.p.) 5 min prior to the platinum drug (▼) or 200 mg/kg WR2721 (i.p.) 5 min prior to the two i.v. injections (days 0 and 7) of 90 mg/kg carboplatin (■) in comparison to untreated (○) and WR2721-treated (+) mice.

Because the −5 min interval offered at least similar protection from weight loss but better protection in our bone marrow studies, we chose the −5 min interval for the antitumour activity studies. WR2721 200 mg/kg 5 min prior to 60 mg/kg carboplatin did not reduce antitumour efficacy. On the contrary, WR2721 caused a significant increase in the antitumour activity of carboplatin (Fig. 2,Table 1). Tumour volumes measured at day 29 were significantly smaller and tumour doubling times were significantly longer in the group treated with WR2721 + 60 mg/kg carboplatin as compared to the group treated with 60 mg/kg carboplatin alone. At the escalated weekly dose of 90 mg/kg carboplatin with WR2721 protection, inhibition of

Table 1. Growth of subcutaneous OVCAR-3 xenografts in female nude mice (n = 6) *treated with carboplatin (i.v.) with or without WR2721 (i.p., 5 min prior to carboplatin) at days 0 and 7*

Carboplatin dose (mg/kg)	WR2721 dose (mg/kg)	T/C* %	t_D† (days)	SGD‡
Experiment I				
0	0		5.0 (± 1.8)	
0	200	116	6.8 (± 3.8)	0.4
60	0	9.4§	41.8 (± 11.6)§	7.4
60	200	2.2§‖	56.6 (± 13.3)§‖	10.3
90	200	1.4§‖	57.7 (± 9.1)§‖	10.5
Experiment II				
60	0	7.0§	38.0 (± 6.5)§	6.6
60	200	5.7§	49.3 (± 11.1)§‖	8.8

*Mean volume of the treated tumours vs. control tumours at day 29. †Mean tumour volume doubling time (± S.D.). ‡Specific growth delay ($t_{D,treated} - t_{D,control}/t_{D,control}$). §Growth of the treated tumours was significantly different ($2\alpha \leq 0.05$) from the control tumours. ‖Growth of the tumours treated with 60 or 90 mg/kg carboplatin and 200 mg/kg WR2721 was significantly different ($2\alpha \leq 0.05$) from the control tumours and tumours treated with 60 mg/kg carboplatin alone.

tumour growth was slightly, but non-significantly, improved as compared to the WR2721 + 60 mg/kg carboplatin treatment group. Because of the unexpected potentiation of the antitumour activity from 60 mg/kg carboplatin by WR2721, the experiment was repeated in two groups of mice receiving 60 mg/kg carboplatin i.v. without and with 200 mg/kg WR2721 i.p.. Again, pretreatment of mice with WR2721 appeared to potentiate tumour growth inhibition by the standard dose of carboplatin (Table 1). In this experiment, differences were smaller but significant ($2\alpha \leq 0.05$) for the SGO between the groups.

DISCUSSION

WR2721 has been demonstrated to protect multiple non-tumour tissues from cisplatin-induced toxicity, including the bone marrow [4–7]. Preliminary results in mice, using the day 4 nadir of white blood cell counts, also suggest a protection of bone marrow from carboplatin-induced myelotoxicity [10], which is dose limiting in the clinic [1]. In our study, we investigated the efficacy of WR2721 as a selective protector of myelotoxicity, and its optimal administration time relative to carboplatin, using direct assays for bone marrow function. Colony forming units (CFU) in bone marrow are presently considered the best estimate of bone marrow toxicity [11–13]. However, these techniques are laborious and time consuming. The use of a microtitre tetrazolium dye assay has proven useful in this study of bone marrow response to growth factors, enabling automisation and a large sample throughput [24]. Because the tetrazolium dye assay quantitates mitochondrial respiration (cellular activity) [24], bone marrow proliferation may be overestimated due to activation phenomena [28]. Therefore, protein staining with SRB was also performed [25].

Both the clonogenic assay and the 6-day *in vitro* proliferation of WBM cells and NACs indicated a decrease in bone marrow function to approximately 15% of the control by 90 mg/kg carboplatin (Fig. 1). This is in accordance with previous results, using a similar clonogenic (granulocyte–macrophage colony forming capacity) assay for carboplatin-treated mice [29]. WR2721 administered 30 or 5 min prior to 90 mg/kg carboplation partly protected mice against carboplatin-induced myelotoxicity when determined by the *in vitro* proliferation of WBM cells or NACs. This protection was best for the −5 min interval. The MTT staining did not present a consistently higher intensity after treatment with carboplatin and/or WR2721 when compared to the SRB staining. Therefore, metabolic activation of WBM cells or NACs from mice treated with carboplatin and/or WR2721 could be excluded. Although the clonogenic capacity of bone marrow from carboplatin-treated mice appeared to be slightly increased when WR2721 was administered 30 or 5 min before carboplatin, protection was small as compared to the *in vitro* proliferation of WBM cells and NACs in liquid culture. Differences did not reach the level of statistical significance. These results indicate that WR2721 is able to reduce carboplatin-induced damage to a rapidly proliferating but non-clonogenic fraction of the bone marrow when administered prior to carboplatin. This fraction may be late haematopoietic progenitors, since the clonogenic capacity of the bone marrow is considered to be due to early haematopoietic progenitors [13]. In our experiments, only 4% of the NACs was able to form colonies, and for WBM cells, this fraction was below 1%. Therefore, NAC and WBM cell fractions consist mainly of non-clonogenic cells. The WBM fraction contains stromal cells (e.g. fibroblasts and macrophages) and early and late progenitor cells. The NACs are enriched for early and late progenitor cells [22]. The protection of later stages of myeloid progenitors, as observed in our study, probably explains why in the study of Green and colleagues [10], the (early) drop in white blood cell counts of carboplatin-treated mice at day 4 could be reduced by the administration of WR2721 30 min prior to carboplatin. The higher dose of WR2721 used in that study (300 mg/kg) may have added to the observed protection. In mice treated with carboplatin and 5-fluorouracil, WR2721 reduced the drop in platelet counts after two (weekly) doses of 60 mg/kg carboplatin [30].

A protection of the clonogenic capacity of mouse bone marrow (CFU) by WR2721 was observed in a previous study [6], with 600 mg/kg WR2721 administered 30 min prior to cisplatin. It is likely that we would also have observed a better protection of the clonogenic capacity with a 3-fold higher dose of WR2721 prior to carboplatin. However, such a high, almost lethal dose of WR2721 causes severe hypothermia [3, 21]. In fact, this hypothermia by itself may protect mice from cisplatin or carboplatin-induced myelotoxicity, since platinum compounds in combination with hyperthermia were shown to be extremely toxic to the bone marrow [31].

When WR2721 was administered 30 min after carboplatin, protection was only observed for *in vitro* proliferation of WBM cells. This may indicate that protection occurs at the level of adherent cells, probably stromal cells which, as a haematopoietic microenvironment, are able to support the *in vitro* proliferation of haematopoietic progenitors by the production of haematopoietic growth factors [32]. An indirect protective effect through stromal cells has been observed for the rescue agent diethyldithiocarbamate administered after carboplatin [32].

Several important conclusions can be drawn from this bone marrow toxicity study. Firstly, WR2721 protected mice from carboplatin-induced myelotoxicity, protection was optimal when WR2721 was administered 5 min prior to carboplatin, and protection appeared to be mainly at the level of later haematopoietic progenitors. Secondly, assaying bone marrow function at different stages of maturation may provide important (additional) information on bone marrow toxicity of treatment regimens.

WR2721 5 min prior to a standard, twice weekly dose of 60 mg/kg carboplatin did not compromise the reduction of OVCAR-3 tumour growth in nude mice, which is in accordance with our expectations, based on the relatively low reaction rates of carboplatin with WR2721 and its main metabolites [16], and the inability of solid tumours to accumulate WR1065 [9]. On the contrary, WR2721 potentiated the tumour growth reduction by carboplatin in this model, while WR2721 itself did not demonstrate tumour growth reduction. This very interesting finding was reproduced in a second experiment in the same tumour model. A similar potentiation of antitumour activity by WR2721 in the case of cisplatin has been suggested earlier [7, 33]. A rationale for this is not easily found. Platinum kinetics in the tumour tissue may have changed due to the administration of WR2721. This question will be the topic of future studies in this and other tumour models, in which the formation of Pt-DNA adducts will be determined. With WR2721 (5 min before carboplatin), the MTD of carboplatin could be increased by a factor of 1.5 to 90 mg/kg. An additional decrease in tumour growth was observed with this increased carboplatin dose, although the difference did not reach the level of statistical significance as compared to the standard (60 mg/kg) dose of carboplatin in combination with WR2721.

It can be concluded that WR2721 partly protected mice from carboplatin-induced myelotoxicity. Protection was best when

WR2721 was administered 5 min prior to carboplatin. Surprisingly, the antitumour activity of carboplatin was potentiated by WR2721 in an OVCAR-3 xenograft nude mouse model, while overall toxicity (weight loss) decreased. Therefore, the platinum dose could be increased and a substantial increase in therapeutic efficacy was achieved.

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