Cancer Chemother Pharmacol (1992) 29: 467–470

Cancer Chemotherapy and Pharmacology

The reversal of cisplatin-protein interactions by the modulating agent WR2721 and its metabolites WR1065 and WR33278*

Marco Treskes, Ulbe Holwerda, Leo G. J. Nijtmans, Herbert M. Pinedo, and Wim J. F. van der Vijgh

Department of Oncology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

Received 20 May 1991/Accepted 14 October 1991

Summary. The reversibility of cisplatin-protein interactions by the modulating agent WR2721, its active thiol-metabolite WR1065, and the symmetrical disulfide WR33278 was studied using the model compounds (Pt(diethylenetriammine) monofunctionally bound to the sulfur in glutathione (Pt(dien)SG) and Pt(diethylenetriammine) monofunctionally bound to the sulfur in S-methylglutathione (Pt(dien)SMeG). Both model compounds could be quantified by high-performance liquid chromatography (HPLC) with UV detection. The Pt-cysteine-like bond in Pt(dien)SG could not be reversed by any of the WR compounds or by the strong nucleophiles thiosulfate (TS) and diethyldithiocarbamate (DDTC). However, the Pt-methionine-like bond in Pt(dien)SMeG could be reversed by WR1065, although the reversal was slow ($k_2 = 0.142\ M^{-1}\ s^{-1}$) as compared with that obtained using the modulating agents TS ($k_2 = 10.1\ M^{-1}\ s^{-1}$) and DDTC ($k_2 = 3.66\ M^{-1}\ s^{-1}$). WR2721 was hardly able to reverse the Pt-S bond in Pt(dien)SMeG ($k_2 = 0.00529\ M^{-1}\ s^{-1}$), and WR33278 showed no capacity to do so. The activity of *cis*-diamminedichloroplatinum(II) (CDDP)-inactivated fumarase was not appreciably restored by any of the WR compounds (16%, 7.7%, and 0 for 20 mM WR1065, WR2721, and WR33278, respectively) in contrast to the strong nucleophile DDTC (61% for 2 mM DDTC). These in vitro studies provide information at the molecular level that may explain why WR2721, in contrast to DDTC, does not provide protection against cisplatin-induced nephrotoxicity when it is given after platinum-containing chemotherapy. The results support the present clinical use of WR2721 prior to the administration of platinum compounds.

* This study was financially supported by the Netherlands Cancer Fund (grant IKA 87-12) and by US Bioscience

Offprint requests to: M. Treskes, Dept. of Oncology – BR232, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II), CDDP] is very active in the treatment of several solid tumors. The antitumor activity of CDDP is most likely the result of its binding to DNA [13]. The success of CDDP treatment is limited by the occurrence of several toxic side effects, among which nephrotoxicity is dose-limiting. However, when the kidneys are sufficiently protected, neurotoxicity and bone marrow suppression also become apparent [9]. CDDP-induced nephrotoxicity can be reduced by hydration, forced diuresis, and the administration of so-called modulating agents. The strong nucleophile sodium thiosulfate (TS), which is rapidly excreted by the kidneys, presumably protects against CDDP-induced nephrotoxicity by inactivating reactive Pt species in the kidney. However, by inactivating active Pt species in the circulation, TS also interferes with the antitumor activity of CDDP. Therefore, TS is mainly successful in two-route regimens whereby the tumor is locally exposed to the Pt compound, whereas TS is given systemically [7]. When it is given 2 h after the Pt drug, the strong nucleophile diethyldithiocarbamate (DDTC) protects rats from CDDP-induced nephrotoxicity without impairing the antitumor activity of the former [3, 4]. The hypothesis that DDTC reverses CDDP-protein interactions responsible for (part of) the toxic side effects, whereas the CDDP-DNA interactions responsible for the antitumor activity are not reversed has been supported by in vitro studies [1]. In a phase I clinical trial, DDTC provided protection against CDDP-induced nephrotoxicity without adversely affecting the antitumor activity of the Pt compound, but the severe neurotoxicity of DDTC discourages its clinical use [10].

WR2721, a prodrug of the radioprotective thiol compound WR1065, given to rodents prior to CDDP protected the animals against CDDP-induced nephrotoxicity [16, 18] and myelotoxicity [17] without producing a negative effect on the antitumor activity. Early clinical trials confirm the selective protection of nontumor tissues [6]. This probably results from the preferential formation and uptake in nontumor tissues of the active nucleophilic thiolmetabolite

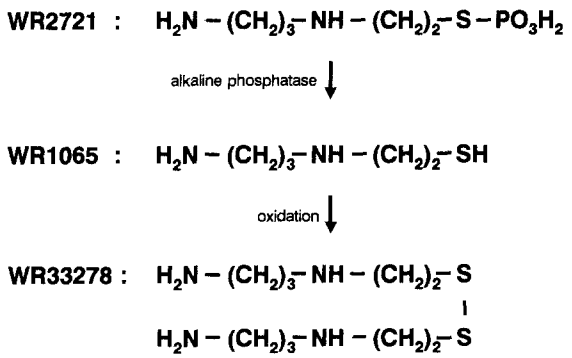

Fig. 1. The molecular structure of WR2721 and its main metabolites

WR1065 [5, 12], which can subsequently inactivate hydrolyzed (activated) CDDP inside the cell before the latter can bind to target molecules (DNA and proteins). Our recent studies showed that the administration of WR2721 after CDDP treatment did not protect mice against nephrotoxicity [16]. To understand our in vivo observations, we started the present study to investigate the reversal of toxic CDDP-protein interactions by WR2721, its thiol-metabolite WR1065, and the symmetrical disulfide WR33278 (Fig. 1) as compared with the rescue agent DDTC. The sulfur-containing amino acids cysteine and methionine show high affinity for Pt(II). Binding of CDDP to methionine and/or cysteine residues in proteins (enzymes) may affect the functionality of these proteins (enzymes) and thus induce toxic side effects. Lempers and Reedijk [8] have studied the stability of Pt-cysteine- and Pt-methionine-like interactions in the presence of the strong nucleophiles DDTC, TS, and thiourea using the complexes formed by chloro(diethylenetriammine)platinum(II) with glutathione [Pt(dien)SG] and with S-methyl-glutathione [Pt(dien)SMeG] (Fig. 2) using nuclear magnetic resonance spectroscopy (NMR). The same group has also studied the ability of these modulating agents to restore the activity of fumarase inactivated by the platination of a methionine in the active center [2]. We used these model systems to determine the ability of WR2721, WR1065, and WR33278 to reverse Pt-protein interactions. The stability of the Pt(dien) complexes was determined using high-performance liquid chromatography (HPLC) with UV detection.

Materials and methods

Chemicals. CDDP was obtained from Bristol Myers Company (Syracuse, N.Y., USA). *cis*-Diamminediaquaplatinum(II) was prepared by incubating CDDP in diluted HNO_3 (pH 3) with 2 molar equivalents of $AgNO_3$ at room temperature in a dark environment for 2 days. The solution was acidified to prevent the formation of hydroxo-bridged polynuclear species. Precipitated AgCl(s) was removed by filtration. WR2721 and WR1065 were obtained from US Bioscience (West Conshohocken, Pa., USA). WR33278 was prepared by bubbling moisturized air through a solution of WR1065 in 10 mM phosphate buffer (pH 7.4) for 24 h. Completion of the reaction was confirmed by electrochemical measurement with a +0.4- to −1.6-V sampled direct-current scan using a PAR303 static mercury-drop electrode along with a PAR 174 potentiostat (EG & G Instruments, Westwood N.J., USA) and a BD100 strip-chart recorder (Kipp & Zonen, Delft, The Netherlands). The mercury-thiol-complex oxidation wave (-0.38 V vs Ag/AgCl) was replaced by a disulfide reduction wave (–0.55 V vs Ag/AgCl). Pt(dien)SG, Pt(dien)SMeG, pig-heart fumarase (Boehringer, Mannheim, FRG), and malic acid (Sigma, St. Louis, Mo., USA) were kindly donated by E. L. M. Lempers (Department of Inorganic Chemistry, Leiden University, The Netherlands). All other chemicals used were of analytical grade.

Fig. 2. The molecular structure of Pt(dien)SG (*left*) and Pt(dien)SMeG (*right*). *G-SH*, Glutathione

Analysis. Pt(dien)SG and Pt(dien)SMeG were quantified by HPLC with UV detection. The system consisted of a spectroflow 400 pump (Applied Biosystems, Maarssen, The Netherlands), a Valco six-port injection valve equipped with a 50-µl loop, a spectroflow 773 variable-wavelength UV/Vis detector (Kratos Analytical Instruments, Westwood N.J., USA) set at 254 nm, and a BD100 strip-chart recorder (Kipp & Zonen, Delft, The Netherlands). The complexes were retained on a partisil ODS3 column (length, 10 cm; inside diameter, 0.3 cm; 5 µm; Phenomenex London, UK). The eluent consisted of 5 mM sodium hexylsulfonate in 10 mM sodium hydrogen phosphate acidified with citric acid to pH 3.5 and 10% (v/v) MeOH. The eluent was degassed by passage through a 0.2-µm filter (Sartorius, Göttingen, FRG) and used at a flow rate of 1.0 ml/min.

Pt(dien)-complex incubations. Pt(dien)-complex (0.1 mM) was incubated for up to 24 h with a 10-fold molar excess of modulating agent in 10 mM phosphate buffer (pH 7.4) at 37° C. Incubations with WR1065 were performed under nitrogen to exclude oxidation to the disulfide. Second-order reaction rate constants (k_2 = k/[modulating agent]) and half-lives ($t_{1/2}$ = In2/k) for the reactions were obtained from the slope (k = slope × In10) of the log[Pt(dien)-complex] vs time plots calculated using the least-squares method.

Fumarase reactivation. Fumarase (10 mg/ml) was inactivated by a 40-fold dilution with 0.4 mM *cis*$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ in 0.1 M phosphate buffer (pH 7.4) at ambient temperature for 1 h. Reactivation of the fumarase by the WR compounds was performed by a 10-fold dilution of the platinated fumarase in 0.1 M phosphate buffer containing a 500-fold molar excess (20 mM) of WR2721 or one of its metabolites in relation to platinum. Reactivation with a 50-fold molar excess (2 mM) of DDTC was carried out as a positive control.

Enzyme activity. Enzyme activity was assayed using a modification of the method of Racker [11]. A substrate solution of 0.05 M malic acid in 0.1 M potassium phosphate buffer was adjusted to pH 7.4 with 0.1 M NaOH. At 1, 30, and 60 min following the addition of modulating agent, 20 µl enzyme solution (diluted 400 times) was mixed with 2.98 ml substrate solution in a 1-cm-path cuvette. The rate of formation of fumaric acid was measured at 240 nm (spectrophotometer model 25; Beckman, Mijdrecht, The Netherlands).

Results

Pt(dien)SG and Pt(dien)SMeG were retained on the solvent-generated cation-exchange column ($k' = 0.58$ and 1.31, respectively) and were easily separated from the small peak at time zero. No interfering peak was observed on the chromatograms for the simple incubation mixtures.

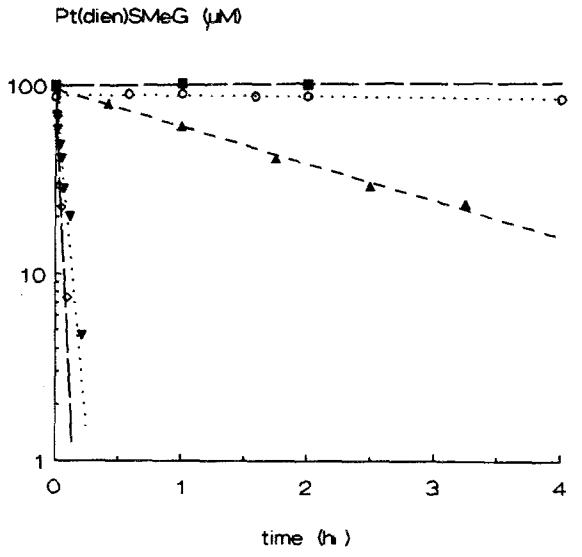

Fig. 3. The disappearance of 0.1 mM Pt(dien)SMeG following incubation with a 10-fold molar excess of modulating agent. ◇, TS; ▼, DDTC; ▲, WR1065; ○, WR2721; ■, WR33278

Quantitation by peak height showed good reproducibility [286 AU $M^{-1} \pm 3.1\%$ ($n = 4$) and 81.3 AU $M^{-1} \pm 2.3\%$ ($n = 5$), respectively].

Incubation of the Pt(dien)SG with the WR compounds failed to result in any reversal of the Pt-cysteine-like bond, even after 24 h. The strong nucleophiles DDTC and TS were also incapable of breaking this Pt-cysteine-like bond. However, the Pt-methionine-like bond in the Pt(dien)SMeG complex could be reversed by some of the modulating agents. In the presence of a 10-fold molar excess of the modulating agents, the initial disappearance of Pt(dien)SMeG exhibited pseudo-first-order kinetics (Fig. 3). Following incubation with WR1065, the rate of disappearance of Pt(dien)SMeG decreased after 4 h, probably due to the uptake of oxygen into the incubation mixture during sampling, causing part of the WR1065 to oxidize to the less reactive symmetrical disulfide WR33278. Therefore, only the measurements recorded during the first 4 h were used for the calculations and shown in the figure. WR1065 reversed the Pt-methionine-like bond of the Pt(dien)SMeG complex, but the reversal was slow ($k_2 = 0.142\ M^{-1}\ s^{-1}$) as compared with that obtained using the strong nucleophiles TS ($k_2 = 10.1\ M^{-1}\ s^{-1}$) and DDTC ($k_2 = 3.66\ M^{-1}\ s^{-1}$). WR2721 hardly reversed the Pt-methionine-like bond, ($k_2 = 0.00529\ M^{-1}\ s^{-1}$), and WR33278 showed no ability to do so. The second-order reaction rate constants, half-lives and correlation coefficients for the log[Pt(dien)SMeG] vs time plots are presented in Table 1.

Fumarase was completely inactivated after a 1-h incubation with 0.4 mM *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$. Its activity was quickly restored by incubation with DDTC at a 50-fold molar excess in relation to platinum (61% after 60 min). However, platinated fumarase was hardly reactivated by any of the WR compounds at a 500-fold molar excess (16%, 7.7%, and 0 after 60 min for WR1065, WR2721, and WR33278, respectively; Table 2). Incubation of active (nonplatinated) fumarase with the modulating agents did not affect the activity of the enzyme.

Table 1. Second-order reaction rate constants and half-lives for the disappearance of 0.1 mM Pt(dien)SMeG following incubation with a 10-fold molar excess of several modulating agents

Modulating agent	k_2 ($M^{-1}\ s^{-1}$)	$t_{1/2}$ (min)	r^2
TS	10.1	1.15	0.995
DDTC	3.66	3.15	0.984
WR2721	0.00529	2187	0.925
WR1065	0.142	81.1	0.996
WR33278	ND	ND	ND
None	ND	ND	ND

ND, No detectable reversal in 24 h

Table 2. Reactivation of *cis*-diamminediaquaplatinum(II)-inactivated fumarase

Modulating agent	Concentration (mM)	Restored activity (%)		
		1 min	30 min	60 min
DDTC	2	35	61	61
WR2721	20	0	0	7.7
WR1065	20	7.8	9.4	16
WR33278	20	0	0	0
None	0	0	0	0

Data represent the percentages of the enzyme activity before platination

Discussion

The use of WR2721 as a modulating agent in platinum chemotherapy has been directly deduced from the radioprotective ability it has shown when given prior to irradiation [6, 12, 16, 17]. The preferential formation and uptake of its thiol metabolite WR1065 in nontumor tissues [12] was expected to result in the protection of these tissues against CDDP-induced toxic side effects through the inactivation of reactive platinum species inside the cell. Indeed, WR2721 given 30 min prior to CDDP did reduce the side effects caused by the latter without interfering with its antitumor efficacy [6, 16, 17, 18]. However, the administration of WR2721 at 30 min after CDDP treatment failed to reduce the nephrotoxicity of the Pt compound [16]. To understand the lack of DDTC-like rescue activity exhibited by this modulating agent, we studied the potential of WR2721 and its main metabolites to reverse platinum-protein interactions, which are purportedly involved in CDDP-induced nephrotoxicity [1, 3].

The inability of the WR compounds and the strong nucleophiles DDTC and TS to reverse the Pt-cysteine-like bond in Pt(dien)SG confirmed the stability of this interaction as previously observed by Lempers and Reedijk [8]. However, the Pt-methionine-like bond in Pt(dien)SMeG could be reversed by DDTC; therefore, the protective action of DDTC given a few hours after CDDP can be explained at least in part by the reversal of Pt-methionine bonds in proteins, resulting in the restoration of their

functionality. As previously found by Lempers and Reedijk [8] using NMR, this reversal was rapid when the strong nucleophiles TS and DDTC were applied. Our HPLC-UV procedure enabled the accurate measurement of the kinetics of these fast interactions. WR1065, the metabolite that is expected to be most reactive toward Pt(II) complexes and responsible for the protective actions of WR2721, reversed the Pt-methionine-like bond in Pt(dien)SMeG but showed low reactivity as compared with the strong nucleophiles TS and DDTC. WR2721, which is not expected to enter the cell in significant amounts, was hardly capable of reversing the Pt-methionine-like bond in Pt(dien)SMeG, and the symmetrical disulfide WR33278 could not do so at all. We presume that other (mixed) disulfides of WR1065 with glutathione or (protein-bound) cysteine are also incapable of reversing this Pt-methionine-like interaction.

The results obtained using the Pt(dien)SMeG model were confirmed by the fumarase assay. The reactivation of platinated fumarase by 20-mM concentrations of the WR compounds was low in comparison to that achieved by only 2 mM DDTC and decreased in the order WR1065>WR2721>WR33278. This order of reactivity corresponds to that previously found using CDDP itself [14]. The reactivation of platinated fumarase by a 50-fold molar excess of DDTC (61%) was lower than that previously observed by Boelrijk et al. (90% [2]), probably due to differences in the fumarase activity remaining after platination (no activity after 1 h in the present study vs 20% activity after 3 h in the study by Boelrijk et al.). In another investigation, we have shown that Pt-DNA adduct formation can be partly prevented by WR2721 and its main metabolites, with WR1065 again being the most active compound [15]. Therefore, we presume that WR2721 offers protection against CDDP-induced toxicities by preventing rather than reversing cellular damage.

It can thus be concluded that WR2721, its active thiol metabolite WR1065, and the symmetrical disulfide WR33278 are slow in reversing Pt-methionine interactions, in contrast to the strong nucleophile DDTC. This may explain why WR2721, as opposed to DDTC, does not provide protection against CDDP-induced nephrotoxicity by restoring protein function as a result of the reversal of Pt-methionine-like bonds following administration of this modulating agent after CDDP treatment. Although care must be taken in extrapolating in vitro model systems to either the in vivo situation or the cellular level, the results of this study explain the lack of protection obtained when WR2721 is given after CDDP and confirm the present clinical use of WR2721 prior to platinum-containing chemotherapy.

Acknowledgements. Drs. A. E. M. Boelrijk, Dr. E. L. M. Lempers, and Prof. J. Reedijk are gratefully acknowledged for their help with the fumarase assay, for the generous gift of Pt(dien) complexes, and for their helpful discussions.

References

1. Bodenner DL, Dedon PC, Keng PC, Borch RF (1986) Effect of dithiocarbamate on *cis*-diamminedichloroplatinum(II)-inducedcytotoxicity, DNA cross-linking, and y-glutamyl transpeptidase inhibition. Cancer Res 46: 2745
2. Boelrijk AEM, Boogaard PJ, Lempers ELM, Reedijk J (1991) Regeneration experiments of the platinated enzyme fumarase by sodium diethyldithiocarbamate, thiourea and sodium thiosulphate. J Inorg Biochem 41: 17
3. Borch RF, Pleasants ME (1979) Inhibition of cis-platinum nephrotoxicity by diethyldithiocarbamate in a rat model. Proc Natl Acad Sci USA 76: 6611
4. Borch RF, Katz JC, Lieder PH, Pleasants ME (1980) Effect of diethyl-dithiocarbamate on tumor response to cis-platinum in a rat model. Proc Natl Acad Sci USA 77: 5441
5. Calabro-Jones PM, Aguilera JA, Ward JF, Smoluk GD, Fahey RC (1988) Uptake of WR2721 derivatives by cells in culture: identification of the transported form of the drug. Cancer Res 48: 3634
6. Glover D, Fox KR, Weiler C, Kligerman MM, Turrisi A, Glick JH (1988) Clinical trials of WR-2721 prior to alkylating agent chemotherapy and radiotherapy. Pharmacol Ther 39: 3
7. Goel R, Cleary SM, Horton C, Kirmani S, Abramson IS, Kelly C, Howell SB (1989) Effect of sodium thiosulphate on the pharmacokinetics and toxicity of cisplatin. J Natl Cancer Inst 81: 1552
8. Lempers ELM, Reedijk J (1990) Reversibility of binding of cisplatin-methionine in proteins by diethyldithiocarbamate or thiourea: a study with model adducts. Inorg Chem 29: 217
9. Ozols RF (1989) Cisplatin dose intensity. Semin Oncol 16: 22
10. Quazi R, Chang AYC, Borch RF, Montine T, Dedon PC, Loughner J, Bennett JM (1988) Phase I clinical and pharmacokinetic study of diethyldithiocarbamate as a chemoprotector from toxic effects of cisplatin. J Natl Cancer Inst 80: 1486
11. Racker E (1950) Spectrophotometric measurement of the enzymic formation of fumaric and *cis*-acetonic acids. Biochim Biophys Acta 4: 211
12. Shaw LM, Glover D, Turrisi A, Brown DQ, Bonner HS, Norfleet AL, Weiler C, Glick JH, Kligerman MM (1988) WR2721 pharmacokinetics. Pharmacol Ther 39: 195
13. Sundquist WI, Lippard SJ (1990) The coordination chemistry of platinum anticancer drugs and related compounds with DNA. Coord Chem Rev 100: 293
14. Treskes M, Holwerda U, Klein I, Pinedo HM, Vijgh WJF van der (1991) The chemical reactivity of the modulating agent WR2721 (ethiofos) and its main metabolites with the antitumor agents cisplatin and carboplatin. Biochem Pharmacol 42: 2125
15. Treskes M, Nijtmans LGJ, Fichtinger-Schepman AMJ, Pinedo HM, Vijgh WJF van der (1992) Effects of the modulating agent WR2721 and its main metabolites on the formation and stability of cisplatin/DNA adducts in vitro. Biochem Pharmacol (in press)
16. Treskes M, Boven E, Holwerda U, Pinedo HM, Vijgh WJF van der (1992) Time dependence of the selective modulation of cisplatin-induced nephrotoxicity by WR2721 (ethiofos) in the mouse. Cancer Res (in press)
17. Wasserman TH, Phillips TL, Ross G, Kane LJ (1981) Differential protection against cytotoxic chemotherapeutic effects on bone marrow CFUs by WR-2721. Cancer Clin Trials 4: 3
18. Yuhas JM, Spellman JM, Jordan SW, Pardini MC, Afzal SMJ, Culo F (1980) Treatment of tumours with the combination of WR-2721 and *cis*-diamminedichloroplatinum(II) or cyclophosphamide. Br J Cancer 42: 574