Session F: Thiol Modulation and Protection

PROTECTION BY WR-2721 OF THE TOXICITY INDUCED BY THE COMBINATION OF CISPLATIN AND 5-FLUOROURACIL

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We evaluated the effects of WR-2721 and its metabolite WR-1065 on *in vitro* growth inhibition by 5-fluorouracil (5FU) and cisplatin (CDDP) and the effect of WR-2721 on *in vivo* toxicity and antitumor effect of 5FU and CDDP. In cell culture both WR-2721 and WR-1065 were not able to reverse growth inhibition caused by either 5FU or CDDP. Administration of WR-2721 i.p. at 525 mg/kg to mice resulted in a severe temperature drop to 27°C; at 200 mg/kg hypothermia was less severe. WR-2721 failed to prevent 5FU toxicity, but the maximum tolerated dose of CDDP in the combination with 5FU (at 100 mg/kg) could be increased from 3 to 7 mg/kg. CDDP at 7 mg/kg enhanced leukopenia caused by 5FU at 100 mg/kg to 20% and thrombocytopenia to 40%; WR-2721 reduced leukopenia and prevented thrombocytopenia induced by the combination. Combination of CDDP, 5FU, and WR-2721 resulted in an enhanced antitumor activity against the murine colon tumor Colon 26 compared to 5FU alone and to 5FU combined with CDDP at their maximum tolerated dose.

5-fluorouracil, Cisplatinum, WR-2721 (ethiofos).

INTRODUCTION

WR-2721 (s-2-(3-aminopropylamino)ethylphosphorothioic acid, ethiofos) is being used as a radioprotector and as a chemoprotector against cytotoxic effects of several cytostatic agents, such as cisplatin (CDDP) (12, 16). The active metabolite of WR-2721, WR-1065, is formed by dephosphorylation catalyzed by the plasma membrane enzyme alkaline phosphatase (6). WR-1065 can prevent DNA damage caused by several chemotherapeutic agents (1). In the clinic WR-2721 has demonstrated to be protective against nephro-, neuro-, and ototoxicity induced by CDDP without loss of antitumor activity (2).

5-Fluorouracil (5FU) is widely used for treatment of solid tumors, such as advanced colorectal cancer, breast cancer, and squamous cell carcinoma of the head and neck. 5FU is active through incorporation into RNA and inhibition of thymidylate synthase (TS) by its metabolite FdUMP, mediated by the formation of a ternary complex between FdUMP, 10-methyltetrahydrofolate, and TS (11).

The combination of 5FU and CDDP is commonly used in cancer chemotherapy of squamous cell carcinoma of the head and neck (4) and is at least additive (7). For CDDP therapy nephrotoxicity is dose limiting, while for 5FU therapy myelotoxicity is dose limiting depending on schedule. The efficacy of the combination can be improved by scheduling and increasing the modulating CDDP dose. The maximal tolerated dose (MTD) for CDDP in the combination is approximately 1/3 of the dose without 5FU. To protect against toxicity induced by an increased CDDP dose, a chemoprotector such as WR-2721 may be used. Here we describe the effects of WR-2721 and WR-1065 on the toxicity induced by CDDP and 5FU as single agents and in combination.

METHODS AND MATERIALS

The effects of WR-2721 and WR-1065* on 5FU and CDDP toxicity have been tested *in vitro* on five different cell lines; C26-10, a murine undifferentiated colon ade-
nocarcinoma (derived from Colon 26); UM-SCC-11B (11B), a human squamous cell carcinoma of the head and neck; HT-29, a human colon carcinoma; and A2780 and OVCAR3, two human ovarian cancer cell lines. Doubling times varied between 20 and 48 hr. The cells were cultured according to standard conditions (3, 8), in Dulbecco's Modified Eagles medium supplemented with 5% heat-inactivated fetal bovine serum at 37°C under 5% CO₂. Drugs were added at day 1 after plating and cells were exposed to drugs continuously for the whole culture period, which was 2 days for C26-10 cells and 3 days for the other cell lines. At day 1 and at the end of the culture periods, the number of cells was enumerated using the sulforhodamine B (SRB) test (14), which is suitable to quantify not only growth inhibition, but also cell kill (3). Shortly, cells were fixed with trichloroacetic acid, cellular protein was stained with SRB, washed with acetic acid and solubilized with unbuffered Tris. The absorbance was read with an ELISA reader at 540 or 510 nm. IC₅₀ values (= concentrations that cause 50% growth inhibition) were calculated from the separate growth curves. For the study of the effects of the WR compounds different schedules have been tested: simultaneously and sequential: WR compounds were added 15 min or 24 hr before the drugs, or 15 min or 24 hr after 5FU. CDDP exposure time was either 1 hr or continuously.

Evaluation of toxicity of the several drugs (alone and in combination) has been performed in female Balb/c and C57Bl/6 mice as described (9, 10) after weekly i.p. administration. Weight loss was used as a parameter for gastro-intestinal toxicity. Hematologic toxicity of the combination of 5FU and CDDP and/or WR-2721 was evaluated in non-tumor bearing C57Bl/6 mice as described (5, 10), by analysis of leucocyte and thrombocyte counts and hematocrit in weekly blood samples obtained by retroorbital bleeding of the same mouse.

The antitumor effect of the combinations was evaluated with the murine colon carcinoma Colon 26 as described (5, 10) using the ratio of the tumor volumes from treated and untreated control animals (T/C values). Values 0.5 < T/C < 1 were considered to represent insensitive; < 0.5, moderately sensitive and < 0.25 sensitive treatment. Values were calculated at several time points after initiation of treatment and maximal values (the lowest) are given. Colon 26 is a tumor causing cachexia to mice resulting in a limited life-span of mice after transplantation of tumors of about 19 days. Treatment was started at day 9 after transplantation. Life-span is calculated from the first day of treatment. The doses of the drugs are defined by their subscripts, so 5FU₁₀₀₀ means 100 mg 5FU/kg and CDDP₁ means 3 mg CDDP/kg.

RESULTS

In vitro

5FU and CDDP were used at concentrations that caused a partial growth inhibition. The IC₅₀ values for 5FU were, 1 µM for C26-10 and 5 µM for HT-29 and 11B cells; for CDDP, 3 µM for 11B, 6 µM for C26-10, 20 µM for HT-29 at continuous exposure; and 5 µM for A2780 and 20 µM for OVCAR3 at 1 hr exposure. In C26-10, HT-29 and 11B, various combinations of CDDP and 5FU have been tested at continuous exposure; however, in this schedule the effects were not more than additive.

Different schedules were used for the combination of 5FU and CDDP with the WR compounds. Pretreatment with WR was used since this is the usual way of clinical administration, a short exposure was used because of the short half-life of not only the WR compounds, but also of the cytostatic agents; continuous co-incubation of the WR-compounds with either 5FU or CDDP was performed to enable a maximal interaction between the drugs. WR-2721 alone did not affect cell growth, but WR-1065 caused a minor growth inhibition at 1000 µM (Fig. 1). At continuous co-incubation neither WR-2721 nor WR-1065 affected the growth inhibition caused by 5FU in C26-10, HT-29 and 11B; or by CDDP in C26-10, HT-29, 11B, A2780, and OVCAR3 (Fig. 1). When the WR compounds were added before or after 5FU or CDDP no interference of WR with growth inhibition caused by either 5FU or CDDP was observed in either cell line.

In vivo

Initial experiments with WR-2721₃₂₅ revealed a striking decrease in body temperature in Balb/c mice from about 37°C to 32°C after 30 min and to 27°C after 2.5 hr. Recovery lasted longer than 24 hr. Other side effects, such as weakness and atonicity, disappeared slowly. Since the hypothermic effect of WR-2721₂₀₀ was less severe (nadir 34°C), this dose was used for all other experiments.

WR-2721₂₀₀ was administered to Balb/c mice i.p. 30 min before 5FU. Weight loss at 5FU₁₀₀₀, the maximum tolerated dose (MTD) of 5FU alone, was less than 5%, that of 5FU₂₀₀ and 5FU₃₀₀ more than 10% (Fig. 2). WR-2721₂₀₀ pretreatment did not result in any protective effects, the weight loss due to 5FU treatment was not prevented, and all mice treated at 5FU₂₀₀ and 5FU₃₀₀ died within 20 days, in contrast to mice treated with 5FU₁₀₀ with or without WR-2721.

In the combination of CDDP and 5FU we considered 5FU as the active agent and CDDP as the modulating agent, thus 5FU was used at 5FU₁₀₀ and the dose of CDDP was escalated. The MTD for the combination was CDDP₃ and 5FU₁₀₀ with a weight loss comparable to that of 5FU alone (Fig. 2). The administration of CDDP₅ and CDDP₇ in combination with 5FU₁₀₀ caused the death of all mice in the second week; mice treated with WR-2721₂₀₀, CDDP₅, and 5FU₁₀₀ survived longer than 15 days and the maximal weight loss diminished (Fig. 2). 5FU₁₀₀, CDDP₇ with WR-2721₂₀₀ was too toxic for mice bearing Colon 26, while 5FU₁₀₀, CDDP₅ with WR-2721₂₀₀ resulted in an acceptable toxicity.

The combination of 5FU₁₀₀ and CDDP₇ resulted in a severe leukopenia (Fig. 3), which was followed by a re-
bound after 18 days. Addition of WR-2721\textsubscript{200} to this combination resulted in a less severe leukopenia and no rebound was observed. The combination of 5FU\textsubscript{100} and CDDP\textsubscript{3} resulted in a decrease of leukocytes equally to WR-2721\textsubscript{200}, CDDP\textsubscript{3} and 5FU\textsubscript{100}. 5FU\textsubscript{100} alone and CDDP\textsubscript{3} alone did not cause thrombocytopenia, but in combination thrombocytes decreased to 44\% (day 4), followed by an increase to 143\%. WR-2721\textsubscript{200} prevented this thrombocytopenia and a rebound of 169\% occurred.

5FU\textsubscript{100} alone caused anemia, but CDDP\textsubscript{3} did not affect hematocrit. 5FU\textsubscript{100} with CDDP\textsubscript{3} caused a comparable decrease in hematocrit, which could not be prevented by WR-2721\textsubscript{200}.

Colon 26 is relatively resistant (T/C > 0.5) against 5FU therapy (5, 10) and therefore suitable for combination studies with 5FU. 5FU\textsubscript{100} treatment resulted in a maximal T/C of 0.6–0.8 and an increase in life-span (ILS) of about 200\%. For 5FU\textsubscript{100} with CDDP\textsubscript{3} the maximal T/C was about 0.4 with an ILS of about 255\%. Both the combinations 5FU\textsubscript{100} CDDP\textsubscript{3} with WR-2721\textsubscript{200} and 5FU\textsubscript{100} CDDP\textsubscript{3} with WR-2721\textsubscript{200} resulted in T/C values between 0.2 and 0.3, but the ILS for the CDDP\textsubscript{3} schedule was only about 150\%, while that for the CDDP\textsubscript{3} was more than 300\%.

![Fig. 1. Effect of continuous co-incubation (3 days) with WR-2721 and WR-1065 on growth inhibition of 5FU and CDDP on HT-29 and 11B cells. The WR-compounds were tested with and without 5FU (5 \(\mu\)M for HT-29 and 11B) or CDDP (3 \(\mu\)M for 11B and 20 \(\mu\)M for HT-29). The optical density (O.D.) at 540 nm is plotted against the concentration of the WR-compounds. Control growth of cells was comparable to that of cells cultured in the presence of the lowest WR concentration. In case the WR compound would protect against the cytotoxic effects of either 5FU or CDDP this would have resulted in an increase of O.D. values towards control growth. Data represent one representative experiment out of 3–4, carried out in quadruplicate. SD of the separate values was less than 5%.](image1)

![Fig. 2. Effect of WR-2721 (WR) on the toxicity induced by various combinations with 5FU and CDDP. The numbers left of the Y-axis denote doses in mg/kg; --, not given. Drugs were administered weekly for 2 weeks, and values (means ± SD) represent the maximal weight loss (in % of the pretreatment weight of the animals) observed in the first week after treatment. *, weight loss in tumor (colon 26) bearing animals; all other data are from non tumor bearing animals.](image2)

![Fig. 3. The effect of WR-2721\textsubscript{200} on leucocyte counts in non-tumor bearing mice treated with 5FU\textsubscript{100} in combination with CDDP. Values denote the nadirs (means ± SE) of the blood counts observed after 4 days. C-7 means CDDP\textsubscript{3}.](image3)
DISCUSSION

Evaluation of the potentially protective effects of WR-2721 was performed both in vitro and in vivo. Our in vitro data demonstrate that both WR-2721 and WR-1065, the dephosphorylated active metabolite, can not reverse or prevent the growth-inhibitory effects of both 5FU and CDDP against tumor cell lines derived from tumors that are treated in the clinic with either 5FU or CDDP or in combination. These data suggest a possible selective effect of WR-2721. Additional studies with non-tumor cells such as fibroblast lines might enable a possible selective effect in vitro to be demonstrated.

Our in vivo experiments were complicated by the severe hypothermia caused by WR-2721 itself. It is not clear what will be the impact of the WR-2721 induced hypothermia, but at a low temperature drug metabolism or pharmacodynamics may be changed, possibly being related to some of the effects described. Comparable effects on body temperature have been described (15) but the mechanisms involved are unclear. Besides the hypothermia, WR-2721 demonstrated some additional toxicity at 5FU/100 leading to a decreased survival. In contrast to previous data (16), we could not demonstrate a protective effect against 5FU induced toxicity both WR-2721 and WR-2721/100. However, WR-2721/100 did protect against CDDP induced toxicity and prevented lethal effects of CDDP in the combination with 5FU, as we could safely increase the CDDP dose in the presence of WR-2721/100, but not without WR-2721/100 pretreatment.

Although the use of peripheral blood cell counts for interpretation of bone marrow toxicity has several limitations, this is the usual method for assessment of myeloid toxicity in patients. In addition, the toxicity pattern was comparable to that observed in patients, myelotoxicity being the dose-limiting toxicity for weekly 5FU (11). From our data it is clear that WR-2721 protected against the additional myelotoxicity caused by CDDP, both leucopenia and thrombocytopenia. Interpretation of the effects is rather complicated, since the decrease in leuko- and thrombocytes was sometimes followed by a rebound. In order to design an optimal scheduling for clinical application, a more detailed analysis of these effects is warranted. This would include the study of the various proliferation lineages in the ontogeny of the blood cell populations in treated and untreated animals.

Initial analysis of the antitumor effect of the combination demonstrated that the antitumor effect of the combination was higher than that of 5FU alone. A complication in these studies was the higher toxicity of the combination in tumor bearing animals compared to non-tumor bearing animals requiring a dose reduction of CDDP. The promising data must be extended to other tumor types such as squamous cell carcinoma of the head and neck for which combination therapy of CDDP and 5FU is commonly used. Our data demonstrate that in the combination of CDDP, and 5FU the dose of CDDP can be enhanced, but dose escalation of CDDP should be carefully guided.

We conclude that WR-2721 can protect against additional CDDP toxicity in the combination with 5FU, leading to a better efficacy of this combination. However, it is not clear whether in vivo this effect is due to a modulating effect of CDDP on 5FU as has been suggested (13) or an additional effect. The overall benefit, however, can be clearly ascribed to the chemoprotective effect of WR-2721.

REFERENCES

13. Scanlon, K. J.; Newman, E. M.; Lu, Y.; Priest, D. G. Biochemical basis of cisplatin and 5-fluorouracil synergism in