The effects of sulfur-containing compounds and gemcitabine on the binding of cisplatin to plasma proteins and DNA determined by inductively coupled plasma mass spectrometry and high performance liquid chromatography–inductively coupled plasma mass spectrometry

Elke E.M. Brouwers[a], Alwin D.R. Huitema[a], Jan H.M. Schellens[b,c] and Jos H. Beijnen[a,c]

The aim of this study was to investigate the effects of sodium thiosulfate (STS), glutathione (GSH), acetylcysteine (AC), and gemcitabine on the platinum–protein (Pt–protein) and platinum–DNA (Pt–DNA) binding of cisplatin in whole blood. This was done to obtain more insight into the platinum (Pt) binding in whole blood and the effects of modulators on this process. STS, GSH, AC, and gemcitabine were added before and after the incubation of whole blood with cisplatin. Pt levels in plasma and plasma ultrafiltrate and the Pt that is bound to DNA in peripheral blood mononuclear cells were determined using inductively coupled plasma mass spectrometry. Additionally, information on the major Pt–DNA adducts was obtained by separation of the Pt–DNA adducts by high performance liquid chromatography with off-line inductively coupled plasma mass spectrometry detection. Results showed that the reactive Pt levels in whole blood are reduced by STS, GSH, and AC. This reduction was demonstrated by a reduced Pt–protein and Pt–DNA binding in the presence of sulfur compounds. Furthermore, STS and AC seemed to be able to release Pt from proteins. The compounds could hardly release Pt from the DNA. Gemcitabine slightly inhibited Pt–DNA binding and did not alter Pt–protein binding. The type of Pt–DNA adducts found were not altered in the presence of the modulators. In conclusion, the results of this study illustrate that STS, GSH, and AC affect Pt binding in whole blood, which suggests that these compounds could affect Pt binding in patients. By interfering with Pt–DNA and Pt–protein binding, the compounds could influence side effects and cytotoxicity. *Anti-Cancer Drugs* 19:621–630 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2008, **19**:621–630

Keywords: cisplatin, gemcitabine, platinum–protein and platinum–DNA binding, sulfur-containing compounds

[a]Department of Pharmacy and Pharmacology, Slotervaart Hospital, The Netherlands Cancer Institute, Louwesweg 6, [b]Department of Medical Oncology, Antoni van Leeuwenhoek Hospital, The Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam and [c]Beta Faculty, Department of Pharmaceutical Sciences, Division of Biomedical Analysis, Section of Drug Toxicology, Utrecht University, Utrecht, The Netherlands

Correspondence to Elke E.M. Brouwers, Department of Pharmacy and Pharmacology, Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam, The Netherlands
Tel: +31 020 512 5513; fax: +31 020 512 4753;
e-mail: Elke.Brouwers@slz.nl

Received 4 February 2008 **Revised form accepted** 6 March 2008

Introduction

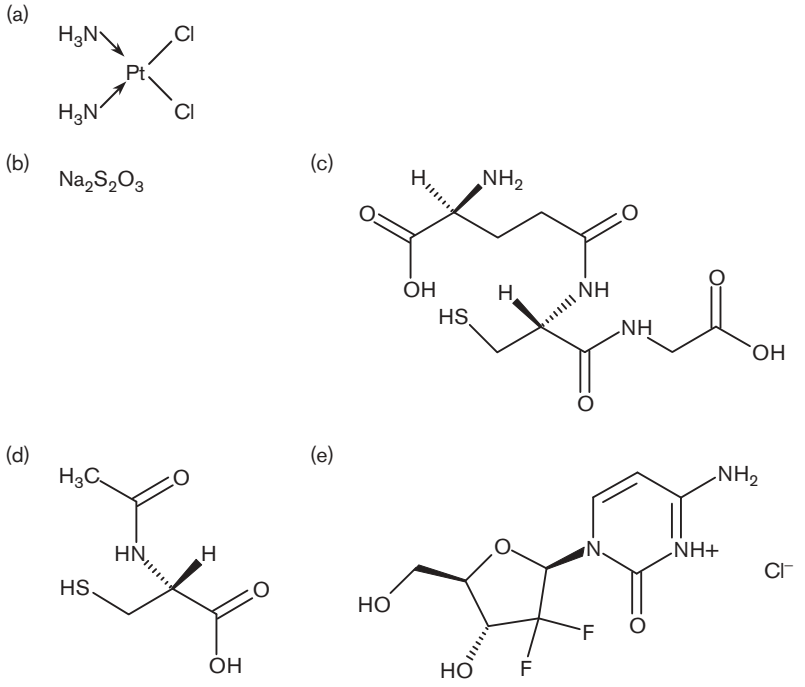
Cisplatin [*cis*-diamminedichloridoplatinum (II)] (Fig. 1) is a successful anticancer drug, which is applied for the treatment of various malignancies. After intravenous infusion, cisplatin and its reactive metabolites become rapidly partitioned into plasma protein-bound platinum (Pt), free plasma Pt, tissue Pt, Pt in peripheral blood mononuclear cells (PBMCs), and erythrocyte-sequestered Pt. As much as 60–95% of cisplatin and its reactive metabolites bind to plasma proteins [1]. The role of the Pt–protein complexes in the mechanism of cytotoxicity is, up to now, unknown and the free Pt fraction is generally considered as pharmacologically active [2,3]. Part of this fraction ultimately enters the cell and cell nucleus and binds to the DNA. Pt–DNA adducts affect DNA replication and transcription and, thereby, inhibit tumor growth.

Unfortunately, the application of cisplatin is impaired by severe side effects, such as nephrotoxicity, ototoxicity, and neurotoxicity. In addition to the direct consequence of these side effects, their persistent nature can seriously affect patients' quality of life. The high incidence of severe side effects has led to the development of treatment strategies aimed to prevent or reduce the side effects without affecting the antitumor activity. As cisplatin has high affinity for binding to sulfur donors, sulfur-containing nucleophiles could serve as rescue agents. Systemically administered sodium thiosulfate (STS), nowadays, is commonly used in combination with locally administered cisplatin in patients with, for example, head–neck carcinoma [4] and intraperitoneal tumors [5–7]. STS has a protective effect against cisplatin-induced nephrotoxicity [5] and ototoxicity [8]. Glutathione (GSH), an endogenous sulfur-containing

0959-4973 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Fig. 1



Structural formulae of cisplatin (a), sodium thiosulfate (b), glutathione (c), acetylcysteine (d), and gemcitabine HCl (e).

compound, also provides protection against cisplatin-induced nephrotoxicity in patients when administered intravenously [9]. In addition to STS and GSH, acetylcysteine (AC), a precursor of GSH, could serve as a cisplatin-neutralizing agent. This compound decreased the toxicity in experimental animals when administered in conjunction with cisplatin [10]. To our best knowledge, to date, no patient studies have been performed on the protective effects of AC against cisplatin-induced toxicity. A major advantage of the use of STS, GSH, and AC is that they are well tolerated in high doses. So far, sulfur-containing compounds were always tested for their ability to prevent side effects. Previous studies, however, showed that sulfur-containing compounds are capable of reversing the Pt–DNA [11] and Pt–protein binding [12]. As persistent side effects might be a consequence of Pt that is accumulated in the body and remains bound to DNA, it is plausible that sulfur-containing compounds could reduce persistent side effects even when administered months after treatment without reducing cytotoxicity. To achieve such effects, however, the compounds should be capable of removing Pt from the cellular and blood compartments. Up to now, this has not been investigated.

In addition to compounds that are administered deliberately to protect against cisplatin-induced toxicity, cytotoxic compounds that are administered with cisplatin in standard combination regimens might also affect the cytotoxic action of cisplatin. Gemcitabine, which was combined with cisplatin for the treatment of nonsmall cell lung cancer, seemed to reduce the formation of Pt–DNA adducts in PBMCs [13].

The effect of the modulators STS, GSH, AC, and gemcitabine on Pt–protein binding and the formation of Pt–DNA adducts can be evaluated in ex-vivo studies. Thereby, information is gained on the potential use of sulfur-containing compounds for the prevention and reduction of side effects *in vivo*. In this study, we investigated the effect of STS, GSH, AC, and gemcitabine (Fig. 1) on the Pt–protein and Pt–DNA binding of cisplatin in whole blood. Pt concentrations in plasma and plasma ultrafiltrate (pUF) were determined using a previously validated inductively coupled plasma mass spectrometry (ICP-MS) method [14]. ICP-MS was also used to assess the total amount of Pt–DNA adducts in PBMCs. Information on the major Pt–DNA adducts, that is, Pt–GG (intrastrand crosslink on pGpG sequences) and Pt–AG (intrastrand crosslink on pApG sequences) was obtained by separation of the Pt–DNA adducts by high performance liquid chromatography (HPLC) with off-line ICP-MS detection.

Experiment

Chemicals

Cisplatin reference standard was purchased from Calbiochem (San Diego, California, USA). Iridium chloride,

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

containing 1000 mg/l iridium in 3.3% HCl, used for internal standardization, was purchased from Inorganic Ventures/IV Labs (Lakewood, New Jersey, USA). Nitric acid (HNO_3) 70% Ultrex II ultrapure reagent was obtained from Mallinckrodt Baker (Philipsburg, New Jersey, USA). Water used for the analyses was sterile water for irrigation (Aqua B. Braun Medical, Melsungen, Germany). Heparinized whole blood was obtained from healthy volunteers. The STS solution (250 g/l) was supplied by the Hospital Pharmacy of Haarlem (The Netherlands). AC was purchased from Zambon (Amersfoort, The Netherlands). Gemcitabine HCl was obtained from Eli Lilly (Houten, The Netherlands). Proteinase K and SDS were acquired from Sigma-Aldrich (Steinheim, Germany). Sodium chloride, EDTA disodium and diammonium salt, potassium hydrogen carbonate, ammonium acetate, and iridium chloride were purchased from Merck (Darmstadt, Germany). Ammonium hydrogencarbonate was purchased from VWR (Fontenay-sous-Bois, France). GSH, zinc chloride, magnesium chloride, nuclease P1, Tris–HCl, phosphate-buffered saline, and Triton X-100 were obtained from Sigma-Aldrich (St Louis, Missouri, USA). DNAse I and alkaline phosphatase were acquired from Roche Applied Science (Basel, Switzerland). The dinucleosides GpG and ApG were purchased from Metabion (Planegg-Martinsried, Germany). Absolute ethanol and methanol were obtained from Biosolve (Valkenswaard, The Netherlands).

Treatment of whole blood with cisplatin and modulators

Two separate experiments were performed to evaluate the effects of STS, GSH, AC, and gemcitabine on Pt–protein and Pt–DNA binding. In the first experiment, modulators were added before cisplatin incubation to assess whether the modulators could prevent Pt–protein and Pt–DNA binding. In the second experiment, modulators were added 3 h after cisplatin incubation to assess whether the modulators were capable of removing Pt from the proteins and DNA. The administered concentrations of the sulfur-containing compounds used in the experiments were calculated by dividing the initial dose generally administered to patients by a blood volume of 5 l [4,15,16]. This was only an approach of the concentrations that will be reached *in vivo*. Volumes of distribution (Vd) of the compounds are 12, 13, and 38 l for STS, GSH, and AC, respectively [16–18]. The cisplatin (Vd = 19.2 l [3]) and gemcitabine (Vd = 40 l [19]) concentrations chosen for the experiments were approximately 30-fold higher than the maximal concentrations reached after a common intravenous infusion [19,20]. This was carried out to achieve Pt–DNA adduct levels that were detectable after HPLC speciation analysis.

For the first experiment, four samples of 30 ml of heparinized whole blood were incubated at 37°C with 3.6 g/l STS, 0.5 g/l GSH, 2.1 g/l AC, or 0.65 g/l gemcitabine. To two 30 ml whole blood samples, no modulator was added. After 30 min, cisplatin was added to all samples up to a final concentration of 0.1 g/l cisplatin (corresponding to 0.065 g/l Pt) and incubation was continued for 6 h.

After 1, 3, and 6 h, aliquots of 10 ml of whole blood were taken from the samples and transferred to 10 ml heparin-containing tubes (Becton Dickinson Vacutainer Systems, Plymouth, UK). After centrifugation for 15 min at 1000*g* and 4°C, the plasma fraction was isolated. PUF was obtained by centrifuging the plasma fraction through 30 kDa cut-off ultrafiltrate filters (Centriplus Millipore Corporation, Bedford, Massachusetts, USA) for 30 min (1000*g*, 20°C). Plasma samples were diluted in a 0.01% (g/v) EDTA diammonium salt and Triton-X solution in water. PUF samples were diluted using a 1% (v/v) HNO_3 solution in water. After the addition of an internal standard, the Pt contents of the plasma and pUF samples were analyzed using ICP-MS.

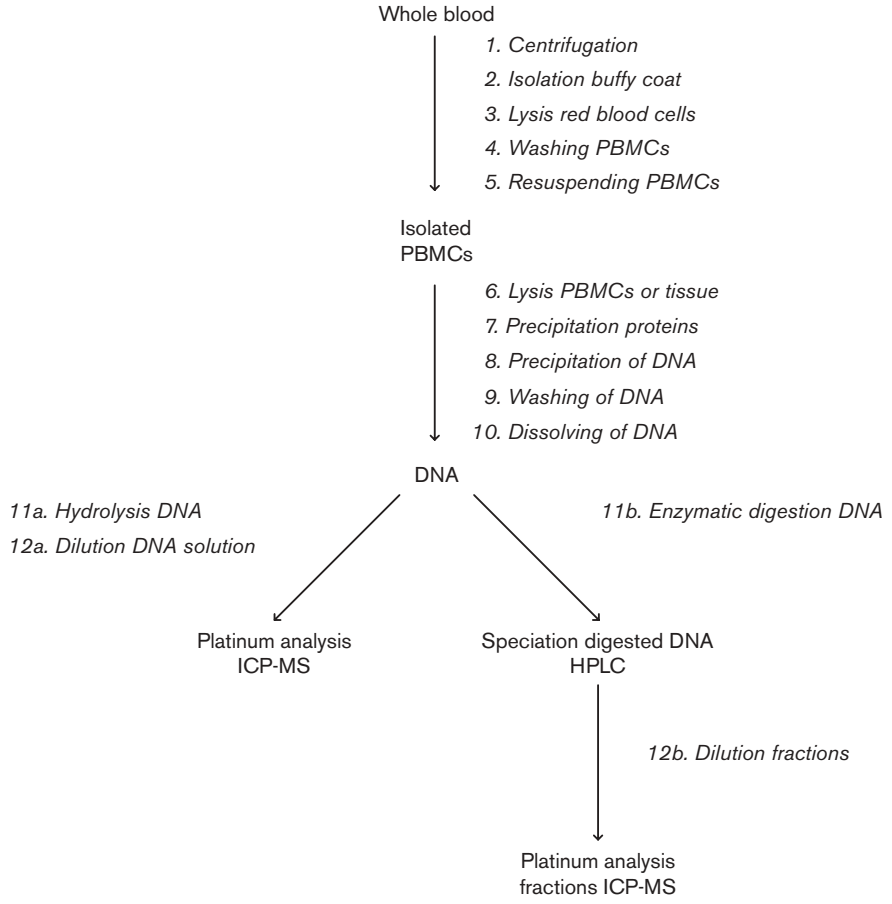
The PBMC fraction was isolated from the centrifuged whole blood as described earlier [21,22]. The sample pretreatment procedure for PBMCs is outlined in Fig. 2. Briefly, contaminating red blood cells were lysed by incubation with 0.83% (w/v) NH_4Cl, 0.1% (w/v) $KHCO_3$, and 1 mmol/l EDTA disodium salt for 20 min at 4°C. PBMCs were washed twice with ice-cold phosphate-buffered saline and resuspended in 9 ml of a buffer containing 10 mmol/l Tris–HCl, 2.3% (w/v) NaCl, and 2 mmol/l EDTA disodium salt at pH 7.3.

DNA was isolated from PBMCs as described previously [21,22]. In brief, 0.9 ml 1.1 mol/l NH_4HCO_3, 0.45 ml 20% (w/v) SDS, and 150 µl 1% (w/v) proteinase K solution were added successively, followed by overnight incubation at 42°C. After the digestion was complete, 3.3 ml of saturated 6 mol/l NaCl was added to each tube and the tubes were shaken vigorously to precipitate proteins. The tubes were centrifuged and the supernatant containing the DNA was transferred to another tube. Subsequently, the supernatant was shaken, centrifuged, and transferred to another tube. After this, 20 ml of absolute ethanol was added to precipitate the DNA. The DNA was washed twice with 75% ethanol and was subsequently dissolved in 1 ml of water. DNA concentrations were analyzed after dilution in 10 mmol/l Tris–HCl (pH 8) by measuring the absorbance at 260 nm using a Biophotometer (Eppendorf, Hamburg, Germany). The purity of the DNA was checked by determining the absorbance ratio at 260 and 280 nm. Ratios between 1.8 and 2.0 were routinely obtained.

Aliquots of 75 µg of DNA were hydrolyzed by incubation in 1% (v/v) HNO_3 at 70°C for 24 h. The resulting solutions were diluted in 1% HNO_3 to concentrations within the calibration range of the ICP-MS. After the

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Fig. 2



Schematic outline of sample pretreatment procedure for PBMCs. HPLC, high performance liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; PBMCs, peripheral blood mononuclear cells.

addition of internal standard, the total amount of Pt–DNA adducts was analyzed by ICP-MS. The total Pt–adduct concentrations were expressed in picogram Pt per microgram DNA. Another 100 µg of DNA was processed to quantitate the individual adducts by HPLC–ICP-MS as described below.

For the second experiment, six samples of 30 ml of whole blood were incubated with 100 mg/l cisplatin at 37°C and after 3 h samples were taken for Pt analyses. Subsequently, STS, GSH, AC, and gemcitabine were added in similar concentrations as described above. To two samples, no modulator was added. The effects of the modulators were evaluated after 1.5 and 3 h. Plasma, pUF, PBMCs, and DNA were obtained and processed as described above.

Enzymatic digestion of DNA

In addition to the determination of the total amount of Pt–DNA adduct, Pt–GG and Pt–AG adducts were assessed using HPLC–ICP-MS. Furthermore, chromatograms were investigated to see whether adduct peaks with deviating retention times were formed in the presence of the modulators compared with the samples containing only cisplatin. Therefore, DNA was digested as described by Pluim *et al.* [21]. Briefly, 100 µg of the DNA was diluted to 500 µl with water. Subsequently, 150 µl ammonium acetate (pH 5) and 6 µl nuclease P1 solution (0.5 U/µl) were added and the solution was incubated for 2 h at 60°C. Then, 12 µl of a solution containing 1 mol/l Tris–HCl, 10 mmol/l $MgCl_2$, and 1 mmol/l $ZnCl_2$ and 5 µl DNAse I (10 U/µl) were added and incubated for 2 h at 37°C. Finally, 10 µl of alkaline phosphatase solution (1 U/µl) was added and incubated overnight at 37°C. The resulting solution, which contains unmodified nucleosides and Pt–DNA adducts, was injected directly into the HPLC system.

Separation of platinum–DNA adducts

Analytical separation of the two major cisplatin–DNA adducts Pt–GG and Pt–AG was carried out with an HPLC system consisting of a 1100 Series liquid chromatograph

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Table 1 High performance liquid chromatography gradient

Time (min)	Buffer A (%)	Buffer B (%)
0–20	100	0
20–40	100→0	0→100
40–41	0→100	100→0
41–50	100	0

binary pump and degasser (Agilent Technologies, Palo Alto, California, USA), a Spectra Series AS3000 autosampler with column oven equipped with a 20 µl injection loop (Thermo Separation Products, Fremont, California, USA), and a photo-diode-array detector Model Waters 996 (Waters Chromatography BV, Etten-Leur, The Netherlands). Separation was achieved using a Polaris 5 C18-A chromsep column (150 × 2 mm ID, particle size 5 µm; Varian BV, Middelburg, The Netherlands). The temperature of the column was kept at 35°C. Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, California, USA). The mobile phase consisted of 5 mmol/l ammonium acetate (pH 4) in 2.5% methanol (buffer A) and in 25% methanol (buffer B). After injection of the digested DNA (approximately 1.5 µg in 10 µl), the analytes were eluted off the column by a gradient increasing from 0 to 100% buffer B (Table 1) with a flow rate of 0.2 ml/min. Pt–DNA adduct concentrations were below the detection limit of the UV detector. Therefore, eluting fractions were collected at intervals of 1 min and the Pt content was analyzed using ICP-MS after a 10-fold dilution in 1% (v/v) HNO3.

The identity of the individual Pt–DNA adducts was confirmed by chromatography of the reaction products of GpG and ApG with cisplatin. Hence 596 and 580 mg/l of the dinucleosides GpG and ApG, respectively, were incubated with 300 mg/l cisplatin (195 mg/l Pt). Adduct concentrations in the Pt–GG and Pt–AG incubation solutions were higher than those in the digested DNA solutions obtained from whole blood. Therefore, adduct peaks could be identified on the basis of absorption spectrum, retention time, and Pt content of the peaks.

Determination of platinum concentrations by inductively coupled plasma mass spectrometry

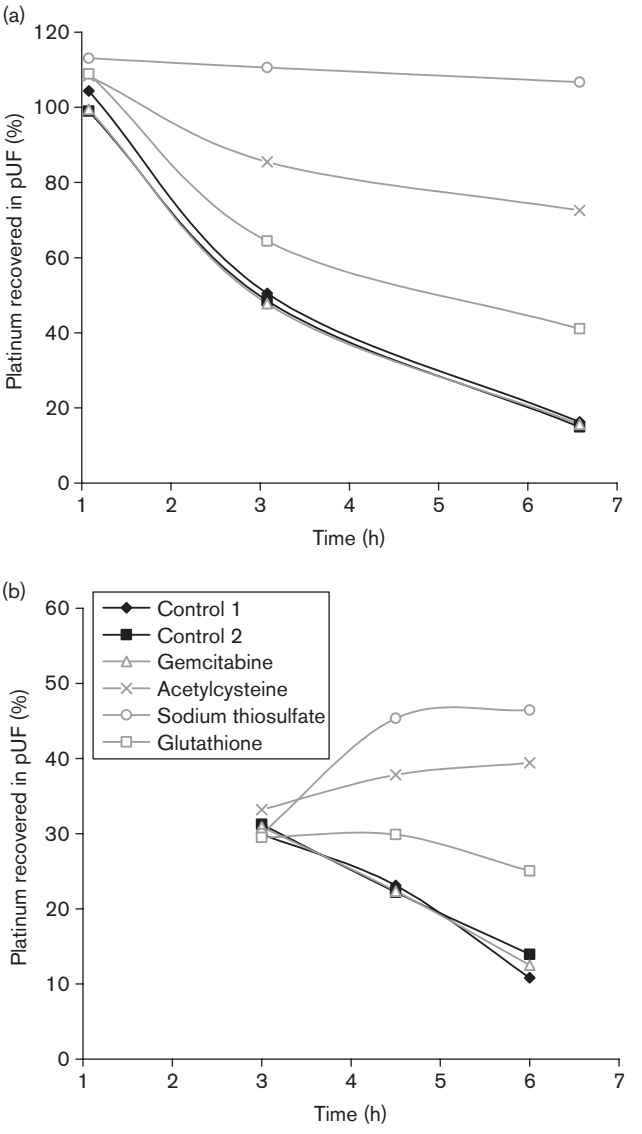
Pt analyses were performed on an ICP-quadrupole-MS (Varian 810-MS) equipped with a 90° reflecting ion mirror (Varian, Mulgrave, Victoria, Australia). The sample introduction system consisted of a Micromist glass low-flow nebulizer (sample uptake 0.4 ml/min), a peltier-cooled (4°C) double-pass glass spray chamber, and a quartz torch. Hoek Loos (Schiedam, The Netherlands) provided argon gas (4.6) with 99.996% purity. Data were acquired and processed using the ICP-MS Expert Software version 1.1 b49 (Varian Mulgrave, Victoria, Australia). The Pt isotope used for calculation of Pt concentrations was ^{194}Pt. Internal standardization was performed on each replicate using iridium (^{191}Ir).

Results

Effect of sodium thiosulfate, glutathione, acetylcysteine, and gemcitabine on platinum–protein binding

The effects of the modulators on the recovery of Pt in pUF when added before cisplatin addition (first experiment) are illustrated in Fig. 3a. Between 1 and 6 h after start of incubation, Pt continued to bind resulting in an ongoing reduction of the ultrafiltrable Pt fraction. After 6 h, the ultrafiltrable fraction in the samples that were solely incubated with cisplatin (controls 1 and 2 in Fig. 3) was reduced to 16%. Gemcitabine did not affect this Pt binding. GSH and AC both seemed to partly prevent

Fig. 3



(a) Percentage of ultrafiltrable Pt (pUF) in plasma versus time after incubation of whole blood with modulators for 0.5 h, followed by cisplatin incubation (first experiment). (b) Percentage of ultrafiltrable Pt in plasma versus time after incubation of whole blood with cisplatin for 3 h followed by the addition of modulators (second experiment).

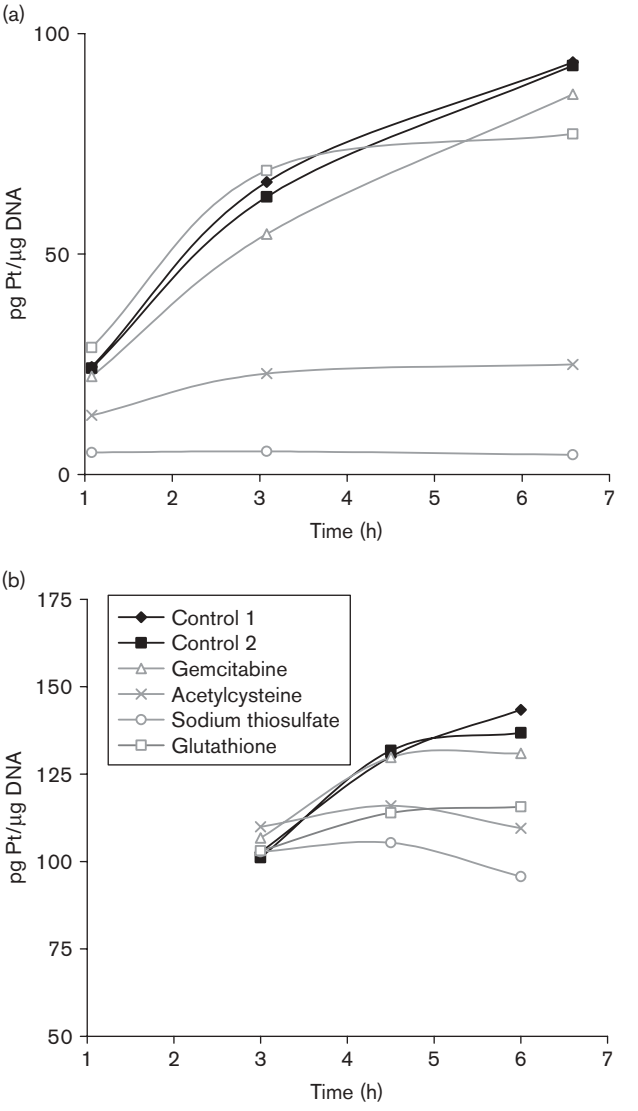
Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Pt–protein binding. The administered GSH and AC concentrations resulted in an increase in ultrafiltrable Pt of 25 and 57%, respectively, at 6 h. Pt–protein binding was almost completely prevented by STS. When the modulators were added 3 h after the samples were incubated with cisplatin (second experiment), initially, 31% of Pt was recovered in the pUF (Fig. 3b). After 6 h of incubation, the samples incubated solely with cisplatin or with cisplatin and gemcitabine showed a Pt recovery in pUF of 12%. Thus, again, gemcitabine did not affect Pt–protein binding. GSH seemed to limit Pt–protein binding by 13% at 6 h, resulting in an ultrafiltrable fraction of 25%. In the AC and STS-containing samples, after 6 h of incubation, the ultrafiltrable Pt fraction was raised from the initial 31% to 39 and 46%, respectively. Interestingly, AC and STS seemed to be capable of releasing Pt from the proteins, resulting in a larger ultrafiltrable Pt fraction.

Effect of sodium thiosulfate, glutathione, acetylcysteine, and gemcitabine on platinum–DNA binding

The total amounts of Pt–DNA adducts in PBMCs were analyzed by ICP-MS without prior separation. The effects of the modulators on the formation of the total amount of Pt–DNA adducts when they were added before cisplatin addition (first experiment) are shown in Fig. 4a. After 1 h of incubation, PBMCs contained 24 pg Pt/μg DNA in the samples solely incubated with cisplatin. The number of Pt atoms bound per nucleotide (*R*b value [23]) was 4.1×10^{-5}. At this time point, gemcitabine and GSH did not affect Pt–DNA binding. AC and STS, however, inhibited Pt–DNA binding with 45 and 80%, respectively, at 1 h. After 3 and 6 h of incubation, samples containing only cisplatin showed a Pt–DNA binding of 65 and 93 pg Pt/μg DNA, respectively, (*R*b values of 11×10^{-5} and 16×10^{-5}, respectively). Gemcitabine seemed to slightly inhibit Pt–DNA binding (7–16%). For GSH, no inhibitory effect was observed after 3 h of incubation. After 6 h, however, Pt–DNA binding seemed to be 18% lower compared with the samples containing only cisplatin. AC, obviously, decreased the extent and rate of Pt–DNA adduct formation. After 6 h, Pt–DNA adduct levels were 80% lower than the levels in the samples with only cisplatin. In the STS-incubated samples, no increase of the Pt–DNA adduct levels with time was observed. When the modulators were added 3 h after the samples were incubated with cisplatin (second experiment), initially, on average 104 pg Pt was bound to 1 μg DNA (*R*b value of 17.8×10^{-5}) (Fig. 4b). After 6 h of incubation, samples with cisplatin alone or cisplatin and gemcitabine contained on average 137 pg Pt/μg DNA (*R*b value of 17.8×10^{-5}). Pt–DNA binding was not affected by gemcitabine. Pt–DNA binding was, similar to the first experiment, inhibited by GSH and after 6 h, Pt–DNA levels were 17% lower than the levels in the samples incubated with cisplatin alone. AC and STS seemed to completely prevent further Pt–DNA binding. After 6 h, Pt–DNA adduct levels even seemed to be 8% lower than those before the addition of the modulating compounds.

Fig. 4



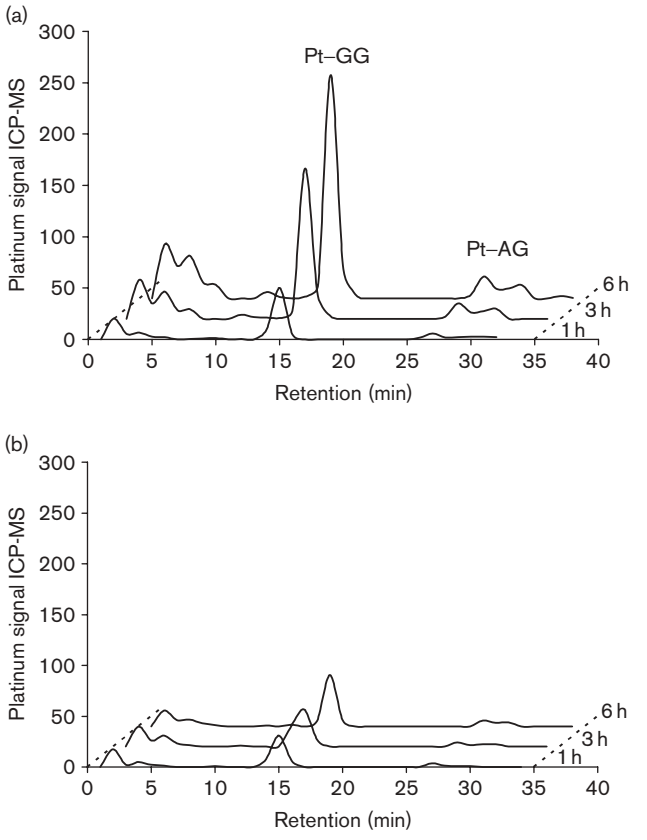
(a) Time-dependent formation of platinum–DNA (Pt–DNA) adducts in peripheral blood mononuclear cells (PBMCs) after incubation of whole blood with modulators for 0.5 h, followed by cisplatin incubation (first experiment). (b) Time-dependent formation of Pt–DNA adducts in PBMCs after incubation of whole blood with cisplatin for 3 h followed by the addition of modulators (second experiment).

In addition to the analyses of the total amount of Pt–DNA adducts, analytical separation of the two major cisplatin–DNA adducts Pt–GG and Pt–AG was applied to obtain information regarding the type of adducts formed in the samples and the ratio of Pt–GG and Pt–AG. Pt–GG and Pt–AG formation was followed through time. The HPLC–ICP-MS chromatograms of the two samples are depicted in Fig. 5. Figure 5a shows the reaction of cisplatin alone with DNA after 1, 3, and 6 h of incubation

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

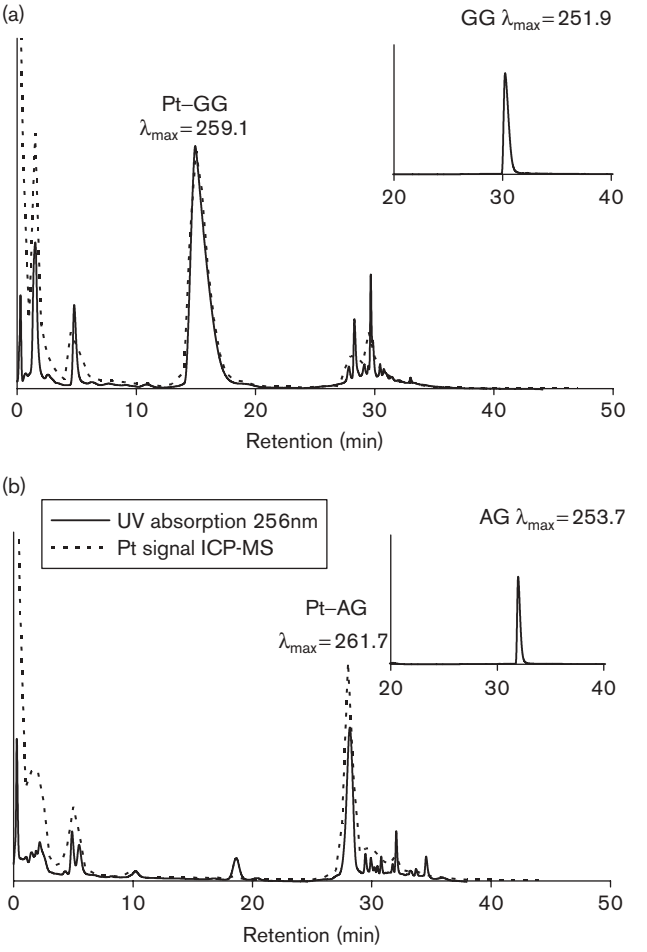
and Fig. 5b shows the reaction of cisplatin with DNA after the addition of AC. The peak at 15 min represents Pt–GG, whereas the small peak at 27 min corresponds to Pt–AG. The increase in the levels of Pt–GG and Pt–AG analyzed by HPLC–ICP-MS with time were in agreement with the increase observed for the total amount of Pt–DNA adducts as analyzed by ICP-MS. Owing to the low injection volume (10 µl) and the dilution before ICP-MS analyses, samples, however, were diluted 250-fold compared with ICP-MS alone. Therefore, unfortunately, sensitivity decreased and for the samples containing low concentrations of adducts, no adequate determination of Pt–GG and Pt–AG could be performed. For that reason, the separation method was used only to gain information on the retention time and the ratios of the adduct peaks formed with or without the presence of modulators. None of the chromatograms revealed a different pattern of peaks and the ratios remained constant, which suggests that the type of adducts formed as similar for all the samples.

Fig. 5



(a) Time-dependent formation of platinum–DNA (Pt–DNA) adducts in peripheral blood mononuclear cells (PBMCs) after incubation of whole blood with cisplatin. (b) Time-dependent formation of Pt–DNA adducts in PBMCs after incubation of whole blood with cisplatin preceded by the incubation with acetylcysteine. ICP-MS, inductively coupled plasma mass spectrometry.

Fig. 6



(a) HPLC-UV including λ_{max} and ICP-MS chromatogram of GpG after in-vitro reaction with cisplatin. The HPLC-UV chromatogram and λ_{max} of GpG are also shown. (b) HPLC-UV including λ_{max} and ICP-MS chromatogram of ApG after in-vitro reaction with cisplatin. The HPLC-UV chromatogram and λ_{max} of ApG are also shown. HPLC-UV, high performance liquid chromatography with ultraviolet radiation; ICP-MS, inductively coupled plasma mass spectrometry

The identities of the Pt–GG and Pt–AG peaks were confirmed by chromatography of the in-vitro reaction products of cisplatin with GpG and ApG. Ultraviolet and ICP-MS chromatograms are shown in Fig. 6a and b. The large figures show the chromatograms of the incubation mixture, whereas the small figures represent the chromatograms of the dinucleosides GpG and ApG. The major peaks visible in the chromatograms correspond to Pt–GG (15.5 min) and Pt–AG (27 min). The large peak at 1 min is caused by cisplatin, whereas the other peaks, most probably, are caused by other adducts formed in the incubation mixtures, such as GG–Pt–GG and AG–Pt–AG. The identities of the Pt–GG and Pt–AG peak were confirmed by the absorption maximum of the peaks, which were shifted to lower energy (higher λ_{max})

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

compared with the unreacted dinucleosides [24]. Furthermore, the retention times of the adduct peaks were shorter than those of the unreacted dinucleosides indicating that the hydrophobicity of the dinucleosides was reduced by cisplatin.

Discussion

Cisplatin has developed into a frequently used chemotherapeutic agent. Its use, however, is hampered by severe toxicities, which can seriously affect the quality of life. Treatment strategies that aim to reduce the side effects without affecting the antitumor activity are frequently tested.

The aim of this study was to investigate the effect of the compounds STS, GSH, AC, and gemcitabine on the Pt–protein and Pt–DNA binding of cisplatin in whole blood. This was done to gain more insight into Pt binding in whole blood and the effect of modulators on this process. Thereby, information is gained on the effect of the modulators on the activity of cisplatin and the potential use of sulfur-containing compounds for the reduction or prevention of side effects and, potentially, for assisting the recovery of these side effects *in vivo*.

Initially, the effect of STS, GSH, AC, and gemcitabine on Pt–protein binding was evaluated. A reduced Pt–protein binding suggests a lower reactivity of Pt present in the bloodstream and this could lead to reduced cytotoxicity. After 6 h of incubation of cisplatin, 85% was bound to blood constituents that were larger than 30 kDa, which is close to the binding of Pt to blood constituents $> 95\%$ in the human body, as calculated from the area under the plasma and free plasma concentration time curves [3]. Gemcitabine did not show an effect on Pt–protein binding, which was expected, because the nitrogen groups in gemcitabine do not have a stronger nucleophilic character than the sulfur groups in proteins [25]. Therefore, Pt–protein binding will be preferred over Pt–gemcitabine binding. GSH and AC, on the other hand seemed to be capable of inhibiting Pt–protein binding to a large extent. Interestingly, Pt–protein binding was found to be almost completely inhibited by STS. A large inhibition (46%) of the ex-vivo Pt–protein binding by STS was also described by Elferink *et al.* [26]. The superior effect of STS on Pt–protein binding compared with GSH and AC may be explained by the electrostatic interaction between the partially positively charged hydrated product of cisplatin and the doubly negatively charged thiosulfate. STS has a more nucleophilic character than GSH and AC [27]. Another issue that might explain the differences in effects between the compounds, could be the deviating molar concentrations used in the current experiments. The STS, GSH, and AC concentrations chosen for the current research were in accordance with the doses of the compounds that are generally administered to patients. Molar ratios were 23 mmol/l STS : 1.6 mmol/l GSH : 13 mmol/l AC.

In addition to the inhibition of Pt–protein binding, AC and STS seemed to be capable of removing Pt from proteins. As the thermodynamic strength of a typical coordination bond such as the Pt–ligand bond is much weaker than a covalent bond, the ligands of Pt compounds can usually be exchanged easily [28]. The observation that Pt in the ultrafiltrable fraction was increased after the addition of AC and STS suggests that these compounds might have shifted the reaction equilibrium of the Pt–protein coordination complex, resulting in a decreased level of Pt–protein complexes. It might be that AC and STS are stronger Lewis bases than most of the sulfur groups present in the proteins or that interactions of AC and STS with Pt are impeded less by stericeffects and are thus thermodynamically more stable than the interactions of proteins with Pt. The possibility of releasing Pt from proteins might result in an increased rate of elimination of Pt from the body and in that way in a reduced toxicity [15]. The ability of STS to remove Pt from proteins shown in this study, however, is in contrast to an earlier ex-vivo investigation by Elferink *et al.* [26], who mentioned that STS was not able to reverse Pt–protein binding to a major extent. Hence, before drawing definite conclusions from the current results, the effect of sulfur-containing compounds on protein structures and proteolysis should be investigated. If sulfur-containing compounds could affect the tertiary protein structure or could induce proteolysis, an increased amount of bound Pt would be recovered in the ultrafiltrate.

The ultrafiltrable Pt fraction observed after incubation with sulfur-containing compounds was expected to contain smaller amounts of reactive Pt compared with those in the samples without sulfur-containing compounds. A previous investigation showed a decreased reactivity of the ultrafiltrable Pt in patients who received coadministration of STS, indicating that the Pt–STS complex was not reactive [29]. The Pt reactivity in this study was measured as the ability of ultrafiltrable Pt to bind to diethyldithiocarbamate. Other investigations reported that the ultrafiltrable fraction after STS coadministration contained significantly less unchanged cisplatin than the fraction without STS treatment [12,30]. Lower concentrations of reactive Pt can, of course, lead to lower toxicity.

To assess the reactivity of Pt present in the samples of the current research, we investigated the Pt–DNA binding reactivity in PBMCs. Pretreatment of the samples with gemcitabine seemed to slightly inhibit Pt–DNA binding. Currently, no explanation for this interaction is known. No effect of gemcitabine on the protein level was observed and the interaction with Pt is

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

expected to occur intracellulary. The highly significant effect as was observed by Crul *et al.* [13], however, was absent in the present investigation. A reason for this inconsistency could be the difference in experimental circumstances. Crul *et al.* [13] evaluated the effect of gemcitabine on Pt–DNA binding *in vivo*, whereas the current experiments were performed *ex vivo*. The Pt–DNA binding observed for GSH and AC were 18 and 80%, respectively, lower than that observed for cisplatin alone. STS seemed to completely prevent Pt–DNA adduct binding. The inhibition of Pt–DNA by the modulators is a relevant parameter for the reactivity of Pt and, thus, for the potential to prevent toxicity. The current data suggest that all of the tested sulfur-containing compounds may reduce toxicity and that STS is the most potent compound. Concurrent administration of cisplatin with sulfur-containing compounds might affect side effects, as well as efficacy. Therefore, dosing regimens and administration routes should be selected carefully, as illustrated by the intra-arterial cisplatin treatment with intravenously administered STS for the treatment of head and neck cancer [4].

In addition to the ability of the modulators to prevent Pt–DNA binding, it was also investigated whether the modulators could release Pt from the DNA. For STS, the distribution of which is limited to the extracellular space [26], it is unlikely that it can directly interact with the Pt–DNA adducts and thereby release Pt. AC, however, is able to enter cells [31] and, thus, might be able to release Pt from the DNA. The current experiments showed that, although AC and STS completely inhibited further Pt–DNA binding when they were administered 3 h after the start of cisplatin incubation, they could hardly release Pt from the DNA. GSH also limited Pt–DNA binding, but did not prevent it. These observations are in line with observations in previous investigations that suggested that platinum species might migrate from S to N donor ligands [32,33], implying that Pt–DNA bonds at GG sites are thermodynamically more stable than Pt–S bonds.

These data imply that, to be protective, the time of administration of the compound is relevant, because none of the compounds could obviously release Pt from the DNA. The cytotoxic protective effect should be initiated before the binding of cisplatin to the DNA. As mentioned before for STS, protection against nephrotoxicity was accomplished only when STS was given within 1 h before and 30 min after cisplatin administration [34]. Furthermore, the observation that no Pt was released from the DNA implies that the tested compounds are probably not capable of reducing persistent toxicity.

In addition to the total amount of Pt–DNA adducts, the different adducts formed after separation using HPLC were studied. Incubation with STS, GSH, AC, and gemcitabine did not result in different adducts. Furthermore, the ratio of Pt–GG and Pt–AG remained constant under all conditions. These results suggest that the DNA binding mode of cisplatin is not modified by STS, GSH, AC, and gemcitabine.

In conclusion, this study showed that the reactive Pt levels in whole blood are reduced by STS, GSH, and AC after ex-vivo incubation of whole blood with cisplatin. This reduction was demonstrated by reduced Pt–protein and Pt–DNA binding in the presence of the sulfur-containing compounds. Consequently, STS, GSH, and AC could prevent cisplatin-induced side effects. It is, however, not expected that these compounds might reduce persistent toxicities, because they were not able to release Pt from the DNA to a large extent. Hence, large activity of the compounds is only expected when they are administered during or shortly after cisplatin treatment, which may have a large impact on cytotoxicity. The minor effect of gemcitabine on Pt–DNA binding needs to be evaluated further in future experiments. The ex-vivo effects observed in this study are expected to be larger than the in-vivo effects, because the exposure time to the modulators will be shorter *in vivo*, owing to elimination of the compounds from the blood. This study, however, provides further insight into the potential effects and use of STS, GSH, AC, and gemcitabine in patients treated with cisplatin.

Acknowledgements

The authors thank The Netherlands Organization for Health Research and Development, ZonMw, for financial support.

References

1 Mandal R, Teixeira C, Li XF. Studies of cisplatin and hemoglobin interactions using nanospray mass spectrometry and liquid chromatography with inductively-coupled plasma mass spectrometry. *Analyst* 2003; **128**: 629–634.
2 Calvert H, Judson I, van der Vijgh WJ. Platinum complexes in cancer medicine: pharmacokinetics and pharmacodynamics in relation to toxicity and therapeutic activity. *Cancer Surv* 1993; **17**:189–217.
3 Graham MA, Lockwood GF, Greenslade D, Brienza S, Bayssas M, Gamelin E. Clinical pharmacokinetics of oxaliplatin: a critical review. *Clin Cancer Res* 2000; **6**:1205–1218.
4 Hoebers FJ, Pluim D, Verheij M, Balm AJ, Bartelink H, Schellens JH, *et al.* Prediction of treatment outcome by cisplatin-DNA adduct formation in patients with stage III/IV head and neck squamous cell carcinoma, treated by concurrent cisplatin-radiation (RADPLAT). *Int J Cancer* 2006; **119**: 750–756.
5 Howell SB, Pfeifle CE, Wung WE, Olshen RA. Intraperitoneal cis-diamminedichloroplatinum with systemic thiosulfate protection. *Cancer Res* 1983; **43**:1426–1431.
6 Markman M, Howell SB, Lucas WE, Pfeifle CE, Green MR. Combination intraperitoneal chemotherapy with cisplatin, cytarabine, and doxorubicin for refractory ovarian carcinoma and other malignancies principally confined to the peritoneal cavity. *J Clin Oncol* 1984; **2**:1321–1326.
7 Van Rijswijk RE, Hoekman K, Burger CW, Verheijen RH, Vermorken JB. Experience with intraperitoneal cisplatin and etoposide and i.v. sodium thiosulphate protection in ovarian cancer patients with either pathologically complete response or minimal residual disease. *Ann Oncol* 1997; **8**: 1235–1241.
8 Madasu R, Ruckenstein MJ, Leake F, Steere E, Robbins KT. Ototoxic effects of supradose cisplatin with sodium thiosulfate neutralization in patients with

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

head and neck cancer. *Arch Otolaryngol Head Neck Surg* 1997; **123**: 978–981.
9 Di Re F, Bohm S, Oriana S, Spatti GB, Zunino F. Efficacy and safety of high-dose cisplatin and cyclophosphamide with glutathione protection in the treatment of bulky advanced epithelial ovarian cancer. *Cancer Chemother Pharmacol* 1990; **25**:355–360.
10 Dickey DT, Wu YJ, Muldoon LL, Neuwelt EA. Protection against cisplatin-induced toxicities by *N*-acetylcysteine and sodium thiosulfate as assessed at the molecular, cellular, and in vivo levels. *J Pharmacol Exp Ther* 2005; **314**:1052–1058.
11 Filipski J, Kohn KW, Prather R, Bonner WM. Thiourea reverses cross-links and restores biological activity in DNA treated with dichlorodiaminoplatinum (II). *Science* 1979; **204**:181–183.
12 Brouwers EEM, Huitema ADR, Beijnen JH, Schellens JHM. Long-term platinum retention after treatment with cisplatin and oxaliplatin. Submitted 2007.
13 Crul M, Schoemaker NE, Pluim D, Maliepaard M, Underberg RW, Schot M, *et al*. Randomized phase I clinical and pharmacologic study of weekly versus twice-weekly dose-intensive cisplatin and gemcitabine in patients with advanced non-small cell lung cancer. *Clin Cancer Res* 2003; **9**: 3526–3533.
14 Brouwers EEM, Tibben MM, Rosing H, Hillebrand MJX, Joerger M, Schellens JHM, *et al*. Sensitive inductively coupled plasma mass spectrometry assay for the determination of platinum originating from cisplatin, carboplatin, and oxaliplatin in human plasma ultrafiltrate. *J Mass Spectrom* 2006; **41**:1186–1194.
15 Leone R, Fracasso ME, Soresi E, Cimino G, Tedeschi M, Castoldi D, *et al*. Influence of glutathione administration on the disposition of free and total platinum in patients after administration of cisplatin. *Cancer Chemother Pharmacol* 1992; **29**:385–390.
16 Prescott LF, Donovan JW, Jarvie DR, Proudfoot AT. The disposition and kinetics of intravenous *N*-acetylcysteine in patients with paracetamol overdosage. *Eur J Clin Pharmacol* 1989; **37**:501–506.
17 Cardozo RH, Edelman IS. The volume of distribution of sodium thiosulfate as a measure of the extracellular fluid space. *J Clin Invest* 1952; **31**:280–290.
18 Bianchi G, Bugianesi E, Ronchi M, Fabbri A, Zoli M, Marchesini G. Glutathione kinetics in normal man and in patients with liver cirrhosis. *J Hepatol* 1997; **26**:606–613.
19 Rademaker-Lakhai JM, Crul M, Pluim D, Sparidans RW, Baas P, Beijnen JH, *et al*. Phase I clinical and pharmacologic study of a 2-weekly administration of cisplatin and gemcitabine in patients with advanced non-small cell lung cancer. *Anticancer Drugs* 2005; **16**:1029–1036.
20 Siegel-Lakhai WS, Crul M, Zhang S, Sparidans RW, Pluim D, Howes A, *et al*. Phase I and pharmacological study of the farnesyltransferase inhibitor tipifarnib (Zarnestra, R115777) in combination with gemcitabine and cisplatin in patients with advanced solid tumours. *Br J Cancer* 2005; **93**:1222–1229.
21 Pluim D, Maliepaard M, van Waardenburg RC, Beijnen JH, Schellens JHM. ^{32}P-postlabeling assay for the quantification of the major platinum-DNA adducts. *Anal Biochem* 1999; **275**:30–38.
22 Ma J, Verweij J, Planting AS, Boer-Dennert M, van Ingen HE, van der Burg ME, *et al*. Current sample handling methods for measurement of platinum-DNA adducts in leucocytes in man lead to discrepant results in DNA adduct levels and DNA repair. *Br J Cancer* 1995; **71**:512–517.
23 Blommaert FA, Saris CP. Detection of platinum-DNA adducts by ^{32}P-postlabelling. *Nucleic Acids Res* 1995; **23**:1300–1306.
24 Hann S, Zenker A, Galanski M, Bereuter TL, Stingeder G, Keppler BK. HPIC-UV-ICP-SFMS study of the interaction of cisplatin with guanosine monophosphate. *Fresenius J Anal Chem* 2001; **370**:581–586.
25 Hay RW, Porter DS. The reaction of sulphur and nitrogen nucleophiles with $[Pt(dien)Cl]^+$. *Transition Met Chem* 1999; **24**:186–188.
26 Elferink F, van der Vijgh WJ, Klein I, Pinedo HM. Interaction of cisplatin and carboplatin with sodium thiosulfate: reaction rates and protein binding. *Clin Chem* 1986; **32**:641–645.
27 Videhult P, Laurell G, Wallin I, Ehrsson H. Kinetics of cisplatin and its monohydrated complex with sulfur-containing compounds designed for local otoprotective administration. *Exp Biol Med (Maywood)* 2006; **231**: 1638–1645.
28 Reedijk J. New clues for platinum antitumor chemistry: kinetically controlled metal binding to DNA. *Proc Natl Acad Sci U S A* 2003; **100**:3611–3616.
29 Goel R, Cleary SM, Horton C, Kirmani S, Abramson I, Kelly C, *et al*. Effect of sodium thiosulfate on the pharmacokinetics and toxicity of cisplatin. *J Natl Cancer Inst* 1989; **81**:1552–1560.
30 Nagai N, Hotta K, Yamamura H, Ogata H. Effects of sodium thiosulfate on the pharmacokinetics of unchanged cisplatin and on the distribution of platinum species in rat kidney: protective mechanism against cisplatin nephrotoxicity. *Cancer Chemother Pharmacol* 1995; **36**:404–410.
31 McLellan LI, Lewis AD, Hall DJ, Ansell JD, Wolf CR. Uptake and distribution of *N*-acetylcysteine in mice: tissue-specific effects on glutathione concentrations. *Carcinogenesis* 1995; **16**:2099–2106.
32 Reedijk J. Why does cisplatin reach guanine-N7 with competing S-donor ligands available in the cell? *Chem Rev* 1999; **99**:2499–2510.
33 Chen Y, Guo ZD, Murdoch PD, Zang EL, Sadler PJ. Interconversion between S- and N-bound L-methionine adducts of $Pt(dien)2^+$ (dien = diethylenetriamine) via dien ring-opened intermediates. *J Chem Soc, Dalton Trans* 1998; **9**:1503–1508.
34 Gandara DR, Wiebe VJ, Perez EA, Makuch RW, DeGregorio MW. Cisplatin rescue therapy: experience with sodium thiosulfate, WR2721, and diethyldithiocarbamate. *Crit Rev Oncol Hematol* 1990; **10**:353–365.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.