Biochemical Pharmacology, Vol. 41, No. 10, pp. 1429–1437, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

THE MECHANISM OF INTERACTION BETWEEN CISPLATIN AND SELENITE

GLENN S. BALDEW,*† JOHANNES G. J. MOL,* FRANS J. J. DE KANTER,‡
BEN VAN BAAR,‡ JEROEN J. M. DE GOEIJ† and NICO P. E. VERMEULEN*§

*Department of Pharmacochemistry, Division of Molecular Toxicology, Free University, De Boelelaan 1083, 1081 HV Amsterdam; †Interfaculty Reactor Institute, Delft University of Technology, Mekelweg 15, 2629 JB Delft; and ‡Department of Organic Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

(*Received* 10 *September* 1990; *accepted* 20 *November* 1990)

Abstract—Cisplatin is a widely used antitumor drug, highly effective in the treatment of several tumors. Cisplatin exerts its antitumor activity through an interaction with DNA, which results in the formation of bidentate adducts. An important side-effect of cisplatin is nephrotoxicity.

Selenite can reduce the nephrotoxicity of cisplatin without reducing the antitumor activity of the drug. We have studied the mechanism of selenite protection against cisplatin-induced nephrotoxicity. The protection correlates with higher levels of selenium in the kidney (about eight times) and with higher levels of glutathione in the kidney, both compared to tumors. Selenite is metabolized into selenols, specifically into methylselenol and glutathionylselenol; this bioactivation of selenite into selenols is a glutathione-dependent process. HPLC with on-line radioactivity detection of ^{195m}Pt showed that methylselenol is capable of forming a complex with cisplatin *in vitro*. ^1H-NMR gave evidence that the complex contains one or more Pt—Se—CH_3 bonds. Attempts to obtain further structural information by Desorption Chemical Ionization and Fast Atom Bombardment mass-spectrometry failed. It is proposed that the formation of a cisplatin–selenol complex also takes place *in vivo*, especially in the kidney, thereby preventing cisplatin exerting its nephrotoxic activity.

The biological activities of the cytostatic drug cisplatin [cis-diamminedichloroplatinum(II)] are governed by chemical reactions between cisplatin and various biological nucleophiles. The antitumor activity of cisplatin results from bifunctional platinum adducts with DNA [1, 2], a reaction probably dependent on the initial formation of reactive aquated platinum(II) complexes [3]. The binding of platinum to protein-sulfhydryl groups is thought to be responsible for the observed nephrotoxicity of cisplatin. Platinum complexes have been shown to react with methionine and cysteine-residues in human α_2-macroglobulin [4]. It has also been demonstrated that glutathione (GSH) plays a role in the resistance of normal and tumor cells to cisplatin [5]. Several sulphur-based compounds have been used to reduce the side-effects such as nephrotoxicity of cisplatin. Nucleophilic substitution reactions of chloride ions by thiols have been proposed to be responsible for the protective effects of diethyldithiocarbamate (DDTC) [3], *S*-2-(3-aminopropylamino)-ethylphosphorothioic acid (WR-2721) [6], and sodium thiosulfate [7] against cisplatin-induced nephrotoxicity. These examples have demonstrated that the toxicity of cisplatin can be manipulated by chemicals which are capable of reaction with cisplatin.

We have demonstrated that administration of sodium selenite 1 hr before cisplatin reduces the nephrotoxicity but not the antitumor activity of cisplatin in mice [8]. The mechanism of the selective protection by selenite is presently unknown. Insight into the molecular mechanism of this protective effect of selenite is required for the design of optimal clinical dosage schedules and modes of administration of selenite. The biological activity of selenite is probably governed by its metabolism. As shown in Fig. 1, selenite is metabolized to hydrogen selenide

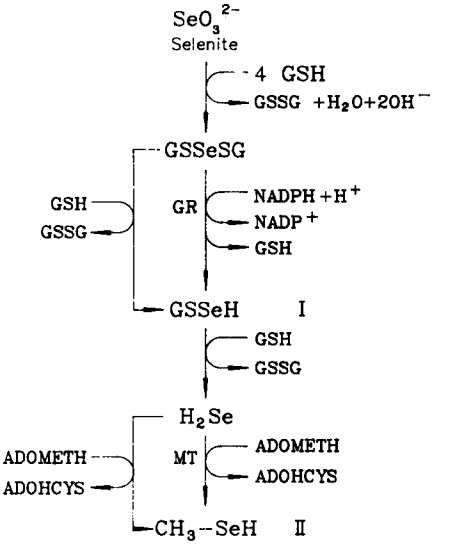

Fig. 1. Metabolism of selenite. Selenite reacts with GSH to bis(glutathione)selenide (GSSeSG) which is subsequently converted to glutathionylselenol (GSSeH) (I), hydrogen selenide (H_2Se) and methylselenol (CH_3SeH) (II). GR = glutathione reductase, MT = methyl transferases, ADOMETH = *S*-adenosylmethionine, ADOHCYS = *S*-adenosylhomocysteine.

§ To whom correspondence should be addressed.

(H_2Se) via bis(glutathione)selenide (GSSeSG). Ganther [9] has postulated that glutathionylselenol (GSSeH) is an intermediate in a reaction between bis(glutathione)selenide (GSSeSG) and GSH. Similar reactions may also take place with other biologically important thiols, such as cysteine (CYS). Hydrogen selenide is sequentially methylated to dimethylselenide and trimethylselenonium via methylselenol (CH_3SeH) [10, 11]. We note that selenols are nucleophilic agents and that as such they play an important role in the protective effect of selenite against cisplatin-induced nephrotoxicity.

The aim of this study was to gain insight into the mechanism of the protective effect of selenite against cisplatin-induced nephrotoxicity. To this end we have studied whether thiol-mediated degradation products of selenite are capable of reacting with cisplatin *in vitro*. The fact that selenite reduces the nephrotoxicity of cisplatin in rodents without affecting the antitumor activity of the drug, might be due to a preferential, thiol-mediated, activation of selenite in kidney cells compared to tumor cells. To test this hypothesis we have also studied the distribution of selenite and GSH in kidneys and tumors of mice.

METHODS

Chemicals. Methylselenol (CH_3SeH) was obtained from Janssen Chimica (Beerse, Belgium). Zinc selenide (ZnSe) was obtained from Johnson Matthey GmbH (Karlsruhe, F.R.G.). Glutathione was obtained from the Sigma Chemical Co. (St Louis, U.S.A.). Sodium selenite, $Na_2SeO_3 \cdot 5H_2O$ p.a., was purchased from Merck (Darmstadt, F.R.G.). Selenous acid, H_2SeO_3 p.a., was purchased from Fluka Chemie AG (Buchs, Switzerland). [^{75}Se]Selenite with a specific activity of 3 GBq/mg selenium was obtained from Dupont de Nemours (Dreieich, F.R.G.). Chemicals for preparing the mobile phase of the chromatographic system were of HPLC grade, all other chemicals used were of analytical grade.

Synthesis of platinum- and selenium-compounds. [^{195m}Pt]Cisplatin with a specific activity of 15 MBq/mg cisplatin, was synthesized as follows: platinum metal, isotopically enriched to 80% ^{194}Pt (Intersales Holland, Hengelo, The Netherlands) was irradiated in a thermal neutron flux of 2.0×10^{14} cm^{-2} sec^{-1} for 168 hr. The resulting ^{195m}Pt was converted to [^{195m}Pt]cisplatin, and the material was tested, according to the procedures described previously [12, 13]. The purity was more than 98%. [^{15}N]Cisplatin was synthesized, using [^{15}N]ammonia (Amersham, Houten, The Netherlands).

Bis(glutathione)selenide (GSSeSG) was synthesized as follows: 1 mM sodium [^{75}Se]selenite was incubated with 4 mM GSH in distilled water at 4° for 30 min. The reaction product was purified with preparative high-performance liquid chromatography (HPLC). Peak fractions were collected, freeze-dried and stored at 4°. Under these conditions the compound was stable for several months. The purity was determined with UV-spectroscopy [14] and ^{75}Se radioactivity measurements and was more than 98%.

HPLC analyses. HPLC analyses were performed on a reversed-phase system, as described previously [12, 15]. Briefly, 20 μL of the incubation mixture was injected on a 100 × 3.0 mm Sherisorb ODS column and eluted at a flow rate of 0.25 mL/min with a gradient from A to B, using the following two mobile phases: (A) 5 mM sodium dodecyl sulphate (SDS), 10 mM sodium phosphate buffer, pH 2.6; (B) 5 mM SDS, 25% 2-propanol, 60 mM sodium phosphate buffer, pH 2.6. Detection was performed with an on-line radioactivity detector and an UV detector (206 nm). Preparative HPLC was performed on a 250 × 22 mm Rsil column, eluted with the mobile phases A and B at a flow rate of 3.5 mL/min.

NMR analyses. All NMR spectra were recorded on a Bruker MSL 400 spectrometer. For ^{77}Se-NMR tubes of 10 mm outer diameter and for ^{1}H-NMR tubes of 5 mm were used. NMR spectra of incubations with methylselenol were recorded in sealed tubes. ^{77}Se-NMR chemical shifts were measured relative to dimethylselenide. Reference for ^{1}H-NMR was tetramethylsilane.

Mass-spectrometric analyses. Mass spectra were recorded on a MAT 90 reversed geometry magnetic sector instrument. Ammonia and isobutane were used as reagent gasses in DCI (direct chemical ionization) experiments, at 150 eV electron energy and at an indicated source pressure of 10^{-4} Torr. In FAB (Fast Atom Bombardment) experiments Xe was used in an Ion Tech saddle field gun, operating at 8 keV and 0.2 mA. Positive and negative ion FAB mass spectra were obtained using a variety of matrix compounds: glycerol, thioglycerol, 2-nitrophenyl-octyl-ether, 3-nitrobenzylalcohol and triethanolamine. FAB experiments were conducted using stainless steel and platinum probe tips.

pH measurements. All pH measurements were performed at 25°. The pH-meter was calibrated with standard buffer solutions of pH 4.00, 7.00 and 10.00 (Merck, Darmstadt, F.R.G.). The pH of deuterated solutions was corrected for deuterium-isotope effects with the equation: pD = pH + 0.40 [16].

Reactions between selenite and thiols. Sodium [^{75}Se]selenite (50 mM) was incubated in distilled water with the thiols L-cysteine and glutathione in a selenite-to-thiol molar ratio of 1:4 at 4° for 30 min. For trapping experiments, sodium [^{75}Se]selenite, 1 mM, was incubated with L-cysteine or glutathione, 100 mM, at 25° in 0.25 M potassium phosphate buffer, pH 7.0. After 90 min, iodoacetic acid (final concentration 0.5 M) was added and the mixture was incubated at 25° for 30 min. Reaction mixtures were analysed by HPLC with on-line ^{75}Se-radioactivity detection [15]. Similar incubations with non-radioactive selenite were used for ^{77}Se-NMR and attempted MS (mass-spectrometric) analyses.

Reactions between metabolites of selenite and cisplatin. Methylselenol was incubated with [^{195m}Pt]cisplatin in distilled water during 24 hr. Incubations of 1 mM cisplatin were performed with several concentrations of methylselenol: 0.1, 1, 2 and 10 mM, at several pH values: 1, 2, 3, 4, 5, 6, and 7. The pH was adjusted with HCl. Incubations were also performed at pH 7.4, in a Tris/HCl buffer, containing 80 mM NaCl. Incubations were performed

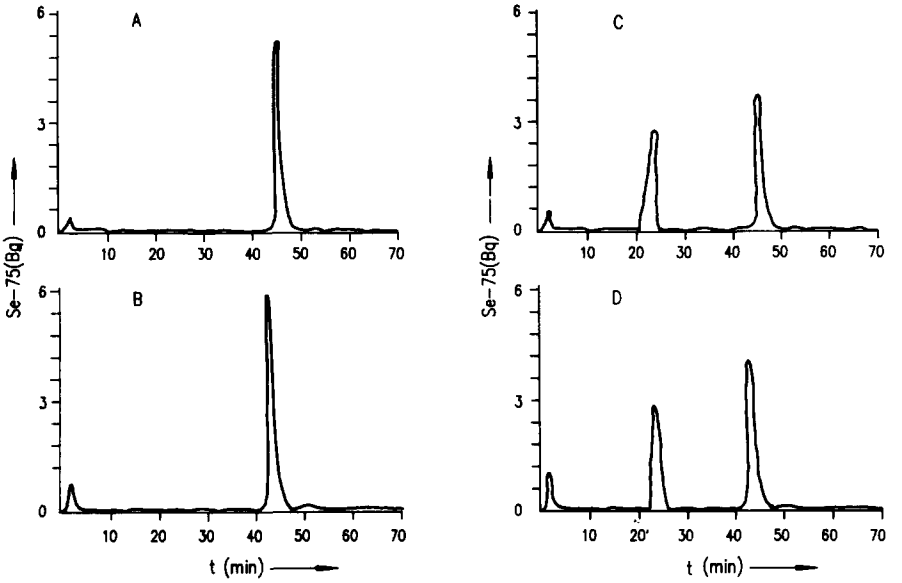

Fig. 2. HPLC monitoring of the reactions between [^{75}Se]selenite and thiols. A and B: incubations were performed at a molar ratio of 1:4 (selenite:thiol), (A) CYS, (B) GSH. C and D: trapping-experiments with iodoacetic acid, molar ratio = 1:100:500 (selenite:thiol:iodoacetic acid), (C) CYS, (D) GSH.

at several temperatures: 4°, 25° and 37°. Incubations of cisplatin with methylselenol were performed in sealed NMR-tubes at 37°.

[^{195m}Pt]Cisplatin, 1 mM, was also incubated with sodium [^{75}Se]selenite, with [^{75}Se]bis(glutathione)-selenide (GSSeSG) or zinc selenide (ZnSe) in cisplatin-to-selenium molar ratios of 1:1 and 1:10 at 37° and several pH values: 2, 3, 5 and 7.4. At several timepoints an aliquot of the incubation-mixture was collected and analysed by HPLC with on-line ^{195m}Pt-radioactivity detection (detection-limit: 3.3×10^{-8} M platinum) and by HPLC with on-line ^{75}Se-radioactivity detection (detection limit = 6.2×10^{-10} M selenium).

Laboratory animals. Female BALB/c mice were obtained from the Central Institute for the Breeding of Laboratory Animals/Harlan Sprague–Dawley (CPB/HSD), Zeist, The Netherlands. The mice were 8 weeks of age and weighed 18–20 g at the start of the experiments. All animals were provided with standard laboratory food (SRMA chow, Hope Farms, Woerden, The Netherlands) and water *ad lib.*

Tumors. Transplantable Prima breast tumor cells were obtained from The Radiobiological Institute TNO, Rijswijk, The Netherlands. The Prima tumor originated as a breast carcinoma, induced by forced breeding in BALB/c mice bearing murine mammary tumor virus. The Prima tumor cell-line was cultured *in vitro* in standard Dulbecco's modification of Minimal Essential Medium (Gibco, Paisley, U.K.), supplemented with L-glutamine (500 mg/L), 2-mercapto-ethanol (60 μmol/L) and 10% fetal calf serum (Sera-Lab Ltd, Sussex, U.K.).

Distribution studies. BALB/c mice were inoculated with 0.5×10^6 Prima breast tumor cells s.c. in the left thigh (Day 0), and used for distribution studies when the tumors were greater than 0.5 g (Day 8). Mice were treated with [^{195m}Pt]cisplatin and sodium [^{75}Se]selenite. At several timepoints, the animals were killed and tumors and kidneys were removed and weighed; ^{195m}Pt- and ^{75}Se-radioactivity and GSH-levels were determined. The following groups (N = 3) were included in the experiment. Group I: sodium [^{75}Se]selenite, 25 μmol/kg, administered in 0.5 mL physiological saline. Group II: [^{195m}Pt]cisplatin, 45 μmol/kg i.p., administered in 1 mL physiological saline. Group III: sodium [^{75}Se]selenite, 25 μmol/kg i.p. and [^{195m}Pt]cisplatin, 45 μmol/kg, 1 hr later. Control groups were treated with the vehicle. Timepoints of measurements: 30, 65, 75, 90 and 120 min after the first injection. Radioactivity measurements were performed on a gamma scintillation counter. GSH in tissue homogenates was determined according to Sedlak and Lindsay [17].

Statistics. Student's *t*-test, unpaired, was used to evaluate the significance of differences between experimental groups. The level of significance was set at $P < 0.05$.

RESULTS

Reactions between selenite and thiols

CYS and GSH were chosen as model compounds to study the reactivity of selenite towards thiols. As shown in the HPLC chromatogram of Fig. 2A, only one major ^{75}Se-containing product was formed when [^{75}Se]selenite was incubated with CYS in a selenite-to-thiol molar ratio of 1:4. Similarly, with GSH also one major ^{75}Se-containing product was observed (Fig. 2B). We have used ^{77}Se-NMR to characterize the products. In both incubations, only one ^{77}Se-resonance was found, namely a singlet at 682 ppm

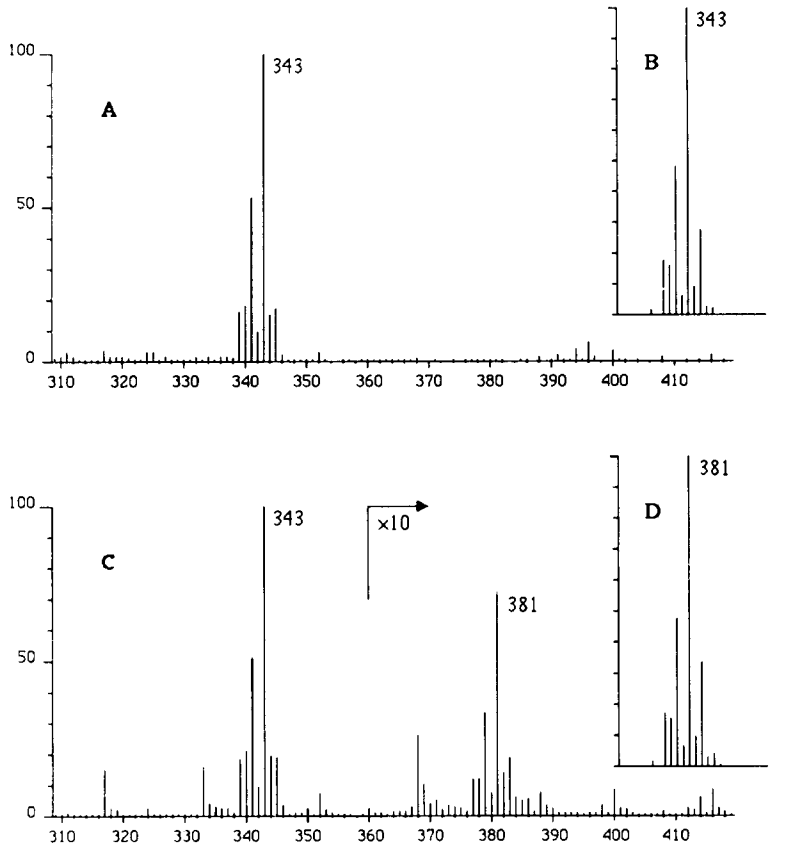

Fig. 3. (A) DCI mass spectrum (NH_3, positive ions) of an incubation of sodium selenite and CYS in water in a molar ratio of 1:4 (selenite:CYS); (B) calculated isotope pattern for $C_6H_{12}N_2O_4S_2SeNa$; (C) as (A) but after addition of KCl; (D) calculated isotope pattern for $C_6H_{11}N_2O_4S_2SeKNa$. The cluster of ions at m/z 343 (A and C) corresponds to the calculated pattern of a (CI protonated) compound with the chemical composition of monosodium bis(L-cysteine)selenide (NaCYSSeCYS). Upon the addition of KCl to the solution an isotope pattern, which corresponds to monopotassium, monosodium bis(L-cysteine)selenide (KNaCYSSeCYS), was observed at m/z 381.

at pH 1.82 for the CYS-incubation and a singlet at 670 ppm, at pH 3.84 for the GSH-incubation. These chemical shifts correspond well with those recently reported for bis(L-cysteine)selenide (CYSSeCYS), 686.74 ppm at pH 2.41, and bis(L-glutathione) selenide (GSSeSG), 673.6 ppm at pH 4.40 [18].

The reaction products were also characterized with DCI-mass spectrometry. Under positive ion conditions and using NH_3 as the reagent gas, 1 μL of the Na_2SeO_3/CYS incubation produces an isotope cluster around m/z 343 with a characteristic Se pattern (Fig. 3A); the isotope pattern corresponds to the pattern which is theoretically calculated for monosodium bis(L-cysteine)selenide (NaCYSSeCYS) (Fig. 3B). After the addition of KCl to the incubation an isotope pattern also occurs at m/z 381 (Fig. 3C); this pattern corresponds to the theoretically calculated pattern for protonated monopotassium, monosodium bis(L-cysteine)selenide (KNaCYSSeCYS) (Fig. 3D). DCI mass spectrometry of incubations of L-cysteine and selenous acid, H_2SeO_3, did not result in the observation of the corresponding acid, bis(L-cysteine)-selenide (CYSSeCYS). This may be due either to instability of the selenide or to suppression of the ion production at different, i.e. low pH. All attempts to identify bis(L-cysteine)selenide or bis(glutathione)selenide (GSSeSG) by DCI and FAB mass spectrometry failed. In most FAB experiments, either with reaction mixtures or with the purified compound, glutathione ions ($[GSH + H]^+$, m/z 308; $[GSH - H]^-$, m/z 306) and diglutathione ions $[GSSG + H]^+$, m/z 613; $[GSSG - H]^-$, m/z 611) were observed. Because of the expected structural similarity between diglutathione (GSSG) and bis(glutathione)selenide (GSSeSG) negative results for FAB mass spectrometry of bis(glutathione)selenide may be due to instability of the selenide towards matrix molecules. Negative results in DCI experiments with bis(glutathione)selenide (GSSeSG) are attributed to the fact that the compound is thermolabile.

As shown in Fig. 2C, in a trapping experiment of CYS and selenite with iodoacetic acid, one new ^{75}Se peak was detected: peak 2. This peak was not observed in incubation mixtures of selenite or bis(L-

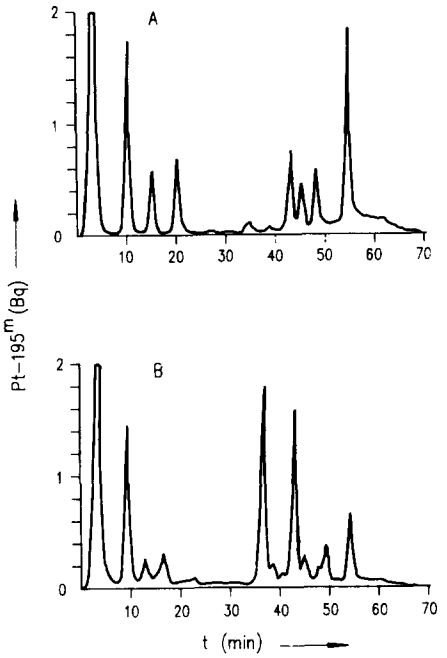

Fig. 4. HPLC monitoring of the reaction between 1 mM [^{195m}Pt]cisplatin and 10 nM methylselenol at pH 2 and 37°. Incubation time: (A) 6 hr, (B) 24 hr.

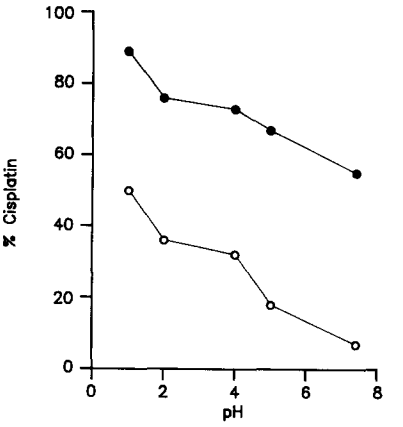

Fig. 5. pH dependency of the reaction between 1 mM [^{195m}Pt]cisplatin and 10 mM methylselenol at 37°. (●) t = 15 min, (○) t = 2 hr.

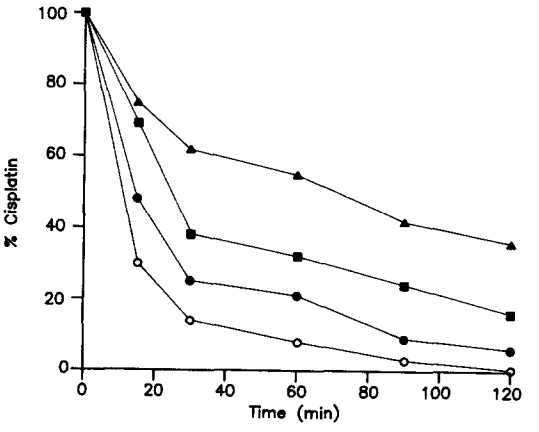

Fig. 6. Decrease of cisplatin concentration after incubation with methylselenol at several pH values and molar ratios (cisplatin: methylselenol). (▲) pH 2 (1:10), (■) pH 2 (4;10), (●) pH 7.4 (1:10), (○) pH 2 (1:40). Temperature = 37°.

cysteine)selenide (CYSSeCYS) with iodoacetic acid and could be assigned to L-cysteine, carboxymethyl-selenide ($CYSSeCH_2COOH$), the carboxymethylated derivative of cysteinylselenol (CYSSeH). Peak 2 elutes, as aspected for L-cysteine, carboxymethyl-selenide in front of bis(L-cysteine)selenide. Identification of peak 2 with ^{77}Se-NMR was not possible, due to the low sensitivity of this technique: the natural isotopic abundance of ^{77}Se is 7.5% [19] and millimolar concentrations of selenium solutions are required for ^{77}Se-NMR spectroscopy. Analoguous experiments with GSH resulted in an additional peak, which could be assigned to $GSSeCH_2COOH$ (Fig. 2D).

Reactions between metabolites of selenite and cisplatin

[^{195m}Pt]Cisplatin and [^{75}Se]selenite were incubated in distilled water, pH 2, at 37° in cisplatin-to-selenite molar ratios of 1:1 and 1:10. Incubations were monitored up till 24 hr. No reaction products were observed. All ^{195m}Pt-radioactivity was detected as unchanged cisplatin and all ^{75}Se-radioactivity was detected as unchanged selenite. No precipitation of radioactivity occurred. In mixtures of incubations, performed at pH values of 3, 5 or 7.4, all cisplatin and all selenite remained unchanged. Similar results were obtained when [^{75}Se]bis(glutathione)-selenide or zinc selenide were used instead of selenite.

The HPLC chromatogram, shown in Fig. 4, demonstrates that several reaction products were formed when methylselenol was incubated with [^{195m}Pt]cisplatin in a cisplatin-to-methylselenol molar ratio of 1:10 at pH 2.0 and 37°. In this reaction a precipitate which contained radioactive platinum was formed and the reaction mixture also contained radioactive platinum compounds. The precipitate was practically insoluble in acidic aqueous solutions, methanol, propanol, dioxane, acetone, diethylether, chloroform, dimethylsulfoxide and hexane. As shown in Fig. 5, the reaction rate increased with increasing pH, which is probably due to an increased hydrolysis of cisplatin [20] and an increased dissociation of methylselenol with increasing pH. At physiological pH (7.4) the highest reaction rate was measured, but no soluble reaction products were found. Similar results were obtained when the incubations were performed at 25° or 4° instead of 37°. Apparently, the stability and the solubility of the reaction products as well as the reactivity of the reactants are influenced by the pH (Fig. 6). The reaction rate was found to be dependent on the

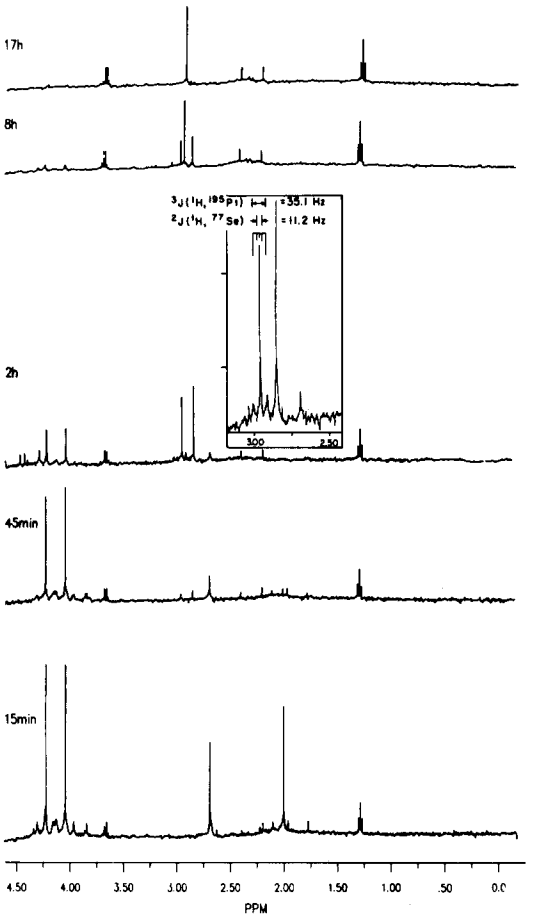

Fig. 7. Reaction between 3.5 mM cisplatin and 10.5 mM methylselenol in D_2O, pD 2.2, monitored with ^{1}H-NMR. Incubations were performed in a NMR tube at 37°.

concentration of both reactants, cisplatin and methylselenol, which is in line with our expectation of a S_N2 nucleophilic substitution of a chloride anion of cisplatin by methylselenol (CH_3SeH).

The identity of the reaction products between cisplatin and CH_3SeH was studied with ^{1}H-NMR and FAB–MS. Figure 7 shows the ^{1}H-NMR spectrum of an incubation mixture of 4 mM [^{15}N]cisplatin with 10 mM methylselenol, in D_2O, pD 2.2, performed in a NMR tube at 37°. ^{1}H-NMR resonances were observed in the range of 0 to 4.75 ppm. The intensity of the signal at 2.00 ppm, assigned to methylselenol, decreased rapidly during incubation of methylselenol with cisplatin. After 90 min the intensity of the signal was reduced to zero. This is probably caused by a reaction of methylselenol with cisplatin and by oxidation of methylselenol (CH_3SeH) to dimethyldiselenide ($CH_3SeSeCH_3$). The intensity of the peak at 2.67 ppm, assigned to dimethyldiselenide, also decreased rapidly probably as a result of a reaction of dimethyldiselenide with cisplatin, through a preceding reduction of dimethyldiselenide to methylselenol. The intensity of the signal at 4.13 ppm, assigned to cisplatin, also decreased and was reduced to zero after 18 hr. Exchange of the hydrogen of the NH_3-moiety with deuterium plays only a minor role, as has been demonstrated by an incubation of cisplatin under the same conditions. As Pt—N bonds are relatively stable it is unlikely that the decrease of the intensity of the signal at 4.13 ppm is due to a replacement of NH_3 by methylselenol. Most probably, this decrease is due to the formation of complexes with one or more H_3N—Pt—Se—CH_3 bonds, which are practically insoluble in D_2O and are precipitating during the incubation. During the incubation, a few other signals, including two with ^{77}Se-satellites at 2.84 ppm and 2.94 ppm, occurred (Fig. 7). The coupling constants $^2J(^1H, ^{77}Se)$ are 11.2 Hz and 8.0 Hz respectively and are in the range expected for $^2J(^1H, ^{77}Se)$ couplings: 4–12 Hz [21]. As shown in Fig. 7, besides the two ^{77}Se-satellites, two signals symmetrically relative to the signal at 2.94 ppm were observed. The coupling constant, $^3J(^1H, ^{195}Pt)$ = 35 Hz, is in the range expected for $^3J(^1H, ^{195}Pt)$ coupling constants: 15–50 Hz [21]. The signal at 2.94 ppm is therefore assigned to a compound with a Pt—Se—CH_3 bond. Identification of the reaction products with ^{195}Pt-NMR or ^{77}Se-NMR was not possible, due to the low sensitivities of these techniques.

FAB–MS did not produce any signal which could be attributed to a platinum containing compound; effective FAB–MS of the insoluble fraction may be precluded by the insolubility of the compounds.

The decrease of the cisplatin concentration proceeded in two distinct phases. In the first phase, the reaction proceeded through first order kinetics with a reaction rate constant, k_{obs}, of 3.25×10^{-4} sec^{-1} ($T_{1/2}$ = 36 min). In this phase, the decrease of the cisplatin signal is probably caused by a reaction of cisplatin with methylselenol. After 90 min, no signal at 2.00 ppm (methylselenol) could be detected. The decrease of the cisplatin concentration proceeded again through first order kinetics, however during this phase with a reaction-rate constant k_{obs}, of 9.29×10^{-5} sec^{-1} ($T_{1/2}$ = 124 min).

Distribution studies

As shown in Table 1, ^{75}Se levels in the kidneys of BALB/c mice, treated with one i.p. injection of [^{75}Se]selenite were consistently higher (4–10 times) than those in the Prima tumors on all timepoints tested. Co-administration of [^{195m}Pt]cisplatin did not influence ^{75}Se levels in tumor or kidneys.

After i.p. administration of [^{195m}Pt]cisplatin, ^{195m}Pt levels in the kidneys were also higher than those in the tumor: the kidney to tumor ratio was 2.3, 60 min after administration of [^{195m}Pt]cisplatin (= 120 min after administration of physiological saline), which is much lower than the ratio of 8 for ^{75}Se levels, observed 65 min after administration of a single dose of sodium [^{75}Se]selenite. Co-administration of sodium [^{75}Se]selenite, 1 hr before [^{195m}Pt]cisplatin did not influence ^{195m}Pt levels in the tumor or the kidneys (Table 1).

GSH levels in the kidneys were higher (2.22 ± 0.54 vs 0.06 ± 0.03 nmol GSH/g tissue, N = 3) than those in the tumors of Prima tumor bearing BALB/c mice.

Table 1. Levels of selenium and platinum in kidney and tumor of mice

Selenite (μmol/kg)	Cisplatin* (μmol/kg)	Time† (min)	Selenium (nmol/g tissue)		Platinum (nmol/g tissue)	
			Kidney	Tumor	Kidney	Tumor
25	0	30	23 ± 14‡	6 ± 3	—	—
25	0	65	32 ± 1	4 ± 1	—	—
25	45	65	32 ± 3	4 ± 1	53 ± 10	14 ± 5
0	45	65	—	—	69 ± 8	14 ± 5
25	0	75	25 ± 8	6 ± 2	—	—
25	45	75	31 ± 8	5 ± 2	52 ± 20	21 ± 11
0	45	75	—	—	67 ± 5	20 ± 2
25	0	90	31 ± 2	5 ± 2	—	—
25	45	90	30 ± 3	5 ± 1	84 ± 20	18 ± 7
0	45	90	—	—	65 ± 13	20 ± 7
25	0	120	32 ± 6	4 ± 2	—	—
25	45	120	31 ± 9	3 ± 1	40 ± 20	11 ± 3
0	45	120	—	—	64 ± 10	28 ± 11

* Cisplatin was administered 1 hr after sodium selenite or physiological saline.
† Time after administration of selenite or physiological saline.
‡ Mean ± SE (N = 3).

DISCUSSION

Reduction of cisplatin-induced nephrotoxicity can be achieved by administration of sodium selenite [8, 22]. The primary aim of this study was to gain insight into the molecular mechanism of this protective effect of sodium selenite. The protection may be due to an interaction between cisplatin or its hydrolysis products and selenite or one of its metabolites. In this respect, nucleophilic metabolites of selenite deserve special attention. An important nucleophilic metabolite of selenite is methylselenol (Fig. 1). The results presented in this paper have demonstrated that methylselenol is capable of reacting with cisplatin *in vitro*. The identity of the reaction products could not be established. However, we have obtained indications that 1 or more Pt—Se—CH_3 bonds were formed, which is in agreement with the expected substitution of chloride ions by methylselenol. Dedon and Borch [23] have shown that cisplatin is capable of forming polymeric complexes with GSH *in vitro*. It is likely that the observed platinum–selenol complexes formed *in vitro* are also polymeric in nature.

Bioactivation of selenite

Using HPLC and trapping experiments with iodoacetic acid, we have also shown that in presence of a large excess of thiols, such as GSH and CYS, nucleophilic selenium compounds are formed from selenite. In this study, mass spectral- and NMR-data have provided evidence for the formation of bis(cysteinyl)selenide (CYSSeCYS) *in vitro*. *In vivo*, glutathionylselenol (GSSeH) might be formed by degradation of bis(glutathionyl)selenide (GSSeSG), either by GSH-reductase or by excess GSH [9]. Up till now, however, the presence of selenols in mammalian tissues have not been established, probably because they are rapidly converted in hydrogen selenide or elemental selenium (Fig. 1).

The protective effect of sodium selenite against cisplatin-induced nephrotoxicity in rodents depend on the formation of selenols in the kidney. Recently, Satoh *et al.* (24) have shown that selenomethionine also provides protection against the nephrotoxicity of cisplatin. Interestingly, selenomethionine is also capable of generating methylselenol *in vivo*. Selenomethionine can be converted via the transsulphuration pathway to selenocysteine, which in turn is converted to hydrogen selenide by the enzyme selenocysteine-lyase [25]. Hydrogen selenide is subsequently converted to methylselenol. Selenomethionine can also be converted to methylselenol via the transamination pathway [25]. The protection of selenomethionine against the nephrotoxicity of cisplatin may also depend on the formation of methylselenol in the kidney. The influence of methylselenol on the antitumor activity of cisplatin, however, is at present unknown.

Selenols may also play an important role in the protective effect of selenite against the toxicity of heavy metals such as mercury and cadmium. The high renal toxicity of methylmercury (CH_3HG^+) is probably the result of interactions between methylmercury and thiolate anions [26]. Iwata *et al.* [27] have shown that selenite is capable of converting albumin-bound methylmercury *in vitro* to bis(methylmercury)-selenide ($CH_3HgSeHgCH_3$) and trimethylmercuryselenonium [$(CH_3Hg)_3Se^+$]. This conversion took place under influence of GSH and protein-bound thiol groups. Hydrogen selenide was shown to be very reactive towards albumin-bound methylmercury *in vitro*, while bis(glutathione)selenide (GSSeSG) was only slightly reactive [27]. The reactivity of bis(glutathione)selenide towards albumin bound methylmercury, however, was increased about 11 times when GSH was added in the incubation mixture. We have shown that selenite and bis(glutathione)selenide can be converted to nucleophilic compounds, probably glutathionylselenol, in the presence of GSH, the selenite induced

detoxification of mercury might also be due to a reaction of methylmercury with selenols or to a selenol-induced liberation of methylmercury from methylmercury–protein bonds. These examples demonstrate that there are probably similarities between the mechanisms of the protective effects of selenite against the toxicity of cisplatin and methylmercury.

Mechanism of protection

The first explanation for the protective effect of sodium selenite against cisplatin-induced nephrotoxicity in rodents is that selenols react with cisplatin in the kidney: this may result in detoxification of cisplatin.

Cisplatin has a high affinity for nucleophilic agents, such as compounds with a thiol- or thiomethyl-moiety. It has been suggested that the crucial event in the nephrotoxic activity of cisplatin is binding of platinum to thiol-groups of enzymes in the kidney [3]. Such covalent binding would result in inactivation of these enzymes and ultimately in kidney dysfunction. *A second possible explanation* of the protective effect of selenite is that selenols may be able to liberate platinum from platinum protein bonds: this may result in restoring of the biological activities of these proteins and protection against the nephrotoxicity induced by cisplatin.

In this study we have shown that selenite is also capable of reacting with thiols. Ganther and Corcoran [28] have shown that selenium can be incorporated into proteins as —S—Se—S—. Therefore, *a third explanation* for the protective effect of selenite may be competition between selenium compounds and platinum compounds for thiol-groups in the kidney: this may prevent the binding of platinum compounds to thiols and thereby the nephrotoxicity induced by cisplatin.

Selectivity of protection

Cisplatin is supposed to exert its nephrotoxic activity by inactivation of thiol-containing enzymes in the kidney, whilst its antitumor activity is based on formation of bifunctional adducts with DNA [3]. Lempers *et al.* [29] have studied *in vitro* the reactions between [PtCl-(dien)]Cl, as model compound for cisplatin, and thiols. They have shown that nucleophiles are capable of abstracting Pt(dien) from thiols. They have suggested that nucleophilic agents should be able to protect against cisplatin-induced nephrotoxicity by abstracting platinum from cisplatin–protein complexes. Bodenner *et al.* [3] have demonstrated that diethyldithiocarbamate protects against cisplatin-induced nephrotoxicity by abstracting platinum from platinum–protein (SH) adducts, but not from platinum–DNA adducts. Diethyldithiocarbamate is also capable of alkylating cisplatin and its hydrolysis products [23]. The selectivity of the protection of WR-2721 against cisplatin-induced nephrotoxicity is probably based on chelation of platinum complexes in the kidney by a nucleophilic metabolite of WR-2721 [6]. WR-2721 is dephosphorylated by a membrane alkaline phosphatase, becoming a free sulfhydryl as it enters the cell. Most tumor cells lack the necessary alkaline phosphatase and therefore do not accumulate WR-2721.

It has been shown that selenite reduces the nephrotoxicity of cisplatin in rodents, without reducing the antitumor activity of the drug [8].

As shown in Table 1, selenium levels in the kidney were about eight times higher than those in the tumor, 65 min after administration of a single dose of sodium selenite, 25 μmol/kg, to tumor bearing BALB/c mice. GSH levels in the kidney were also higher than those in the tumor. These data are in agreement with those reported by Lee *et al.* [30]. As can be seen in Fig. 1, GSH is required for the bioactivation of selenite to selenols. It has been shown that the kidney is also a major organ for the enzymic conversion of selenite to dimethylselenide [31]. Selenols are very reactive alkylating compounds, which implies that they will probably primarily exert their biological activity in cells in which they are generated. In our distribution experiments a selenite dose was used which has previously been shown to provide protection against cisplatin-induced nephrotoxicity in BALB/c mice, when administered 1 hr before a single injection of cisplatin, but not when administered 1 hr thereafter [8]. The events responsible for the nephrotoxicity of cisplatin, however, occur shortly after administration of cisplatin [32]. The selectivity of selenite is therefore most probably due to the accumulation of selenite and selenols in the kidney, shortly after administration of selenite and cisplatin.

Clinical implications

Unfortunately, the clinical application of sodium selenite as chemoprotector against cisplatin-induced nephrotoxicity is limited by its toxicity. The toxicity of sodium selenite and other selenium compounds, such as selenomethionine, is caused by the bioavailability of their selenium moiety and by their bioactivation to hydrogen selenide [33]. Hydrogen selenide is considered to be the most toxic selenium-metabolite. The present study suggests that selenols, formed through a chemical reaction with glutathione (GSH) and other thiols in principle may be able to react chemically with platinum complexes, similar to the reaction of methylselenol with cisplatin. The effects of thus formed selenols on the nephrotoxicity of cisplatin therefore would appear to be worthy of further examination.

In conclusion, we have demonstrated that methylselenol, a nucleophilic metabolite of selenite, is capable of forming a complex with cisplatin *in vitro*. Bioactivation of selenium compounds, such as selenite, to selenols requires GSH or other thiols. This bioactivation of selenite is more predominant in kidney cells than in tumors as a result of higher levels of GSH and selenium in kidneys than in tumors. Most likely selenols are responsible for the protective effect of sodium selenite against the nephrotoxicity of cisplatin: a protection which is obtained without reduction of the antitumor activity of cisplatin. These findings open new perspectives for a mechanism-based selection of clinically applicable seleno-chemoprotectors against cisplatin-induced nephrotoxicity.

Acknowledgements—We would like to thank Dr P.

Lelieveld, Radiobiological Institute TNO, Rijswijk, The Netherlands, for providing Prima breast tumor cells. We are grateful to K. J. Volkers for synthesis of platinum compounds. This work was supported by a grant from the Dutch Cancer Society, Amsterdam, The Netherlands.

REFERENCES

1. Roberts JJ and Thomson AJ, The mechanism of action of antitumor platinum compounds. *Prog Nucleic Acid Res Mol Biol* **22**: 71–133, 1979.
2. Reedijk J, The mechanism of action of platinum antitumor drugs. *Pure Appl Chem* **59**: 181–192, 1987.
3. Bodenner DL, Dedon PC and Borch RF, Effect of diethyldithiocarbamate on cis-diaminedichloroplatinum (II)-induced cytotoxicity, DNA cross-linking, and γ-glutamyl transpeptidase inhibition. *Cancer Res* **46**: 2745–2750, 1986.
4. Pizzo SV, Roche PA, Feldman SR and Gonias SL, Further characterization of the platinum-reactive component of the α_2-macroglobulin-receptor recognition site. *Biochem J* **238**: 217–225, 1986.
5. Hamilton TC, Winker MA, Louie KG, Batist G, Behrens BC, Tsurno T, Grotzinger KR, McKoy WM, Young RC and Ozols RF, Augmentation of adriamycin, melphalan and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem Pharmacol* **34**: 2583–2586, 1985.
6. Yuhas JM, Active versus passive absorption kinetics as the basis for selective protection of normal tissues by *S*-2-(3-aminopropylamino)-ethylphosphorothioic acid. *Cancer Res* **40**: 1519–1524, 1980.
7. Pfeifle CE, Howell SB, Felthouse RD, Woliver TBS, Andrews PA, Markman M and Murphy MP, High-dose cisplatin with sodium thiosulfate protection. *J Clin Oncol* **3**: 237–244, 1987.
8. Baldew GS, van den Hamer CJA, Los G, Vermeulen NPE, de Goeij JJM and McVie JG, Selenium-induced protection against cis-diamminedichloroplatinum (II) nephrotoxicity in mice and rats. *Cancer Res* **49**: 3020–3023, 1989.
9. Ganther HE, Reduction of the selenotrisulfide derivative of glutathione to a persulfide analog by glutathione reductase. *Biochemistry* **10**: 4089–4098, 1971.
10. McConnell KP and Portman OW, Excretion of dimethylselenide by the rat. *J Biol Chem* **195**: 277–282, 1952.
11. Palmer IS, Fischer DD, Halverson AW and Olson OE, Identification of a major selenium excretory product in rat urine. *Biochim Biophys Acta* **177**: 336–342, 1969.
12. Baldew GS, Volkers KJ, de Goeij JJM and Vermeulen NPE, Determination of cisplatin and related platinum complexes in plasma ultrafiltrate and urine by high-performance liquid chromatography with on-line radioactivity detection. *J Chrom Biom Appl* **491**: 163–174, 1989.
13. Hoeschele JD, Butler TA, Roberts JA and Guyer CE, Analysis and refinement of the microscale synthesis of the ^{195m}Pt-labeled antitumor drug, cis-dichlorodiammineplatinum(II), cis-DDP. *Radiochim Acta* **31**: 27–36, 1982.
14. Ganther HE, Selenotrisulfides. Formation by the reaction of thiols with selenious acid. *Biochemistry* **7**: 2898–2905, 1968.
15. Baldew GS, de Goeij JJM and Vermeulen NPE, Determination of ^{75}Se-labelled selenite and metabolites in plasma and urine by high-performance liquid chromatography with on-line radioactivity detection. *J Chromatogr Biom Appl* **496**: 111–120, 1989.
16. Pleasants JC, Guo W and Rabenstein DL, A comparative study of the kinetics of selenol/diselenide and thiol/disulfide exchange reactions. *J Am Chem Soc* **111**: 6553–6558, 1989.
17. Sedlak T and Lindsay AR, Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* **25**: 192–205, 1968.
18. Rabenstein DL and Tan K, ^{77}Se-NMR studies of bis(alkylthio)selenides of biological thiols. *Magnetic Resonance Chem* **26**: 1079–1085, 1988.
19. McFarlane HCE and McFarlane W, In: *NMR of Newly Accessible Nuclei* (Ed. Laszlo P), Vol. 2, pp. 276–290. Academic Press, New York, 1983.
20. Pinto A and Kippard SJ, Binding of the antitumor drug cis-diamminedichloroplatinum(II) (cisplatin) to DNA. *Biochim Biophys Acta* **780**: 167–180, 1985.
21. Harris RK and Mann BE (Eds). *NMR and the Periodic Table*, Academic Press, New York, 1978.
22. Baldew GS, Volkers KJ and van den Hamer CJA, Reduction of cisplatin nephrotoxicity by selenium: does metallothionein play a role? *Arch Toxicol Suppl* **12**: 171–174, 1988.
23. Dedon PC and Borch RF, Characterization of the reactions of platinum antitumor agents with biological and nonbiological sulfur-containing nucleophiles. *Biochem Pharmacol* **36**: 1955–1966, 1987.
24. Satoh M, Naganuma A and Imura N, Optimum schedule of selenium administration to reduce lethal and renal toxicities of cis-diamminedichloroplatinum in mice. *J Pharmacobio-Dyn* **12**: 246–253, 1989.
25. Ip C, Differential effect of dietary methionine on the biopotency of selenomethionine and selenite in cancer chemoprevention. *J Natl Cancer Inst* **80**: 258–262, 1988.
26. Rabenstein DL, The chemistry of methylmercury toxicology. *J Chem Educ* **55**: 292–296, 1978.
27. Iwata H, Masukawa T, Kito H and Hayashi M, Involvement of tissue sulfhydryls in the formation of a complex of methylmercury with selenium. *Biochem Pharmacol* **30**: 3159–3163, 1981.
28. Ganther HE and Corcoran C, Selenotrisulfides. II. Cross-linking of reduced pancreatic ribonuclease with selenium. *Biochemistry* **8**: 2557–2563, 1969.
29. Lempers ELM, Inagaki K and Reedijk J, Reactions of [PtCl(dien)]Cl with glutathione, oxidized glutathione and *S*-methyl glutathione. Formation of an *S*-bridged dinuclear unit. *Inorg Chim Acta* **152**: 201–207, 1988.
30. Lee FYF, Allalunis-Turner MJ and Siemann DW, Depletion of tumour versus normal tissue glutathione by buthionine sulfoximine. *Br J Cancer* **56**: 33–38, 1987.
31. Diplock AT, Metabolic aspects of selenium action and toxicity. *CRC Crit Rev Toxicol* **4**: 271–329, 1976.
32. Heidemann HTh, Gerkens JF, Jackson EK and Branch RA, Attenuation of cisplatinum-induced nephrotoxicity in the rat by high salt diet, furosemide and acetazolamide. *Arch Pharmacol* **329**: 201–205, 1985.
33. Muller A, Gabriel H, Sies H, Terlinden R, Fischer H and Romer A, Biotransformation of ebselen in perfused rat liver. *Biochem Pharmacol* **37**: 1103–1109, 1988.