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Biochemical Modification of the Toxicity and the Anti-tumour Effect of 5-Fluorouracil and cis-Platinum by WR-2721 in mice

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WR-2721 (ethiofos) was tested on Balb/c mice for its chemoprotective capacity against 5-fluorouracil (5FU) monotherapy. In this combination WR-2721 was not active, but WR-2721 pretreatment allowed an elevation of the cisplatin (CDDP) dose in 5FU/CDDP combination therapy in these mice. Thrombocytopenia caused by the 5FU/CDDP (100 and 7 mg/kg, respectively) therapy was prevented by WR-2721 (200 mg/kg) and a partial protection against leukopenia was observed in C57Bl/6 mice. Various WR-2721/CDDP/5FU combinations were tested on two murine colon tumour models. The best antiproliferative effect against Colon 26 (in Balb/c mice) and the lowest toxicity were found with 5FU (100 mg/kg) and CDDP (5.5 mg/kg) delivered together 30 min after WR-2721 (200 mg/kg). The increased efficacy of WR-2721/CDDP/5FU both in Colon 26 and Colon 38 (in C57Bl/6 mice) compared to single 5FU or 5FU/CDDP treatment at the same dose could not be explained by enhanced inhibition of thymidylate synthase (TS), the 5FU target enzyme. The protection by WR-2721 against toxicity of CDDP/5FU might enable the use of high doses of CDDP in this combination.
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INTRODUCTION

s-2(3-AMINOPROPYLAMINO)ETHYL PHOSPHOROTHIOTIC ACID (WR-2721, ethiofos), has initially been developed and used as a radioprotector [1, 2]. Lately it has been investigated for its chemoprotectivity against toxic side-effects of cis-platinum (CDDP) and other agents [3, 4]. WR-2721 has to be activated to its metabolite WR-1065 by dephosphorylation catalysed by alkaline phosphatase (AP), a plasma membrane enzyme and as such responsible for membrane passage and hydrolysis of WR-2721 [5, 6]. WR-1065 is the active derivative, which protects DNA against several chemotherapeutic agents [6, 7]. A high uptake of WR-1065, after administration of labelled WR-2721 has been shown in kidney, liver, bone marrow, heart and spleen; low uptake occurs in the central nervous system, muscle and tumour [8–11]. Selective protection of WR-2721 in non-tumour tissue against cytotoxic effects can be explained by difference in absorbtion due to bad vascularisation and low AP activity in the tumour [12, 13]. In the clinic WR-2721 has been demonstrated to be protective against nephro-, neuro- and ototoxicity induced by CDDP without loss of anti-tumour activity [14].

5-Fluorouracil (5FU) is widely used for the treatment of solid tumours, such as advanced colorectal cancer, breast cancer and squamous cell carcinoma of the head and neck. 5FU has to be metabolised and converted to the nucleotide level to become active [15]. The metabolite 5-fluoro-2′ deoxyuridine-5′-monophosphate (FdUMP) inhibits thymidylate synthase (TS), leading to a cessation of DNA synthesis. In the presence of 5,10-

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methylene-tetrahydrofolate (CH_2-THF) this inhibition is enhanced [16]. Next to TS inhibition, 5FU metabolites can exert a cytotoxic effect via the incorporation into RNA or DNA, although the latter, which can induce DNA strand breaks, is not considered to be an important mechanism of cytotoxicity [15, 17].

The combination of 5FU and CDDP is commonly used in cancer chemotherapy of squamous cell carcinoma of the head and neck [18, 19]. The mechanism of this combination, which is thought to be at least additive, remains unsolved. DNA double strand breaks or a stabilizing effect on the inhibited TS by CDDP are proposed mechanisms [20]. Nephrotoxicity is the dose limiting factor of CDDP therapy, while myelotoxicity is another important side-effect [21]. Depending on schedule, myelotoxicity is the dose-limiting factor of 5FU therapy [15]. The efficacy of the combination of 5FU and CDDP can be improved by scheduling and increasing the modulating CDDP dose. The maximum tolerated dose (MTD) for CDDP in combination with 5FU is approximately 1/3 of the MTD of single CDDP. A chemoprotector such as WR-2721 may be used to protect against cytotoxicity induced by an increased CDDP dose. Here we describe the effect of WR-2721 on the toxicity and antitumour activity induced by the combination CDDP and 5FU, in two murine colon tumour models, Balb/c mice bearing Colon 26, an undifferentiated colon carcinoma and C57Bl/6 mice bearing Colon 38, a colon adenocarcinoma. These models have a very different sensitivity for 5FU and, although the sensitivity of these models for CDDP is expected to be low, we have used them because the models have shown their validity in biochemical modulation studies [22–24]. Since TS seems to be a crucial factor in the antitumour effect of 5FU we determined the effect of the combination on TS inhibition.

MATERIALS AND METHODS

Drugs

WR-2721 was obtained from U.S. Bioscience (West Conshohocken, USA). The drug was solubilised with sterilised water to a concentration of 50 mg/ml. 5FU (ABIC, Netanya, Israel) was formulated as a 50 mg/ml solution. This stock solution was diluted with sterilised and pyrogen-free PBS to a final concentration of 10 mg/ml. Platinol (CDDP), (Bristol-Myers Squibb, Woerden, The Netherlands) was used at a concentration of 0.5 mg/ml. The drugs were administered weekly intraperitoneally. The doses of CDDP and 5FU are defined by their subscripts, so $5FU_{100}$ means 100 mg 5FU/kg and $CDDP_3$ means 3 mg CDDP/kg. Unless stated differently, drugs were administered simultaneously.

Tumours

The murine colon tumours Colon 26 and Colon 38, maintained in Balb/c mice and C57Bl/6 mice (Harlan/Olac, Zeist, the Netherlands), respectively, were obtained from Dr P. Lelieveld of the REPGO-TNO Institute, Rijswijk, The Netherlands. Current characteristics of these tumours have been described elsewhere [22, 23]. Tumours were transplanted subcutaneously in both flanks in the thoracic region in small fragments of 1–5 mm^3. When tumours had reached a volume of 50–150 mm^3, treatment was started. Tumour size was determined by caliper measurement (length × width × height × 0.5) twice a week. The volume of the tumours was expressed relative to that on the first day of treatment (day 0). Before treatment, mice were randomised in groups, one as a control group and the other for treatment. Each group consisted of at least six mice. Mice were treated by intraperitoneal injection once a week for 4 weeks. Anti-tumour activity was evaluated by calculation of the relative tumour size of treated vs. control mice (T/C).

Toxicity and survival

Toxicity of the treatment was assessed on the basis of body weight and survival rate after two courses of treatment. The body weight values obtained on the day of first treatment (day 0), were set at 100% (absolute value: about 20 g). Mice were subsequently weighed at least four times a week, always starting on the first day after treatment. At the end of the treatment period the number of survivors was determined for each group.

Temperature changes were monitored after WR-2721 administration by measuring body temperature intrarectally by a Lameris Ellab-Instruments (Lameris, the Netherlands) device.

Haematological effects of the combination of 5FU and CDDP and/or WR-2721 were evaluated in non-tumour-bearing C57Bl/6 mice. Long-term experiments would not be possible in tumour-bearing mice, since such mice would die too soon due to cachexia caused by the tumour and would not survive therapy at the MTD in combination with serial blood sampling. Haematological toxicity was assessed by determining the haematocrit value and performing leucocyte and thrombocyte counts, as described previously [23, 24]. Briefly, blood samples (80–150 μl) were collected with heparinised haematocrit capillaries by retro-orbital puncture under slight ether anaesthesia 3 days before and 4 days after therapy. The samples were transferred into Eppendorf reaction vials containing 20 μl diluted EDTA. Drugs were administered intraperitoneally weekly during 2 weeks. Blood sampling was continued until values returned to normal.

Enzyme assays

Tumours for evaluation of biochemical effects of treatment were removed 3 days after the first treatment and immediately frozen and subsequently stored in liquid nitrogen. TS inhibition has been measured with a ligand binding assay, to determine the number of free FdUMP binding sites of TS; and with a ^3H-release assay determining the conversion of dUMP into dTMP and thereby the catalytic activity of TS. Assays on TS catalytic activity were performed at two substrate concentrations: 1 μM dUMP (K_m) and 10 μmol/l dUMP (saturating concentration). Both the ligand binding assay and the catalytic activity assay were performed with saturating folate concentrations, according to published methods [16, 25, 26]. Tumours of untreated mice served as control samples.

RESULTS

Temperature

After administration of a dose of 525 mg/kg WR-2721 to non-tumour-bearing female Balb/c mice, the rectal temperature of the mice was measured. The average body temperature before therapy of about 37°C decreased rapidly (Fig. 1). The lowest temperatures were observed after 2.5 h. The recovery was very slow and a normal temperature was not observed for the next 24 h. In addition, in the first 3–4 h the animals were weak, atonic, did not react to pain stimuli and bradypnea was also seen. After 3–4 h these symptoms slowly disappeared, animals started to move slowly but were feeble for the next 2–3 h. Two out of five mice treated with WR-2721 died during the next 2 days. At the lower dose of 200 mg/kg WR-2721, the hypothermic effect was less severe, but total recovery was almost as slow as after the high dose administration. The general symptoms were very mild.

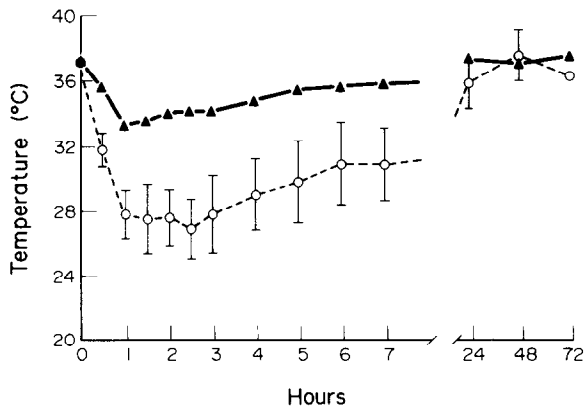

Fig. 1. Temperature changes after administration of WR-2721 (200 mg/kg, -▲- and 525 mg/kg, -○-) intraperitoneally to female Balb/c mice. Values are means (SD) (for 200 mg/kg SD are within the symbols), $n = 5$.

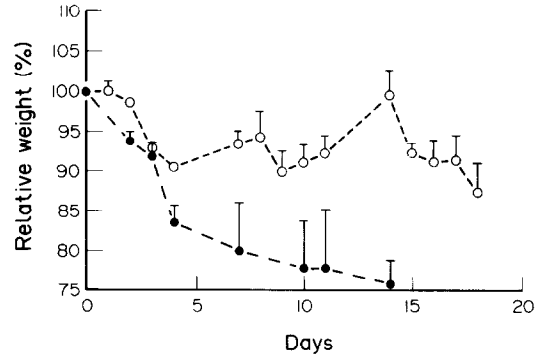

Fig. 2. Comparison of the weight loss in tumour (Colon 26)-bearing (-●-) and non-tumour-bearing (-○-) Balb/c mice after weekly intraperitoneally treatment for 2 weeks with $WR\text{-}2721_{200}/CDDP_7/5FU_{100}$. Values are means of 4–6 mice (SD).

Survivals and weight loss

Initially a dose of 525 mg/kg of WR-2721 was used for non-tumour bearing Balb/c mice and administered intraperitoneally 30 min before 5FU varying from the maximum tolerated dose (MTD) for 5FU (for single 5FU in this schedule, 100 mg/kg) to three times the MTD. These doses were derived from the literature [4, 23]. The WR-2721 pretreatment did not result in any protective effects, all mice died after 2 weeks. Also, the 5FU induced weight loss was not prevented by WR-2721 pretreatment. The weight loss for the combination WR-2721 and 200 mg/kg 5FU was about 15%, comparable to single $5FU_{200}$. Because of the hypothermic and lethal effects of 525 mg/kg WR-2721, a dose of 200 mg/kg WR-2721 was used for subsequent experiments. This dose was combined with 100 and 200 mg/kg 5FU; WR-2721 was again given 30 min before 5FU, but did not increase the life span of these mice. The MTD could not be increased with WR-2721. There was no protective effect of WR-2721, at this dose, on weight loss (data not shown).

In the combination WR-2721/CDDP/5FU, we considered 5FU as the active agent and CDDP as the modulating agent; therefore, we used 5FU at its MTD and escalated the dose of CDDP from 3 to 7 mg/kg. The weight loss for the MTD single 5FU (100 mg/kg) was less than 10% [23, 24]. We used non-toxic CDDP doses far below the MTD of weekly single agent CDDP (9 mg/kg), which did not cause weight loss or decreased survival. The MTD for the combination without WR-2721 was $CDDP_3$ and $5FU_{100}$ (Table 1). When a pretreatment with $WR\text{-}2721_{200}$ (30 min) was given, the dose of CDDP could be increased to 7 mg/kg, while maintaining the dose of $5FU_{100}$. Pretreatment of WR-2721 decreased the toxicity of this schedule considerably. The administration of $CDDP_5$ and $CDDP_7$ in combination with $5FU_{100}$ caused the death of all mice in the second week; the mice treated with WR-2721, $CDDP_7$ and $5FU_{100}$ survived longer (Table 1). The weight loss caused by low doses of CDDP and $5FU_{100}$ was less than 10%. At the dose of $CDDP_7$ in combination with $5FU_{100}$ the weight loss exceeded 10%. The co-administration of WR-2721 diminished the weight loss. Tumour-bearing mice (Balb/c bearing Colon 26) did not tolerate the combination with $CDDP_7$ (Fig. 2) and were treated with a lower dose of 5.5 mg/kg.

Table 1. Toxicity of 5FU and CDDP in combination with WR-2721

5FU	Dose (mg/kg) CDDP	WR2721	Median life span (days)	Maximum weight loss (%)*	Survival at day 20/*n*
100	3	—	>15	6.7	6/6
100	5	—	13	4.4	0/3
100	7	—	11	18.9	0/3
100	7	200	>15	9.5	5/6

Life span of non-tumour-bearing Balb/c mice treated with 5FU and escalating doses of CDDP combined with a pretreatment of WR-2721 (200 mg/kg) was determined after two weekly treatment courses.
*Maximal weight loss after the first treatment.

Haematological toxicity

In C57Bl/6 mice we studied whether WR-2721 could prevent bone marrow suppression induced by the therapeutic dose of $5FU_{100}$ in combination with a high dose of $CDDP_7$. We studied the combination of 5FU and CDDP at the MTD ($5FU_{100}$ and $CDDP_3$), single 5FU at the MTD (100 mg/kg) and single CDDP at the high dose (7 mg/kg). Control counts are not shown, but they did not change much during the experiment.

Administration of single $CDDP_7$ resulted in a decrease in leucocytes to 50% and single $5FU_{100}$ treatment caused a severe leukopenia of 22%. The combination therapy of $5FU_{100}$ and $CDDP_7$ resulted in severe leukopenia with a nadir of 19% after 11 days; a marked rebound was observed at day 18 (Fig. 3a). Administration of WR-2721 intraperitoneally 30 min before the combination resulted in a less severe leukopenia and no rebound was observed. The combination therapy of $5FU_{100}$ and $CDDP_3$ resulted in a decrease of leucocytes comparable to treatment with WR-2721, $CDDP_7$ and $5FU_{100}$. All nadirs occurred at the 11th day after the first treatment (Fig. 3a).

Administration of single $5FU_{100}$ did not cause a thrombocytopenia, but a 2-fold increase was observed (Fig. 3b). These rebounds were observed at the 18th day after the first treatment. Single $CDDP_7$ treatment did not significantly alter thrombocyte counts. The combination therapy of $5FU_{100}$ and $CDDP_3$ resulted in an immediate increase of thrombocytes resulting in a peak at the 11th day. Values remained high during the time period of measurements. The combination therapy of $5FU_{100}$ and $CDDP_7$ without WR2721 resulted in a decrease of thrombocytes to 44% with the nadir at the fourth day after the first treatment, followed by a rebound (Fig. 3b). WR-2721 pretreatment prevented the thrombocytopenia but an increase occurred.

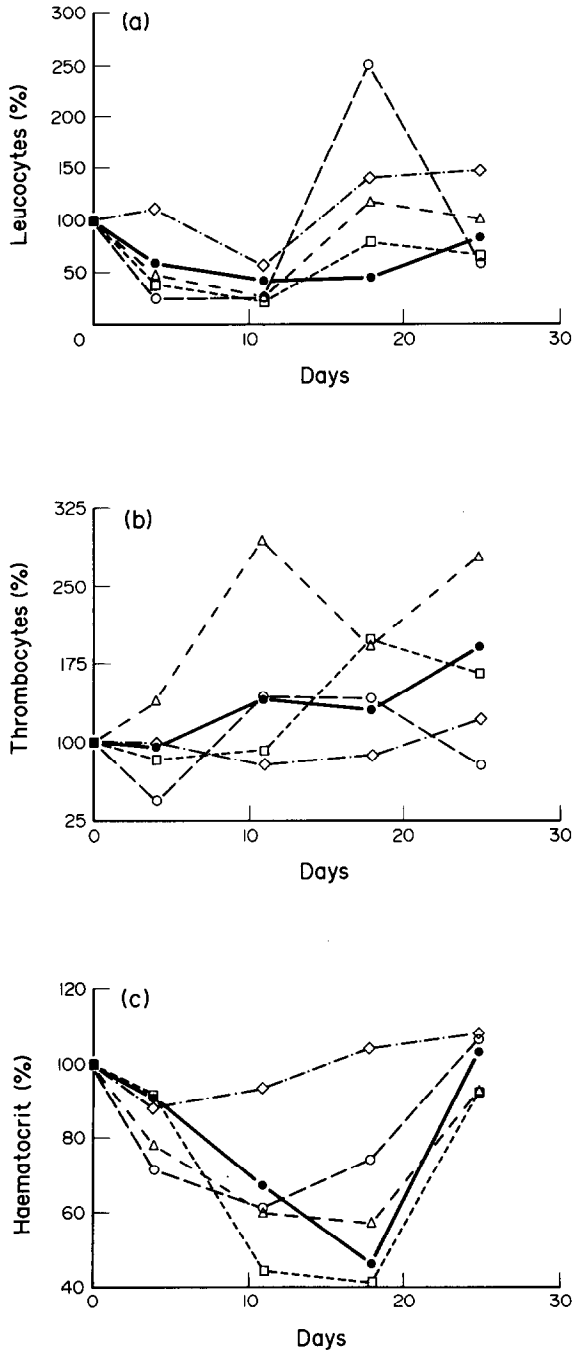

Fig. 3. Effect of WR-2721 on haematological toxicity caused by CDDP and 5FU, (a) leucocytes, (b) thrombocytes and (c) haematocrit. Values were calculated relative to pretreatment values in the same female C57Bl/6 mice. Pretreatment values were leucocytes 7.6 (2.3) × 10^6 cells/ml; thrombocytes 1100 (440) × 10^6 cells/ml; haematocrit 44 (3)%; values are means (S.D.) of 24 mice. Mice were treated with: $5FU_{100}$ (-□-), $CDDP_7$ (-◇-), $CDDP_3$ + $5FU_{100}$ (-△-), $CDDP_7$ + $5FU_{100}$ (-○-), $WR\text{-}2721_{200}$ + $CDDP_7$ + $5FU_{100}$ (-●-). n = 4–7 and SD was less than 15%.

Statistics at day 11 (nadir)—leucocytes: sequence of toxicity, $CDDP_7$ < $WR\text{-}2721_{200}$ + $CDDP_7$ + $5FU_{100}$ < $CDDP_7$ + $5FU_{100}$ = $CDDP_3$ + $5FU_{100}$ = $5FU_{100}$; the latter three groups were significantly different from the first two groups ($P < 0.01$). Thrombocytes: $5FU_{100}$ less toxic than $CDDP_3$ + $5FU_{100}$ or $CDDP_7$ + $5FU_{100}$, ($P < 0.001$); $CDDP_7$ less toxic than $CDDP_3$ + $5FU_{100}$ or $CDDP_7$ + $5FU_{100}$ ($P < 0.01$), $WR\text{-}2721_{200}$ + $CDDP_7$ + $5FU_{100}$ significantly less toxic than $CDDP_7$ + $5FU_{100}$ ($P < 0.01$). Haematocrit: $5FU_{100}$ significantly more toxic than all other treatments ($P < 0.01$) $CDDP_7$ + $5FU_{100}$ more toxic than $CDDP_7$ ($P < 0.001$); $CDDP_7$ + $5FU_{100}$ comparable with $WR\text{-}2721_{200}$ + $CDDP_7$ + $5FU_{100}$.

Table 2. Anti-tumour effect of 5FU combinations with CDDP and WR-2721 on Colon 26 and Colon 38

Treatment schedules	Maximum TC (%) (day)	ILS (%)
Colon 26		
1. $5FU_{100}$	47 (7)	200
2. $CDDP_3$ + $5FU_{100}$	39 (7)	257
3. $CDDP_{5.5}$	44 (7)	>300
4. $WR\text{-}2721_{200}$ → $CDDP_{5.5}$ + $5FU_{100}$	31 (7)*	257
5. $5FU_{100}$ → $WR\text{-}2721_{200}$ → $CDDP_{5.5}$	20 (7)	100
6. $5FU_{100}$ → ($WR\text{-}2721_{200}$ → $CDDP_{5.5}$ Q2W)†	21 (7)	157
7. $WR\text{-}2721_{200}$ → $CDDP_7$ → $5FU_{100}$	21 (7)	127
8. ($WR\text{-}2721_{200}$ → $CDDP_7$, Q2W) + $5FU_{100}$)†	35 (7)	157
Colon 38		
9. $5FU_{100}$	2.0 (17)	>300
10. $CDDP_3$ + $5FU_{100}$	2.0 (17)	>300
11. $WR\text{-}2721_{200}$ → $CDDP_{5.5}$ + $5FU_{100}$	1.0 (17)	>300

Maximum T/C: (Relative tumour volume, treated mice/relative tumour volume, control mice) × 100%. Values of the first three groups are means of 3 to 5 experiments.

ILS = increase of median life span: calculated as (median life span treated mice)/(median life span untreated mice) × 100%; the first day of treatment was used as day 0. Median life span of controls is about 15 days. For Colon 38 no endpoint of lifespan was obtained, since mice were terminated when tumour size exceeded 2000 mm^3. For treatment schedule No. 3 of Colon 26 50% of the mice had a complete response. Values are means for Colon 26 treatment schedule Nos 1–4.

*$WR\text{-}2721_{200}$ → $CDDP_{5.5}$ + $5FU_{100}$ was significantly better than $5FU_{100}$ ($P<0.05$).

Arrows indicate a time interval; $5FU_{100}$ was administered 24 h before $WR\text{-}2721_{200}$ and $CDDP_{5.5}$, while WR-2721 was given 30 min before CDDP.

†Mice were treated weekly for 4 weeks; Q2W, means that CDDP was given every 2 weeks, starting at the second course of 5FU.

Figure 3c shows that single $5FU_{100}$ treatment resulted in an anemia at the 11th day after the first treatment and that treatment with $CDDP_7$ did not alter haematocrit values significantly. In the combination therapy at the MTD, the nadir (57%) was at the 18th day and a higher CDDP dose caused an anaemia at the 11th day. Pretreatment with WR-2721 could not prevent this effect.

Anti-tumour activity

The colon tumour Colon 26 is relatively resistant against 5FU therapy and is, therefore, suitable to study a possible modulating effect of CDDP in combination with 5FU. For single 5FU (Table 2, no. 1), the MTD is 100 mg/kg. At increasing CDDP doses we studied whether in the presence of WR-2721, the anti-tumour effect would be potentiated (Table 2). The MTD for the combination 5FU and CDDP without pretreatment of WR-2721 in tumour-bearing mice is the same as in non-tumour-bearing mice, so we used $5FU_{100}$ and $CDDP_3$ (Table 2, no. 2). WR-2721, $5FU_{100}$ and $CDDP_7$ (Table 2, no. 7) resulted in a strong anti-tumour effect, unfortunately accompanied by a high toxicity, in contrast to non-tumour-bearing mice (Fig. 2). The median life span (MLS) did not increase and was not significantly different from the control group. It appeared that this combination is too toxic for tumour-bearing mice. Several approaches have been studied in order to control this unexpected toxicity. Using an alternating schedule, the mice were allowed to recover

from the high CDDP dose (Table 2, no. 8). Still this schedule was too toxic, so we decreased the CDDP dose to 5.5 mg/kg, which was tolerated by the mice. The treatment with WR-2721_{200}, $CDDP_{5.5}$ and $5FU_{100}$ (Table 2, no. 7) resulted in an increase of the MLS. The anti-tumour activity in this schedule was increased compared with other schedules (Fig. 4). We also tested these drugs in a different setting with 5FU administration 24 h prior to WR-2721 and CDDP (Table 2, no. 5). This appeared to have a very good anti-tumour effect, but its toxicity was unacceptable. An alternating schedule was tested consisting of weekly $5FU_{100}$ administration with, on day 7 and 21, WR-2721_{200} and $CDDP_{5.5}$ given 24 h after 5FU (Table 2, no. 6). This approach did not result in a reduced toxicity, while the anti-tumour effect decreased compared with weekly treatment with all drugs. We also tested a treatment with single $CDDP_{5.5}$ (Table 2, no. 8). The anti-tumour effect of $CDDP_{5.5}$ was remarkable, the MLS was prolonged over 300% compared with the control.

The most optimal schedule WR-2721_{200}/$CDDP_{5.5}$/$5FU_{100}$ (Table 2, no. 11) was also tested on Colon 38. The anti-tumour effect was very good but the treatment appeared to be too toxic in this mouse strain. The growth inhibitory effect of a combination therapy of $CDDP_3$ and $5FU_{100}$ (Table 2, no. 10) on this tumour, was comparable to that of single $5FU_{100}$ (Table 2, no. 9).

TS inhibition

Inhibition of TS is a major factor which may determine the antitumour activity of 5FU. Treatment with $5FU_{100}$ resulted in a reduced FdUMP binding to TS, 3 days after treatment compared with control (Table 3). This time point was chosen because previous experiments had shown that TS inhibition is maximal in Colon 26, 72 h after 5FU administration [27]. Treatment with $CDDP_3$ and $CDDP_{5.5}$ also affected the number of free FdUMP binding sites of TS. For a combination of $5FU_{100}$ and $CDDP_3$ or $CDDP_{5.5}$ treatment, the FdUMP binding in Colon 26 was lower than that of single $5FU_{100}$ treatment. Administration of single WR-2721_{200} also decreased the number of free FdUMP binding to TS in Colon 26, compared with control. A combination treatment of $CDDP_{5.5}$, $5FU_{100}$ and WR-2721_{200} resulted in a FdUMP binding that was not significantly different from FdUMP binding due to single $5FU_{100}$ treatment. Administration of WR-2721 combined with $CDDP_3$ or $CDDP_{5.5}$ was comparable to $CDDP_3$ or $CDDP_{5.5}$ treatment.

Table 3. Effect of different combinations of 5FU, CDDP and WR-2721 on the activity of TS in Colon 26 tumours

Treatment	FdUMP binding (fmol/mg protein)	Catalytic activity (pmol/mg protein/h)	Ratio activity binding
Control	1834 (458)	2324 (720)	1.7 (0.8)
5FU	575 (184)	674 (18)	2.0 (1.1)
$CDDP_3$	816 (166)	3804 (1036)	4.6 (0.4)
$CDDP_{5.5}$	587 (26)	1673 (329)	2.7 (0.8)
$CDDP_3$ + 5FU	267 (136)	1239 (239)	3.8 (0.4)
$CDDP_{5.5}$ + 5FU	294 (20)	780 (40)	2.7 (0.2)
WR-2721 → $CDDP_{5.5}$ + 5FU	591 (121)	2600 (817)	4.4 (0.9)
WR-2721 → $CDDP_3$	864 (120)	4642 (1650)	5.3 (1.4)
WR-2721 → $CDDP_{5.5}$	568 (153)	1837 (167)	3.4 (0.7)
WR-2721	894 (30)	2959 (176)	3.3 (0.3)
WR-2721 → 5FU	433 (151)	1206 (201)	3.0 (1.0)

The 5FU and WR-2721 doses were fixed at 100 and 200 mg/kg, respectively. WR-2721 was administered 30 min prior to CDDP or 5FU. Values are means (SD) of at least three tumours, the ratios were calculated from the separate tumours.

Statistics—FdUMP binding; all treatments were significantly different from control ($P<0.001$), $CDDP_3$ + 5FU and $CDDP_{5.5}$ + 5FU were significantly lower than 5FU and WR-2721 → $CDDP_{5.5}$ + 5FU ($P < 0.01$). Catalytic activity; 5FU, 5FU + WR-2721, $CDDP_3$ + 5FU and $CDDP_{5.5}$ + 5FU were significantly lower than control ($P < 0.01$). $CDDP_3$, WR-2721 + $CDDP_3$ were significantly higher than control ($P < 0.01$). All values were significantly higher than 5FU ($P < 0.01$). Ratio; control vs. $CDDP_3$, WR-2721 + $CDDP_3$, $CDDP_3$ + $5FU_{100}$, WR-2721 + $CDDP_{5.5}$ + 5FU, $P < 0.001$.

TS catalytic activity was clearly inhibited by treatment with $5FU_{100}$ (Table 3). Combination therapies of $5FU_{100}$ and $CDDP_{5.5}$ or $5FU_{100}$ and WR-2721_{200} had an effect comparable to that of single $5FU_{100}$ treatment. Single WR-2721_{200} or WR-2721_{200} and $CDDP_{5.5}$ administration did not inhibit TS catalytic activity, while $CDDP_{5.5}$ had a slight inhibitory effect. However, treatment with $CDDP_3$ alone and in combination with WR-2721_{200} increased the catalytic activity of TS compared to control. Another striking effect was seen after WR-2721_{200}/$CDDP_{5.5}$ $5FU_{100}$ administration; although $5FU_{100}$ was given, no inhibition of TS catalytic activity was observed.

A ratio was calculated between the activities of 1 and 10 μmol/l 2′ deoxyuridine-5′ monophosphate (dUMP), in order to detect changes in enzyme kinetics, which might occur during treatment. This ratio was [mean (SD)] 2.2 (0.3) for control and comparable for all other treatments. However, the ratio between the FdUMP binding of a tumour and its TS catalytic activity at 10 μmol/l dUMP changed markedly (Table 3). The values for $CDDP_3$-containing treatments and WR2721/$CDDP_{5.5}$/$5FU_{100}$ were significantly higher than the control value ($P<0.001$).

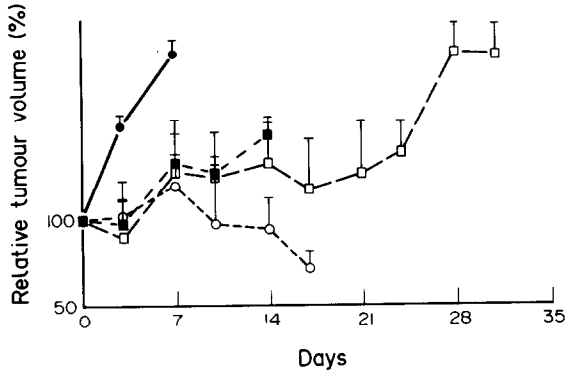

Fig. 4. The effect of WR-2721 and CDDP on the antitumour activity of 5FU against Colon 26. Mice were treated weekly; for 4 weeks WR-2721 was administered 30 min before 5FU and CDDP; control (-●-), $5FU_{100}$ (-■-), WR-2721_{200} + $CDDP_{5.5}$ + $5FU_{100}$ (-□-) and WR-2721_{200} + $CDDP_7$ + $5FU_{100}$ (-○-). Values are means (SEM) of 8–12 tumours.

DISCUSSION

Our data demonstrate that WR-2721 protected against weight loss, haematological toxicity and mortality of combined 5FU/CDDP treatment. WR-2721 pretreatment allowed an elevation of the CDDP dose in the combined 5FU/CDDP treatment

of murine colon tumours, leading to a better anti-tumour effect than 5FU alone. Biochemically, this improved anti-tumour effect cannot be explained by an increased extent of TS inhibition.

In the initial *in vivo* experiments WR-2721 was given in a too high dose (525 mg/kg), which caused severe hypothermia. The mechanisms involved in this hypothermia, observed in WR-2721-treated mice [28, 29] and rats [30], are still unknown. The effect is probably related to mouse strain and species since 600 mg/kg intraperitoneally could safely be given to LAF_1 mice, while only 525 mg/kg could be administered to Balb/c mice [4]. For intravenous administration to Balb/c mice, a dose of 500 mg/kg has been used and no side-effects were reported [11]. In patients, hypotension was observed after WR-2721 treatment [14]. This could occur in the mice as well and lead to hypothermia. If we take into account their high body surface/volume ratio compared with man, mice will cool down much faster, when they have low blood pressure. Locomotive decrement was considered the main side-effect of WR-2721 at lower doses [31, 32]. This toxicity was of an acceptable degree in our experiments with $WR\text{-}2721_{200}$.

The dose of 5FU could not be increased in combination with $WR\text{-}2721_{200}$. From this we concluded, in contrast to previous data [4], that WR-2721 did not have any protective capacities against the toxic effects of 5FU, expressed as weight loss and survival. This was consistent with *in vitro* data [33]. However, WR-2721 was able to protect against CDDP toxicity, since in the combination treatment of CDDP with 5FU the dose of CDDP could be doubled. A possible explanation for this drug-dependent chemoprotective effect could be that 5FU toxicity may be on the RNA level [23], while WR-2721 protects on the DNA level, against formation of CDDP intra-strand links [7].

Analysis of the haematological toxicity of WR-2721/5FU/CDDP combination therapies revealed that WR-2721 prevented enhancement of leukopenic and thrombocytopenic effects of $CDDP_7$ in the combination with $5FU_{100}$. It was clear that anaemia was caused by $5FU_{100}$ alone. WR-2721 could not protect against this toxic effect of 5FU. It has been shown that 5FU spares haemopoietic stem cells which are responsible for long-term repopulation [34], but CDDP is more toxic for earlier haemopoietic stem cells, than for more mature cells [35, 36, Treskes, personal communication]. The haemopoietic stem cells could eventually be responsible for the rebound in leucocytes and thrombocytes after leukopenia and thrombocytopenia, respectively. Haematological toxicity was observed after peripheral blood cell counting, but the number of these cells does not give a valid indication of changes in the number or quality of progenitor cells in the bone marrow and is more likely to reflect toxicity to more mature hemapoietic cells [37]. However, determination of the numbers of these cells in the blood is still the way that haematological effects of cytotoxic treatments are evaluated. It allows the determination of the endpoint of haematological toxicity, being the number of functional erythrocytes, leucocytes and thrombocytes essential for normal functioning of the immune system [37]. The protection of WR-2721 pretreatment against myelotoxicity of $CDDP_7/5FU_{100}$ indicates that this is not the cause of death. Most likely, gastrointestinal toxicity is dose limiting.

Toxicity and growth inhibitory effects of combination therapies of CDDP and 5FU appeared to be schedule dependent, but the scheduling is a controversial issue. *In vitro* pre-exposure to CDDP increased 5FU activity on ovarian and head and neck carcinoma cells [20, 38]. *In vivo* tests with mice showed that 5FU followed by CDDP had a better anti-tumour effect and lower toxicity than CDDP administered before 5FU [39–42]. In our tumour model, this schedule resulted in unacceptable toxicity. However, all combination therapies were more effective than single $5FU_{100}$, which is most likely to represent an additional effect of CDDP. We observed that at a higher CDDP dose with WR-2721 pretreatment, the efficacy of the combination CDDP/5FU was better, but the effect is certainly not synergistic. Single $CDDP_{5.5}$ treatment had a better anti-tumour effect than each of the CDDP/5FU combinations that were tested, while CDDP treatment was expected to have little effect on colon tumours. The relatively high antitumour activity of CDDP therapy described for this and a few other murine colon tumour models [41, 42] might be due to higher drug peak plasma levels in mouse than in man [43].

The antiproliferative effect of 5FU was reflected in the inhibition of FdUMP binding to TS and the catalytic activity of TS. The inhibitory effect of $CDDP_3$ and $CDDP_{5.5}$ administration observed in the FdUMP binding assay and of $CDDP_{5.5}$ in the catalytic assay of about 50–60% was consistent with *in vitro* data [44]. This was not a direct effect of the drug on TS protein, for when CDDP was added to cell-free extracts, the enzyme activity was not inhibited [44]. It is known that platinum can form complexes with essential and non-essential sulphydryl groups of TS [45], but these CDDP–sulphydryl complexes did not inhibit TS. This was assayed with a spectrophotometric method. We used two radioactive methods to measure more long-term effects of CDDP on the activity of TS. CDDP treatment resulted in inhibition of FdUMP binding to TS, but hardly decreased the catalytic activity of the enzyme. CDDP interaction with TS might result in a block of the binding of FdUMP by a change in the ternary structure of the enzyme, without affecting binding of the natural substrate dUMP. Since CDDP did not contribute to the extent of TS inhibition in the combination with 5FU, another mechanism of CDDP cytotoxicity might play a more important role. Thymidine starvation caused by TS inhibition due to 5FU might decrease the repair of CDDP-induced strand breaks and fragmentation of DNA, resulting in an increased DNA damage [46].

WR-2721 itself had a slight inhibitory effect on FdUMP binding to TS, but no effect on the catalytic activity of TS. However, in combination with $5FU_{100}$ and $CDDP_{5.5}$, which both have an inhibitory effect on the catalytic activity, no inhibition of catalytic activity was observed. A similar tendency, decrease of inhibition of catalytic activity, was seen when WR-2721 was combined with $CDDP_3$ or $CDDP_{5.5}$. How interactions of TS and WR-2721 should be explained is still unclear. Possibly, the thiol form of WR-2721, WR-1065 interacts or protects the sulfhydryl groups of TS, because it has been shown that WR-1065 can form disulfides with (protein-bound) cysteine and it inactivates reactive platinum complexes [7].

The increased efficacy of WR-2721/CDDP/5FU in Colon 26 compared with single 5FU seems promising, but it has also been observed that WR-2721 pretreatment might reduce the antitumour effect of single CDDP in this tumour (unpublished results). Since combination therapies of CDDP/5FU for the treatment of colon cancer have a limited value in the clinic [47], it would be more interesting to test WR-2721/CDDP/5FU combinations in head and neck cancer [48]. In this tumour type the combination CDDP/5FU is more active, with a response rate of 20–70% [18, 19]. Addition of folinic acid to this combination could also improve the anti-tumour effect according to *in vitro* data [38], but clinical reports are less conclusive [49, 50]. WR-

2721 pretreatment creates the possibility of using higher CDDP doses in the CDDP/5FU combination treatment without increased toxicity.

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The Effect of a Low-fat Diet on Hormone Levels in Healthy Pre- and Postmenopausal Women: Relevance for Breast Cancer

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It has been postulated that differences in the levels of circulating hormones may be the explanation for the epidemiological link between per capita dietary fat intake and the incidence of breast cancer. We have investigated this possible relationship in 19 postmenopausal, and 18 premenopausal women who completed a 4-week period on a diet aiming to reduce fat intake to around 20% of total kilocalories. 7-day dietary records revealed a significant decrease in dietary fat intake in both the pre- and postmenopausal groups (from 37.2% of calories from fat to 23.2% and from 37.9 to 24.3%, respectively). There was a minor increase in the level of sex hormone-binding globulin, and a small decrease in prolactin in the postmenopausal group, which were of borderline significance. There were no significant changes in total oestradiol (E2), or non-protein-bound (free) E2 concentrations. In the premenopausal group there were no significant changes in any of the hormone levels investigated.
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INTRODUCTION

THERE ARE marked international variations in the incidence of breast cancer [1]. This, combined with the increases in breast cancer incidence in populations migrating from countries with a low incidence of breast cancer to those with a high incidence [2, 3] suggest that environmental factors may influence the incidence of the disease. One such factor is dietary fat consumption, and there is a good correlation between average per capita fat intake and breast cancer incidence worldwide [4, 5]. Animal studies have confirmed the relationship between dietary fat intake and mammary tumorigenesis, and have also demonstrated that tumour formation may be hormonally dependent [6].

In man, there is a wealth of evidence to implicate oestrogens in the aetiology and pathogenesis of breast cancer. Recent interest has concentrated on the proportion of non-protein-bound (free) oestradiol (E2), and several studies have demonstrated an increase in the levels of free E2 in patients with breast cancer [7–9]. There are also differences in the levels of non-protein-bound E2 between British and Japanese women [10], and this may partly explain the differences in breast cancer rates between the two countries.

Several dietary intervention studies have found that investigating the effects of a low-fat diet in both pre- and postmenopausal women include the suppression of plasma oestrogen levels [11–13]. It is possible, therefore, that the relationship between dietary fat intake and breast cancer may be dependent on changes in oestrogenic stimuli to the breast. In the current study we have measured plasma E2 levels, the fractional binding of E2, and prolactin levels in closely monitored groups of pre- and postmenopausal volunteers before and during their adherence to a low-fat diet for a period of 4 weeks.

POPULATION AND METHODS

73 women were interviewed regarding participation in a study involving "dietary modification, and its possible effects on

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