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THE CHEMICAL REACTIVITY OF THE MODULATING AGENT WR2721 (ETHIOFOS) AND ITS MAIN METABOLITES WITH THE ANTITUMOR AGENTS CISPLATIN AND CARBOPLATIN

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Abstract—The antitumor agents cisplatin [*cis*-diamminedichloroplatinum(II), CDDP] and carboplatin [*cis*-diammine(1,1 - cyclobutanedicarboxylato)platinum(II), CBDCA] can react with a nucleophilic agent by a direct ligand exchange of the (labile) anionic ligands or through hydrolysis of these ligands followed by a fast reaction of the hydration product with the nucleophile. At pH 7.4 and 37°, CDDP and CBDCA were incubated with several molar excesses of the modulating agent WR2721, its active thiol metabolite WR1065 or the symmetrical disulphide WR33278. The reaction rate constants for the hydrolysis and the direct inactivation by the WR-compounds were obtained from the pseudo first-order disappearance of the intact Pt-drug, with or without the WR-compounds at molar ratios of 50, 100 and 200. The hydrolysis of carboplatin ($k_{aq,CBDCA} = 2 \times 10^{-8}\,M^{-1}\,sec^{-1}$) was 100-fold less rapid than that of cisplatin ($k_{aq,CDDP} = 2 \times 10^{-6}\,M^{-1}\,sec^{-1}$). However, direct inactivation by WR2721, WR1065 and WR33278 was only 4-, 4- and 22-fold less rapid for carboplatin than for cisplatin, respectively. This direct inactivation was slow compared to the strong nucleophiles thiosulphate (TS) and diethyldithiocarbamate (DDTC) and decreased for both Pt-drugs in the following order: WR1065 ($k_{WR1065/CDDP} = 49.1 \times 10^{-4}\,M^{-1}\,sec^{-1}$, $k_{WR1065/CBDCA} = 12.4 \times 10^{-4}\,M^{-1}\,sec^{-1}$) > WR2721 ($k_{WR2721/CDDP} = 25.3 \times 10^{-4}\,M^{-1}\,sec^{-1}$, $k_{WR2721/CBDCA} = 6.07 \times 10^{-4}\,M^{-1}\,sec^{-1}$) > WR33278 ($k_{WR33278/CDDP} = 8.60 \times 10^{-4}\,M^{-1}\,sec^{-1}$, $k_{WR33278/CBDCA} = 0.39 \times 10^{-4}\,M^{-1}\,sec^{-1}$). Thus for CDDP, the hydrolysis-mediated interaction with the WR-compounds contributed more to the disappearance of intact platinum antitumor agent than it did for CBDCA. Considering the relatively low reactivity of WR2721 and its main metabolites with the platinum antitumor agents, in addition to their pharmacokinetic behavior, a significant inactivation of the plantinum antitumor drugs by WR2721 and its main metabolites is, in contrast to TS, not expected in the circulation.

Cisplatin (CDDP†) and its most promising second generation analog carboplatin (CBDCA) (Fig. 1) are potent cytostatic drugs used against several types of solid tumor [1]. Their efficacy is limited by several side effects [2, 3]. A lot of effort has been put into combining them with so-called modulating agents to decrease the toxic side effects of the platinum antitumor agents without interfering with their antitumor activity [4, 5]. These modulating agents generally contain sulfur which, as a strong nucleophilic moiety, has a high affinity for platinum(II). Protection against toxic side effects allows an increase of the dose which means that due to the steep dose–response curve of platinum antitumor agents, a considerable increase in antitumor efficacy could be achieved [1].

TS is mainly successful in two-route regimens, i.e. the platinum antitumor agent is administered locally (i.a. or i.p.) to the tumor while TS is given systemically to protect the non-tumor tissues [6–9]. DDTC is given several hours after the platinum antitumor agent to selectivity reverse Pt-protein lesions responsible for (part of) the nephrotoxic side effects [10–13]. DDTC may also lower myelotoxicity by stimulation of stromal cells in the bone marrow [14]. However, clinical studies were disappointing due to toxic side effects of DDTC itself and

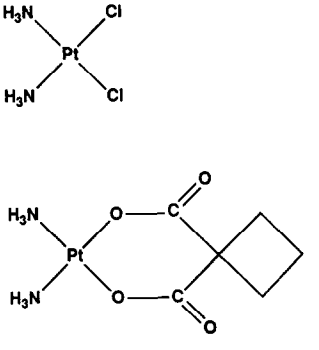

Fig. 1. Structural formulas of the antitumor agents cisplatin (top) and carboplatin (bottom).

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† Abbreviations: CDDP, *cis*-diamminedichloroplatinum(II), cisplatin; CBDCA, *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II), carboplatin; TS, thiosulphate; DDTC, diethyldithiocarbamate; WR2721, *S*-2-(3-aminopropylamino)ethylphosphoro-thioic acid, ethiofos; GSH, glutathione.

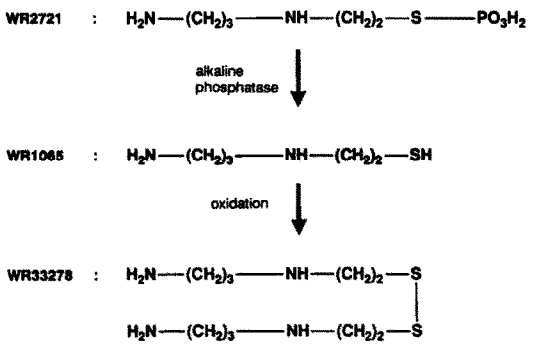

Fig. 2. Structural formulas of the modulating agent WR2721 and its conversion into the main metabolites WR1065 and WR33278.

insufficient protection against CBDCA-induced myelotoxicity [15, 16].

WR2721 was developed as a radioprotecting agent [17]. When administered prior to radiotherapy it selectively protects non-tumor tissue against radiation-induced damage. This selectivity may be the result of the preferential formation and uptake of the dephosphorylated thiol metabolite WR1065 in non-tumor tissues [18, 19]. WR1065 can be oxidized, forming mixed disulphides with endogenous thiols or the symmetrical disulphide WR33278 with a second molecule of WR1065 (Fig. 2). Due to the nucleophilic nature of the thiol WR1065 and its preferential accumulation in non-tumor tissue, WR2721 was also expected to protect selectively against the toxic side effects of platinum antitumor agents. Indeed, animal studies [20, 21] and early clinical trials [22] demonstrated this selective protection.

To gain understanding of the mechanisms behind the protective action of WR2721 and to contribute to rational administration schedules of WR2721 in combination with platinum antitumor agents, we investigated the direct inactivation of CDDP and CBDCA by the modulating agent WR2721, its thiol metabolite WR1065 and the symmetrical disulphide WR33278.

MATERIALS AND METHODS

Chemicals. CDDP and CBDCA were obtained from the Bristol Myers Co. (Syracuse, NY, U.S.A.). WR2721 and WR1065 were obtained from US Bioscience (West Conshohocken, PA, U.S.A.). WR33278 was prepared by bubbling moisturized air through a solution of WR1065 (0.1 M) in a 10 mM phosphate buffer of pH 7.4 for 24 hr. 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 with a PAR 174 potentiostat (EG&G Instruments, Nieuwegein, The Netherlands) and a strip chart recorder (model BD100, Kipp & Zonen, Delft, The Netherlands). The oxidation wave of the thiol–mercury complex (−0.38V vs Ag/AgCl) was replaced by the reduction wave of the disulphide (−0.55V vs Ag/AgCl). Sodium thiosulphate (Ph. Eur. grade) was purchased from Brocacef b.v. (Maarssen, The Netherlands). All other chemicals used were of analytical grade.

Analysis. CDDP and CBDCA were quantitated by HPLC with UV detection. the HPLC system consisted of a Spectroflow 400 solvent delivery system (Separations Analytical Instruments, N.I. Ambacht, The Netherlands), a Valco six port injection valve equipped with a 50 μL loop, a Spectroflow 773 variable wavelength UV/Vis detector (Separations Analytical Instruments) set at 213 nm and a strip chart recorder (model BD100, Kipp & Zonen). The platinum drugs were retained on a 10 × 46 cm i.d. stainless steel column (Analytica b.v., Rijswijk, The Netherlands) slurry-packed with MCI gel-CDR10 from Mitsubishi Chemical Industries (Analytica b.v.) [23]. NH_4Cl, 0.1 M, pH 6.0 was degassed by passage through a 0.2 μm membrane-filter (Sartorius, Breukelen, The Netherlands) and used as the eluent at a flow rate of 1.0 mL/min. The separation was carried out at 40°.

Incubations. CDDP or CBDCA (0.1 mM) was incubated with an excess of WR2721, WR1065 or WR33278 in 10 mM phosphate buffer, pH 7.4 at 37°. WR-compounds were present at molar ratios of 0, 50, 100 and 200. To compare with existing literature [24, 25], the rate for the direct interaction between TS and CDDP or CBDCA was also determined with our system under the above mentioned conditions.

Kinetics. The Pt-drug can react with the modulating agent by a direct interaction or through a rate-limiting hydrolysis step followed by a rapid reaction of the hydrolysis species with the modulating agent. Rate constants were determined for the disappearance of the intact Pt-drug in the presence of 5, 10 and 20 mM WR-compound (a molar excess of 50, 100 and 200, respectively). The rate of hydrolysis was measured by incubating the platinum antitumor agent (0.1 mM) in the same buffer without modulating agent. With H_2O and the modulating agent being present at a molar excess ≥50, pseudo first-order kinetics are expected for the disappearance of the intact Pt-drug. From the log[Pt-drug] vs time plot the reaction rate k_{obs} (= −slope × 2.3) is obtained. Plotting k_{obs} against the concentration of the modulating agent [MA], a linear relationship ($k_{obs} = k_{aq}[H_2O] + k_2[MA]$) is expected with an intercept equal to $k_{aq} \times [H_2O]$ (in which $[H_2O]$ = 55.5 M) and a slope representing the second-order rate constant (k_2) for the direct inactivation of the Pt-drug by the modulating agent.

RESULTS

Retention of both CDDP and CBDCA on the MCI gel-CDR10 was sufficient to separate them from interfering components in the (relatively simple) incubation matrix.

The disappearance of both platinum antitumor agents obeyed pseudo first-order kinetics in all incubations. As an example, Fig. 3 shows the log[Pt-drug] vs time plots for CDDP and CBDCA (0.1 mM) incubated with 10 mM of each modulating agent. In Table 1, the pseudo first-order reaction rates and the corresponding half-life times ($T_{1/2} = \ln 2/k_{obs}$)

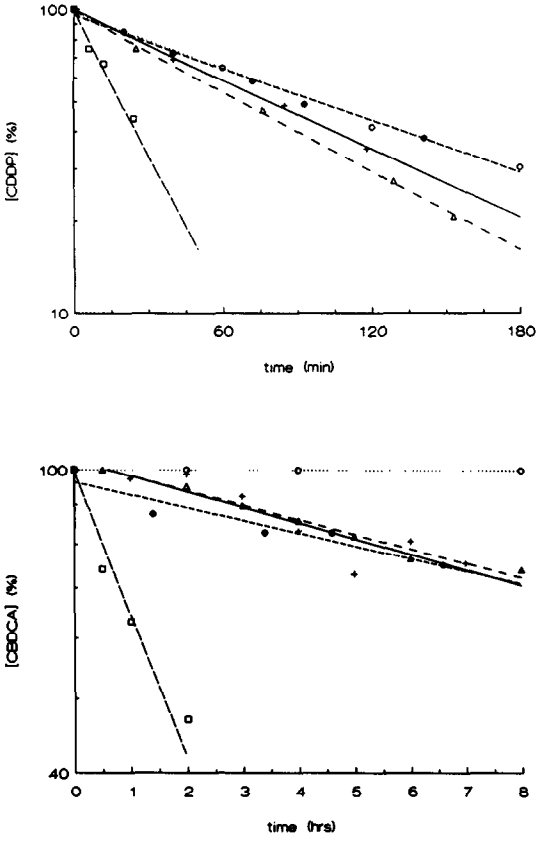

Fig. 3. Log[Pt-drug] vs time curves for CDDP (0.1 mM, top) and CBDCA (0.1 mM, bottom) when incubated alone (○) or with a 10 mM of WR2721 (+), WR1065 (△), WR33278 (●) or TS (□).

are presented as well as the coefficients of determination for the log[Pt-drug] vs time plots calculated by the linear least squares method. In contrast to CDDP, no measurable hydrolysis of CBDCA could be observed within 8 hr. For both platinum antitumor agents there was an increase in the disappearance of parent drug when incubated with TS or the WR-compounds, demonstrating a direct interaction of the modulating agents with the platinum antitumor agents. When k_{obs} was plotted against the modulating agent concentration, an excellent linear relationship was obtained for each modulating agent with both platinum drugs. The reaction rate constants k_{aq} and k_2 are presented in Table 2 together with the coefficients of determination for the linear k_{obs} vs [WR-compound] plots calculated by the least squares method. The hydrolysis of CBDCA, calculated from the intercept, was 100-fold slower than the hydrolysis of CDDP. The direct interaction of all modulating agents with CDDP was also faster than with CBDCA. However, the direct interaction by WR2721, WR1065, WR33278 and TS was only 4-, 4-, 22- and 7-fold less rapid for CBDCA than for CDDP, respectively. For both platinum antitumor agents, the rate of the direct interaction decreased in the following order: TS ⩾ WR1065 > WR2721 > WR33278.

DISCUSSION

WR2721 is one of the modulators presently under investigation to prevent the toxic side effects of CDDP and CBDCA. It differs from TS and DDTC by the preferential formation and uptake of its main metabolite WR1065 by non-tumor tissues. With WR2721, the only mechanistic studies performed concerned the metabolism and uptake of WR2721 by tumor and non-tumor tissues *in vivo* and *in vitro*,

Table 1. Observed reaction rates (k_{obs}) and half-life times ($T_{1/2}$) of the pseudo first-order disappearance of CDDP and CBDCA (0.1 mM) in the presence of TS, WR2721 and its metabolites WR1065 and WR33278 at several concentrations

		CDDP			CBDCA		
Compound	Concentration (mM)	$k_{obs} \times 10^5$ (sec^{-1})	$T_{1/2}$ (hr)	r^{2*}	$k_{obs} \times 10^5$ (sec^{-1})	$T_{1/2}$ (hr)	r^{2*}
WR2721	5	13.4	1.44	0.995	0.319	60.3	0.817
	10	14.7	1.31	0.999	0.658	29.2	0.920
	20	17.2	1.12	0.995	1.24	15.6	0.972
WR1065	5	14.0	1.38	0.999	0.733	26.3	0.948
	10	16.9	1.14	0.999	1.30	14.8	0.978
	20	21.4	0.898	0.999	2.58	7.45	0.997
WR33278	5	10.1	1.92	0.996	0.365	52.9	0.939
	10	11.0	1.75	0.996	0.585	33.0	0.965
	20	11.5	1.68	0.994	0.950	20.2	0.992
TS	5	44.9	0.428	0.998	3.97	4.85	0.985
	10	66.4	0.290	0.990	8.14	2.37	0.988
	20	108	0.179	0.999	16.4	1.17	0.991
Control	—	9.25	2.08	0.996	0	—	—

* The coefficient of determination for the log[Pt-drug] vs time plots.

Table 2. The second-order rate constants for the hydrolysis (k_{aq}) and the direct interaction (k_2) of CDDP and CBDCA obtained from the k_{obs} vs [modulator] plots

	CDDP			CBDCA		
Compound	$k_{aq} \times 10^6$ ($M^{-1} sec^{-1}$)	$k_2 \times 10^4$ ($M^{-1} sec^{-1}$)	r^{2*}	$k_{aq} \times 10^8$ ($M^{-1} sec^{-1}$)	$k_2 \times 10^4$ ($M^{-1} sec^{-1}$)	r^{2*}
WR2721	2.19	25.3	0.998	0.55	6.07	0.999
WR1065	2.11	49.1	1.00	1.68	12.4	1.00
WR33278	1.78	8.60	0.923	3.23	0.39	0.999
TS	4.40	416	1.00	0.0	82.4	1.00

* The coefficient of determination for the k_{obs} vs [WR-compound] plots.

mostly related to their radiation protection [6–8, 26–28], and its influence on the structure of DNA [27]. To contribute to an understanding of the actions of WR2721 in combination with platinum antitumor agents and a rational design of treatment schedules, we studied the direct chemical interaction of WR2721 and its main metabolites with CDDP and CBDCA.

CBDCA with its bidentate leaving ligand is less reactive than CDDP. With WR1065, WR2721 and WR33278 the second-order rate constants differ by a factor of 4, 4 and 22, respectively, while the rate of hydrolysis differs by a factor of 100 (Table 2). Thus for CDDP, the hydrolysis-mediated interaction with the WR-compounds contributed more to the disappearance of the intact platinum antitumor agent than it did for CBDCA. The second-order rate constant for the hydrolysis of CDDP ($167 \times 10^{-8} M^{-1} sec^{-1}$), as calculated from the observed hydrolysis, is in good agreement with the mean second-order rate constant for the hydrolysis of CDDP calculated from the intercept in the k_{obs} vs [modulator] plots ($188 (\pm 35) \times 10^{-8} M^{-1} sec^{-1}$, Table 2) and with earlier findings ($141 \times 10^{-8} M^{-1} sec^{-1}$, [24]). The slightly faster hydrolysis rate we observed might be caused by a direct interaction of CDDP with the weak phosphate ligand (10 mM), which was not taken into account. This assumption is supported by the even faster hydrolysis rate observed in a 50 mM phosphate buffer ($198 \times 10^{-8} M^{-1} sec^{-1}$, [25]). For CBDCA, no significant hydrolysis was observed within 8 hr. The mean second-order rate constant for the hydrolysis of CBDCA calculated from the k_{obs} vs [WR-compound] plots ($1.82 (\pm 1.35) \times 10^{-8} M^{-1} sec^{-1}$) was about 100 times lower than that of CDDP, which is also in good agreement with earlier findings [25]. Variations in the hydrolysis rate constants, as calculated from the intercepts, are rather large. This may be expected for linear fits with large slopes and small intercepts. This uncertainty was very pronounced in the case of the steep k_{obs} vs [TS] plot for CBDCA; this intercept has not, therefore, been taken into account.

The second-order reaction rate constants of CDDP and CBDCA with the WR-compounds are low compared to the modulating agent TS ($k_{CDDP/TS} = 577 \times 10^{-4} M^{-1} sec^{-1}$, $k_{CBDCA/TS} = 82.4 \times 10^{-4} M^{-1} sec^{-1}$). These second-order reaction rate constants for TS are in good agreement with previous findings [24, 25]. Therefore, it seems possible to correlate our results to the results from these studies with other physiologically important thiols [24]. Thus, it can also be concluded that the WR-compounds react slowly with CDDP and CBDCA, when compared to DDTC ($k_{CDDP/DDTC} = 614 \times 10^{-4} M^{-1} sec^{-1}$, $k_{CBDCA/DDTC} = 76.2 \times 10^{-4} M^{-1} sec^{-1}$, [24]).

WR1065, which is considered to be the protective species, is the most reactive WR-compound towards CDDP and CBDCA. In mice treated with WR2721, tissue levels of WR1065 are maximal in the mM range after 5–15 min and WR1065 is subsequently cleared rapidly from most tissues [26]. GSH, which reacts at a faster/similar rate with CDDP/CBDCA ($k_{CDDP/GSH} = 132 \times 10^{-4} M^{-1} sec^{-1}$/$k_{CBDCA/GSH} = 9.15 \times 10^{-4} M^{-1} sec^{-1}$, [14]) compared to WR1065, is present inside the cell in the millimolar range [26]. Therefore, WR1065 is not expected to add much to the cytoplasmic inactivation of CDDP (and maybe hydrolysed species) by GSH. Because WR1065, in contrast to GSH, is concentrated near DNA [27], the inactivation of reactive Pt-species near the DNA could explain the protective action of WR2721 [20–22] if toxic side effects are, at least in part, the result of damage to the DNA. If this is the case, then the selective protection of non-tumour tissues by WR2721 can be understood. Furthermore, a relative lack of formation and uptake of WR1065 by the tumour is then expected to be crucial for the selectivity of protection.

In the clinic, WR2721 is given 30 min prior to the platinum drug as a 15 min infusion to allow (preferentially) non-tumor tissues to accumulate protective WR1065 before exposing the body to the platinum antitumor agent. WR2721 (740 mg/m^2, 15 min infusion) attains rapidly steady state plasma levels in the 0.1 mM range and it is cleared rapidly from plasma ($T_{1/2} < 5$ min, [19]). In a patient receiving 750 mg/m^2 WR2721 as five repeated injections over 15 min, WR2721 fluctuated around 0.3 mM during the first 15 min and decreased rapidly thereafter. WR1065 plasma levels increased over 15 min to 0.1 mM and decreased slowly thereafter, probably due to a sustained release of WR1065 from a pool of mixed disulphides [28]. Plasma levels of (mixed) disulphides have not been reported yet. The low reactivity of WR33278 may be expected to be representative of the reactivity of mixed disulphides of WR1065 (e.g. with free or even protein-bound cystein).

CDDP is cleared rapidly from the circulation by

renal excretion and protein binding ($T_{1/2\beta}$ = 31.6 min, $k = 3.66 \times 10^{-4}\,sec^{-1}$, [29]). CBDCA is mainly cleared by renal filtration ($T_{1/2\beta}$ = 120 min, $k = 9.63 \times 10^{-5}\,sec^{-1}$, [30]). Even with 0.1 mM of the thiol metabolite WR1065 constantly present in plasma, the plasma half-life times (and with first-order kinetics, also the AUCs) of CDDP and CBDCA would decrease by less than 1%. Therefore, a noticeable inactivation of intact Pt-drug by WR2721 or one of its metabolites is not expected in the circulation even when the Pt-drug and WR2721 are administered at the same time. This is in contrast to the simultaneous administration of CDDP with TS which was shown to inactivate a substantial part of the platinum drug [7–9]. This is in accordance with the calculated 19% decrease in $T_{1/2\beta}$ (and AUC for first-order kinetics) of CDDP at a steady state plasma level of 1.5 mM TS [7] ($k_{CDDP/TS} = 577 \times 10^{-4}\,M^{-1}\,sec^{-1}$).

The administration of 4 g/m^2 DDTC 45 min after 100 mg/m^2 CDDP did not change the total and ultrafilterable Pt kinetics [31]. Using the second-order rate constant ($614 \times 10^{-4}\,M^{-1}\,sec^{-1}$, [24]) and the mean plasma DDTC level of 0.466 mM [31], $T_{1/2\beta}$ of CDDP in the circulation is expected to decrease by 7%. This might have been shown by an increase in (inactive) ultrafiltrate Pt and a subsequent decrease in protein-bound Pt. This was not observed [31] but interpatient variability may have obscured the small difference in $T_{1/2\beta}$. Because the infusion of DDTC starts 45 min after finishing the CDDP infusion [31], the effect of DDTC on the total AUC of CDDP will be much smaller than the estimated 7% reduction in $T_{1/2\beta}$.

Considering (a) the expected low level of Pt-drug inactivation in the circulation and (b) the rapid uptake and subsequent rapid decrease of WR1065 in non-tumor tissues, the question arises as to whether the protection of non-tumor tissues by WR2721 prior to CDDP, without interfering with antitumor activity, can be improved by administering WR2721 close to or even concomitantly with the platinum antitumor agent. To test this hypothesis we are presently studying the effect of WR2721 on the toxicities and antitumor activities of CDDP and CBDCA when administered shortly before the Pt-drug.

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