β-Lyase-Dependent Attenuation of Cisplatin-Mediated Toxicity by Selenocysteine Se-Conjugates in Renal Tubular Cell Lines

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ABSTRACT

Cisplatin [cis-diaminedichloroplatinum(II)] is a widely used antitumor drug with dose-limiting nephrotoxic side effects due to selective toxicity to the proximal tubule. In the present study, the chemoprotective potential of three selenocysteine Se-conjugates, Se-methyl-L-selenocysteine, Se-(2-methoxyphenyl)-L-selenocysteine, and Se-(2-chlorobenzyl)-L-selenocysteine, belonging to three structural classes, against the nephrotoxic effects of cisplatin was investigated. Selenocysteine Se-conjugates have previously been proposed as kidney-selective prodrugs of pharmacologically active selenols because of their active uptake and bioactivation by cysteine conjugate β-lyases in the kidney. To elucidate whether chemoprotection is β-lyase-dependent wild-type LLC-PK1 cells, possessing a very low β-lyase activity, and LLC-PK1 cells stably transfected with full-length cDNA coding for rat kidney cysteine conjugate β-lyase/glutamine transaminase K (R1J) were used. The results indicate that all three selenocysteine Se-conjugates were able to attenuate the cisplatin-induced loss of viability in R1J cells but not in the parental LLC-PK1 cells, as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and neutral red uptake. In addition, cisplatin-induced reactive oxygen species (ROS) production was determined using 2′,7′-dichlorodihydrofluorescein diacetate. The selenocysteine Se-conjugates were able to decrease ROS levels after cisplatin exposure in both cell types. However, this ROS-protective effect was more profound in R1J cells. Se-Methyl-L-selenocysteine provided the strongest protection. The protective activity against cisplatin-induced cytotoxicity and ROS generation was blocked by aminooxyacetic acid, a selective inhibitor of pyridoxal 5′-phosphate-dependent cysteine conjugate β-lyases, further supporting the role of β-lyase in the observed chemoprotection. The precise molecular mechanism by which selenols, generated by β-lyase, provide protection against cisplatin-induced cytotoxicity, however, remains to be established.

Several selenocysteine Se-conjugates (SeCys-conjugates; Fig. 1) have recently been shown to be very potent chemopreventive agents in rat tumor models (Ip, 1998; Ip et al., 1999). Se-Allyl-DL-selenocysteine has even been claimed to be the most active chemopreventive selenium compound so far identified. After dietary administration at 2 ppm, a reduction of almost 90% of methylnitrosourea-induced mammary tumors in rats was achieved (Ip et al., 1999). In addition to this cancer prevention activity, SeCys-conjugates, of which Se-methyl-DL-selenocysteine is most extensively investigated, inhibit cell proliferation and induce apoptosis in several tumor cells (Ip, 1998; Sinha et al., 1999; Kim et al., 2001).

Furthermore, Se-methyl-DL-selenocysteine inhibits angiogenesis in mammary cancer at chemopreventive levels of intake (Jiang et al., 1999). Despite their promising pharmacological properties the mechanism of action of SeCys-conjugates remains to be elucidated. Bioactivation of these compounds resulting in the formation of their corresponding selenols, selenenic acids, and/or seleninic acids is considered to be essential for the pharmacological activity (Andreadou et al., 1996a; Ip, 1998). Enzymes involved in the bioactivation of SeCys-conjugates into selenols are pyridoxal 5′-phosphate (PLP)-dependent β-lyases, such as cysteine conjugate β-lyase/glutamine transaminase K (β-lyase/GTK) (Fig. 1; Commandeur et al., 2000) and amino acid oxidases (Rooseboom et al., 2001b). In addition, flavin-containing monoxygenases have been shown to bioactivate SeCys-conjugates into their corresponding selenenic acids via a

ABBREVIATIONS: SeCys, selenocysteine Se; PLP, pyridoxal 5′-phosphate; β-lyase/GTK, cysteine conjugate β-lyase/glutamine transaminase K; ROS, reactive oxygen species; PTC, proximal tubular cell; AOAA, aminooxyacetic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MSeCys, Se-(2-methoxyphenyl)-L-selenocysteine; H2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; MPSeCys, Se-(2-chlorobenzyl)-L-selenocysteine; TFE-Cys, S-(1,1,2,2-tetrafluoroethyl)-L-cysteine; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; ANOVA, analysis of variance.
selenol oxidation syn-elimination mechanism (Rooseboom et al., 2001a).

Besides cancer prevention and antitumor activity, another potential application of SeCys-conjugates may be their use as chemoprotectors against toxic side effects of drugs and other xenobiotics. SeCys-conjugates were proposed as kidney-selective prodrugs (Andreadou et al., 1996a) because they are expected to be actively transported into the proximal tubular cells and bioactivated locally in the kidney due to the high renal β-lyase activity (Commandeur et al., 1995). Therefore, in the present study cisplatin [cis-diaminedichloroplatinum(II)] was selected as a model compound to delineate the chemoprotective potential of SeCys-conjugates against nephrotoxins.

Cisplatin is a widely used drug for the treatment of a variety of human neoplasms; however, severe nephrotoxicity and peripheral neuropathy are dose-limiting side effects (Walker and Walker, 1999). Renal toxicity is observed after several days, mainly in the S2-region of the proximal tubule and bioactivated locally in the kidney due to the high renal β-lyase activity (Trimmer and Essigmann, 1999). Numerous agents have been evaluated as chemoprotectors against cisplatin-induced toxicity, however, none of them are used clinically (for review, see Treskes and van der Vlijgh, 1993; Walker and Walker, 1999). Furthermore, antioxidant enzymes and several antioxidants were shown to protect against cisplatin-induced renal damage, indicating an important role for reactive oxygen species (ROS) in the cisplatin-induced cytotoxicity (McGinness et al., 1978; Dobyan et al., 1986; Lieberthal et al., 1996). The selenium compounds sodium selenite and ebselen have also been shown to protect against the nephrotoxicity of cisplatin without reducing its antitumor activity (Baldew et al., 1989, 1990, 1991). As a mechanism of action, glutathione-dependent bioactivation leading to selenol derivatives and subsequent covalent binding to or reduction of reactive cisplatin metabolites has been proposed. Although this indicates that selenium compounds can serve as chemoprotectors, sodium selenite and ebselen are highly reactive thiol agents. Because SeCys-conjugates, which are not activated by reaction with thiols, are also prodrugs of selenols, it might be possible that these compounds reduce the nephrotoxicity of cisplatin similarly to, for example, ebselen.

In the present study, LLC-PK1 cells were used as an in vitro model to investigate whether SeCys-conjugates are able to protect against the cytotoxicity of cisplatin, taken as a model compound. This cell line retains many characteristics of proximal tubular cells and has been used previously to study the nephrotoxicity of cisplatin and the effect of chemoprotectors (Gstraunthaler et al., 1985; Montine and Borch, 1990; Baldew et al., 1992). To study the role of bioactivation of SeCys-conjugates into their corresponding selenols, we used parental LLC-PK1 cells as well as R1J cells. R1J cells are LLC-PK1 cells stably transfected with full-length cDNA coding for rat cysteine conjugate β-lyase/GTK in which the β-lyase activity is comparable with freshly isolated proximal tubular cells (PTCs) (Goldfarb et al., 1996). This cell line has previously been used to investigate the nephrotoxicity of S-(1,2-dichlorovinyl)-l-cysteine, which is bioactivated into the corresponding toxic thiol by this particular enzyme (Commandeur et al., 1995; Goldfarb et al., 1996). The effect of SeCys-conjugates on several toxic responses, including ROS response and cytotoxicity, after exposure to cisplatin was evaluated in the two cell models.

**Materials and Methods**

**Chemicals.** Aminoxyacetic acid (AOAA) was purchased from Aldrich Chemical Co. (Beers, Belgium). Cisplatin, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red, Se-methyl-l-selenocysteine (MSeCys), ipegal CA-630, sodium deoxycholate, phenylmethylsulfonyl fluoride, aprotinin from bovine lung (5–10 U/ml), sodium orthovanadate, and α-keto-γ-methylbutyric acid were obtained from Sigma-Aldrich (St. Louis, MO). o-Phenylendiamine was from Janssen Chimica (Geel, Belgium). Sodium dodecyl sulfate was purchased from Fluka (Buchs, Switzerland). 2′,7′-Dichlorodihydrofluorescein diacetate (H2DCF-DA) was obtained from Molecular Probes (D-399; Leiden, The Netherlands). Se-(2-Methoxyphenyl)-l-selenocysteine (MPSeCys, yield 57%, purity >98%) and Se-(2-chlorobenzyl)-l-selenocysteine (CBSsCys, yield 39%, purity >98%) were synthesized by Rooseboom et al. (2000). S-((1,2,2-Tetrafluoroethyl)-l-cysteine (TFE-Cys) was prepared as described by Commandeur et al. (1988). Medium 199, DMEM/F12, and cell culture supplements [penicillin, streptomycin, and fetal calf serum (FCS)] were purchased from Invitrogen (Breda, The Netherlands). All other chemicals were of the highest grade commercially available.

**Cell Culture.** LLC-PK1 cells (proximal tubule, pig kidney; Hull et al., 1976) were obtained from American Type Culture Collection (CL-101; Manassas, VA). R1J cells were a generous gift from Prof. Dr. G. G. Gibson (University of Surrey, Surrey, UK). LLC-PK1 and R1J cells were maintained as monolayer cultures in medium 199 supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete culture medium). Cells were routinely seeded at 4 × 10⁵ cells/cm² and were subcultured every 6 to 7 days. Two days before experiments, cells were plated at a density of 2.7 × 10⁶ cells/cm² of culture area. Cells were always maintained in a 5% CO₂ humidified atmosphere at 37°C. All test compounds were dissolved in dimethyl sulfoxide and diluted with complete culture medium resulting in a final concentration of 0.1% dimethyl sulfoxide.

**Western Blotting.** Cells were seeded on 100-mm culture dishes at 2 × 10⁶ cells/dish and cultured for 48 h. Subsequently, cells were rinsed with cold PBS and the cells were scraped in lysis buffer consisting of 1% (v/v) ipegal CA-360, 0.5% (v/v) sodium deoxycholate, and 0.1% (v/v) sodium dodecyl sulfate in PBS supplemented with 100 μg/ml phenylmethylsulfonyl fluoride, 30 μl/ml aprotinin, and 1 mM sodium orthovanadate. Samples were homogenized and centrifuged (12,000g, 5 min, 4°C), and protein from the supernatant (30 μg) from the respective samples was separated on SDS-polyacrylamide gels in a Mini-Protein 3 Cell (Bio-Rad, Veenendaal, The Netherlands) as described by Laemmli (1970) and electroblotted onto a polyvinylidene difluoride membrane (Immobilon P; Millipore, Etten-Leur, The Netherlands) according to the method of Towbin et al. (1979). Rabbit antiserum raised against highly purified rat β-lyase/GTK, kindly provided by Dr. A. Yamauchi (Kobe University, Kobe, Japan), was used for immunological staining (Commandeur et al., 2000). Densitometric analyses of Western blots were performed using Molecular Analyst software, version 1.5, from Bio-Rad (Hercules, CA).
confluence. Cells were scraped, collected in 2 ml of cold PBS, and stored at −20°C until analysis. After thawing the cell suspensions, the cells were lysed by repetitive freeze thawing in liquid nitrogen. The cell homogenates were dialyzed overnight against a 20-fold excess of potassium phosphate buffer (20 mM, pH 7.4), and the protein content was determined as described below. β-Lyase activity was measured by incubating substrates (final concentration 1 mM) in a total volume of 100 μl containing 0.5 mg of protein/ml of cell homogenate and 0.5 mM α-keto-γ-methylbutyric acid (cofactor) in 50 mM sodium borate buffer, pH 8.6. After 0 and 20 min, reactions were terminated by adding 500 μl of 0.14% α-phenylenediamine in 3 N HCl. Samples were derivatized, and pyruvate contents were analyzed as described previously (Roosseboon et al., 2000). To determine the role of PLP-dependent cysteine conjugate β-lyases, incubations were also performed in the presence of 1 mM aminooxyacetic acid. Similarly to experiments performed previously in cytosolic fractions, nonenzymatic degradation was not observed (Roosseboon et al., 2000).

**Determination of Cell Viability.** Cell viability was determined using the MTT and neutral red uptake assay. The MTT assay is based on the reduction of MTT to formazan by the mitochondrial enzyme succinate dehydrogenase (Denizot and Lang, 1986). Following the incubations described below, MTT solution (3 mg/ml in PBS) was added to the wells, resulting in a final concentration of 0.6 mg/ml. After 2 hours at 37°C in a humidified atmosphere, the medium was discarded, and the formazan product in each well was solubilized in 50 μl of acidic isopropanol (0.1 M HCl). Absorbance at 595 nm was determined on a microplate reader (Bio-Rad 3550) equipped with a spectrophotometer (Bio-Rad, Veenendaal, The Netherlands). Cell viability was expressed as (A<sub>540</sub>-treated cells/A<sub>540</sub> of appropriate control) × 100% after correction for background absorbance (100% cytoxicity).

The neutral red uptake assay was based on the lysosomal uptake of neutral red as described by Riddell et al. (1986). Briefly, following the incubation described below, neutral red solution (0.25 mg/ml) was added to the cell cultures at a final concentration of 50 μg/ml neutral red. After 2 hours at 37°C cells were rinsed twice with PBS. Subsequently, 50 μl of destaining buffer consisting of 1% (v/v) acetic acid, 50% (v/v) ethanol, and 49% (v/v) distilled water was added to the cells, and the culture plates were placed for 15 min on an orbital shaker. Lysosomal uptake of neutral red was determined spectrophotometrically at 540 nm on a microplate reader (Bio-Rad 3550) to quantify neutral red uptake. Viability was expressed as (A<sub>540</sub>-treated cells/A<sub>540</sub> of appropriate control) × 100% after correction for background absorbance (100% cytoxicity).

**Determination of ROS Production.** Cells were incubated with 20 μM H<sub>2</sub>DCA-DA as a probe to detect ROS in complete culture medium (supplemented medium 199). After 2-hour incubation at 37°C cells were rinsed once with PBS and exposed to the test compounds in phenol red-free culture medium (DMEM/F12) supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin as described below. ROS production was measured after 24 hours on a CytoFluor 2300 fluorescence measurement system (Millipore Corporation, Bedford, MA) with extinction wavelength of 485 nm and emission wavelength of 538 nm. ROS production was corrected for background fluorescence (control wells).

**Exposure of LLC-PK<sub>1</sub> and R1J Cells to TFE-Cys.** Cells were seeded in 96-well plates (1.0 × 10<sup>4</sup> cells/well) and allowed to grow for 48 hours. At that time, LLC-PK<sub>1</sub> and R1J cells were approximately 80% confluent. LLC-PK<sub>1</sub> and R1J cells were exposed to increasing concentrations of TFE-Cys (0, 0.125, 0.25, and 0.5 mM) in complete culture medium (supplemented medium 199). After 24 hours, cell viability was assessed by measuring cellular succinate dehydrogenase activity in the MTT assay (see above). For ROS determination cells were incubated with the ROS probe described above before a 24-hour TFE-Cys exposure in phenol red-free complete culture medium (supplemented DMEM/F12). The contribution of PLP-dependent β-lyase/GTK to TFE-Cys cytotoxicity was investigated by exposure of the cells to 0.25 mM aminooxyacetic acid simultaneously with TFE-Cys. To investigate whether α-keto-γ-methylbutyric acid, previously suggested to increase the toxicity of S-(1,2-dichlorovinyl)-L-cysteine (Stevens et al., 1986; Elfarra et al., 1987), can increase the cytotoxicity of TFE-Cys, and LLC-PK<sub>1</sub> and R1J cells were also exposed as described above and in the presence of this α-keto acid (5 mM). α-Keto-γ-methylbutyric acid itself had no effect on the cell viability.

**Effect of SeCys-Conjugates on Cisplatin-Induced Toxicity in LLC-PK<sub>1</sub> and R1J Cells.** Cells were seeded in 96-well plates (1.0 × 10<sup>4</sup> cells/well) and allowed to grow for 48 hours. At that time, LLC-PK<sub>1</sub> and R1J cells were approximately 80% confluent. After preincubation of the cells for 24 hours with increasing concentrations (0, 6.25, 12.5, 25, and 50 μM) of SeCys-conjugates in complete culture medium (supplemented medium 199), cells were rinsed with PBS. Subsequently, cells were exposed to increasing concentrations of cisplatin (0, 12.5, 25, and 50 μM) in complete culture medium. Cell viability was assessed after 24 hours by MTT, and neutral red uptake assay was assessed as described above. To determine ROS formation, cells were incubated with SeCys-conjugates and were subsequently exposed to the ROS probe as described above before cisplatin (0 and 50 μM) exposure in phenol red-free complete culture medium (supplemented DMEM/F12). The role of PLP-dependent β-lyase/GTK in the chemoprotection by SeCys-conjugates against cisplatin-induced loss of cell viability and ROS production was determined. Cells were preincubated for 24 hours with 50 μM SeCys and 0.25 mM aminooxyacetic acid in complete culture medium. To assess cell viability, cells were rinsed after 24 hours with PBS and exposed to 25 μM cisplatin for 24 hours, and viability was determined as described above. To assess ROS formation, cells were exposed to the ROS probe after the preincubation, as described above, and exposed to 50 μM cisplatin for 24 hours. Cells were also incubated simultaneously with cisplatin and SeCys-conjugates.

**Protein Determination.** The BCA protein assay (catalog no. 23225; Pierce Chemical, Rockford, IL) was used to determine the protein contents of the samples. The assay was performed according to the manufacturer’s instructions.

**Statistical Analysis.** Statistical evaluation of the results was performed using the Student’s t test. Differences were considered significant if P was less than 0.05 (*), 0.01 (**), and 0.001 (***) comparisons between the SeCys-conjugates as an effect of dose were performed by two-way ANOVA, and differences were considered significant if P was less than 0.05.

**Results**

**Western Blotting and β-Lyase Activity in Cell Homogenates of LLC-PK<sub>1</sub> and R1J Cells.** The presence of functional β-lyase/GTK in R1J cells was evaluated by Western blot analysis and determination of β-lyase activity. The membranes were developed with rabbit antiserum raised against the monomer of highly purified rat β-lyase/GTK. As shown in Fig. 2, a protein with identical gel mobility as β-lyase/GTK (48.5 kDa) was stained in R1J cells and in freshly prepared rat PTCs. No immunoreactive protein was observed in homogenates of LLC-PK<sub>1</sub> cells because these pig kidney epithelial cells do not express rat β-lyase/GTK and porcine β-lyase/GTK does not cross-react with antibody to the rat enzyme. In addition to Western blotting, β-lyase activity was measured in cell homogenates of LLC-PK<sub>1</sub> and R1J cells. TFE-Cys was bioactivated in cell homogenates of the R1J cells as measured by the formation of pyruvate (Table 1). This bioactivation was blocked by addition of aminooxyacetic acid, indicating that this bioactivation was completely dependent on PLP-dependent β-lyases (Table 1). In homogenates of the LLC-PK<sub>1</sub> cells, the rate of pyruvate formation from TFE-
Cys was much lower compared with that in R1J cells (Table 1). Similar results were obtained regarding the bioactivation of SeCys-conjugates by homogenates of the R1J cells (Table 1). LLC-PK1 cell homogenates showed 8- to 12-fold lower bioactivity toward these conjugates compared with R1J cell homogenates. MSeCys was most efficiently bioactivated by LLC-PK1 and R1J cell homogenates (Table 1).

Cytotoxicity of TFE-Cys in LLC-PK1 and R1J Cells.
To compare the sensitivity of parental LLC-PK1 and R1J cells toward \( \beta \)-lyase-dependent nephrotoxins, both cells were exposed to increasing concentrations of TFE-Cys. A similar experiment to compare the sensitivity of these two cell types toward nephrotoxins was previously performed with \( S \)-(1,2-dichlorovinyl)-L-cysteine as a substrate (Goldfarb et al., 1996). Exposure of LLC-PK1 cells and R1J cells to TFE-Cys resulted in a concentration-dependent loss in cell viability as determined by the MTT assay (Fig. 3, A and B). Aminoxyacetic acid and TFE-Cys exposure did not result in ROS formation in either cell line (data not shown).

Cisplatin-Induced Cytotoxicity and ROS Production.
Cisplatin was cytotoxic to both cell lines because the formation of formazan by succinate dehydrogenase (MTT assay) was concentration-dependently decreased following exposure of the cells to cisplatin for 24 h (Fig. 4, A–F). Comparable results were obtained measuring lysosomal neutral red uptake after cisplatin exposure, as has been shown previously (Table 2). Cisplatin treatment also resulted in a concentration-dependent increase in ROS production (data not shown). This ROS response, which was demonstrated previously in the LLC-PK1 cell line upon cisplatin exposure (Schaaf et al., 2002), was observed to the same extent in both cell lines.

Cytotoxicity of SeCys-Conjugates. SeCys-conjugates themselves were not cytotoxic at concentrations up to 50 \( \mu \)M and
Fig. 4. Effect of treatment (preincubation) with 0, 6.25, 12.5, 25, and 50 μM SeCys-conjugates on the cisplatin-mediated loss of viability in LLC-PK1 and R1J cells. Treatment of LLC-PK1 cells with MSeCys (A), LLC-PK1 cells with MPSeCys (B), LLC-PK1 cells with CBSeCys (C), R1J cells with MSeCys (D), R1J cells with MPSeCys (E), and R1J cells with CBSeCys (F). Cells were preincubated with SeCys-conjugates or medium for 24 h, followed by exposure to cisplatin for 24 h. Cell viability was determined by the MTT assay, as described under Materials and Methods. Results are presented as mean (±S.D., <5%) from quadruplicate measurements from at least three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared with cisplatin-treated control. Comparisons between SeCys-conjugates were performed by two-way ANOVA and were only performed for R1J cells because attenuation of cell viability by SeCys-conjugates was only observed in these cells: MSeCys (D) and MPSeCys (E), P < 0.0001; MSeCys (D) and CBSeCys (F), P = 0.38; and MPSeCys (E) and CBSeCys (F), P < 0.0001.
Cytotoxicity of cisplatin, SeCys-conjugates, or combinations in LLC-PK₁ and R1J cells, as determined by lysosomal neutral red uptake

Experiments were performed as described under Materials and Methods with 50 μM cisplatin and 50 μM SeCys-conjugate. Results are presented as mean value ± S.D. from quadruplicate measurements from at least three independent experiments.

Effects of SeCys-Conjugates on Cisplatin-Induced Cytotoxicity. In initial experiments LLC-PK₁ and R1J cells were exposed to cisplatin and SeCys-conjugates simultaneously; however, the cisplatin-mediated loss of viability was not affected. Therefore, a 24-h preincubation was performed with the SeCys-conjugates before cisplatin exposure. For LLC-PK₁ cells, 24-h preincubation with SeCys-conjugates did not result in any protection against cisplatin-induced loss of viability (Fig. 4, A–C). However, 24-h preincubation of R1J cells with all three SeCys-conjugates tested, MSeCys, MPSeCys, and CBSeCys, up to 50 μM (Fig. 4, D–F). The most toxic SeCys-conjugate was MPSeCys, resulting in 60% loss of viability at 50 μM (Fig. 4E). Exposure of both cells to the SeCys-conjugates did not result in a change in the ROS formation and lysosomal neutral red uptake compared with the untreated control (Table 2).

Effects of SeCys-Conjugates on Cisplatin-Induced ROS Response. Initially, LLC-PK₁ and R1J cells were exposed to cisplatin and SeCys-conjugates simultaneously; however, the cisplatin-mediated loss of viability was not affected. Therefore, a 24-h preincubation was performed with the SeCys-conjugates before cisplatin exposure. For LLC-PK₁ cells, 24-h preincubation with SeCys-conjugates did not result in any protection against cisplatin-induced loss of viability (Fig. 4, A–C). However, 24-h preincubation of R1J cells with all three SeCys-conjugates tested, concentration-dependently attenuated the loss of viability after exposure to 12.5, 25, and 50 μM cisplatin (Fig. 4, D–F). As was shown for the TFE-Cys experiments α-keto-γ-methiolbutyric acid did not influence the protective effect of SeCys-conjugates in either cell line (data not shown). MSeCys provided the strongest protection, as determined by the attenuation of the cisplatin-induced loss of viability (Fig. 4D). After pretreatment with 50 μM MSeCys and 12.5 μM cisplatin the viability was 93% of solvent-treated controls, which is much higher than after cisplatin exposure alone (62%). CBSeCys was slightly less effective than MSeCys in R1J cells, whereas MPSeCys preincubation provided the weakest protection (Fig. 4, E and F). Aminoxyacetic acid, a potent inhibitor of PLP-dependent β-lyases, was used to study the role of PLP-dependent β-lyases in the attenuation of cisplatin-induced loss of cell viability. R1J cells preincubated with MSeCys in the presence and absence of aminoxyacetic acid were subsequently exposed to cisplatin. As shown in Table 3, this potent PLP-dependent β-lyase inhibitor completely abolished the protective effects of MSeCys against cisplatin-induced loss of viability. In addition to the results obtained with the MTT assay, indicating mitochondrial damage, also the attenuation of lysosomal neutral red uptake, a cytotoxicity parameter, was determined. Preincubation of the R1J cells with MSeCys and CBSeCys for 24 h also partially protected these cells from a cisplatin-induced cytotoxicity (Table 2). However, MPSeCys was not able to protect the cells against cisplatin-mediated cytotoxicity, as determined by neutral red uptake (Table 2). All three SeCys-conjugates were ineffective in preventing the cisplatin-induced loss of lysosomal neutral red uptake in LLC-PK₁ cells (Table 2).

**TABLE 2**

Cytotoxicity of cisplatin, SeCys-conjugates, or combinations in LLC-PK₁ and R1J cells, as determined by lysosomal neutral red uptake

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LLC-PK₁</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>12.4 ± 1.7</td>
</tr>
<tr>
<td>MSeCys</td>
<td>97.2 ± 4.9</td>
</tr>
<tr>
<td>Cisplatin + MSeCys</td>
<td>11.9 ± 1.4</td>
</tr>
<tr>
<td>MPSeCys</td>
<td>96.0 ± 2.8</td>
</tr>
<tr>
<td>Cisplatin + MPSeCys</td>
<td>10.4 ± 0.9</td>
</tr>
<tr>
<td>CBSeCys</td>
<td>100.5 ± 6.4</td>
</tr>
<tr>
<td>Cisplatin + CBSeCys</td>
<td>10.0 ± 1.9</td>
</tr>
</tbody>
</table>

*Significantly different from cisplatin-treated R1J cells (P = 0.001).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viabilitya</th>
<th>ROSb %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>25 ± 0.6</td>
<td>100 ± 5.2</td>
</tr>
<tr>
<td>Cisplatin + 50 μM MSeCys</td>
<td>42 ± 2.3</td>
<td>9 ± 0.9</td>
</tr>
<tr>
<td>Cisplatin + 50 μM MSeCys + AOAA</td>
<td>25 ± 0.5</td>
<td>100 ± 7.7</td>
</tr>
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</table>

a Percentage of untreated control; MTT assay; 25 μM cisplatin.
b Percentage of cisplatin-treated control; 50 μM cisplatin.

**TABLE 3**

Effect of AOAA on the cisplatin protection by MSeCys in R1J cells

Experiments were performed as described under Materials and Methods. Results are presented as mean value ± S.D. for four preparations.

**Discussion**

The present study was performed to elucidate whether SeCys-conjugates are able to protect renal tubular cells (LLC-PK₁ cells) against cisplatin-induced cytotoxicity. In addition to the wild-type LLC-PK₁ cell line, cells stably expressing rat kidney β-lyase/GTK (R1J cells) were used. The latter cells were constructed by Goldfarb et al. (1996) by transfection of full-length cDNA coding for rat β-lyase/GTK (Perry et al., 1993) into the LLC-PK₁ cell line. Expression of immunoreactive β-lyase/GTK in R1J cells was confirmed by Western blotting. In agreement with this, the R1J cells showed an 8- to 12-fold higher β-lyase activity compared with the LLC-PK₁ cells, depending on the substrate used. A similar effect was reported previously by Goldfarb et al. (1996) using S-(1,2-dichlorovinyl)-L-cysteine as a substrate, although the β-lyase...
activity in R1J cells was up to 100-fold that of the parental cell line. As shown in the present study, the R1J cells showed a 3-fold higher sensitivity toward the nephrotoxic cysteine S-conjugate TFE-Cys at 0.5 mM compared with the parental cell line. In line with our present findings, MSeCys was previously shown to be nontoxic in freshly prepared rat proximal tubular cells (Andreadou et al., 1996b), although this compound induced apoptosis in several tumor cell lines (Ip, 1998; Sinha et al., 1999; Kim et al., 2001).

The results of the present study indicate that the tested SeCys-conjugates are able to attenuate the cisplatin-induced cytoxicity in R1J cells, but not in LLC-PK1 cells. Furthermore, all three tested SeCys-conjugates prevented the cisplatin-induced elevation of ROS levels. Accumulating evidence points to the contribution of radicals, such as superoxide anion and hydroxyl radicals, to cisplatin-mediated toxicity as we and others have reported (Masuda et al., 1994; Lieberthal et al., 1996; Matsushima et al., 1998; Shiraishi et al., 2000; Schaff et al., 2002). Correspondingly, antioxidant enzymes and several antioxidants were shown to protect against cisplatin-induced renal damage (McGinness et al., 1978; Dobyan et al., 1986; Lieberthal et al., 1996). Whether the observed attenuation of cisplatin-induced ROS levels by SeCys-conjugates results from the induction of antioxidant enzymes or from a direct antioxidant effect of SeCys-conjugates remains to be elucidated. Cisplatin has also been shown to bind to proteins and to deplete glutathione levels. Selenium compounds have been shown to interfere with protein thiol-groups by reaction of selenols with cysteine clusters (Ganter, 1999). Further research is needed to delineate whether a release of cisplatin-protein adducts by selenols, derived from SeCys-conjugates by β-lyases, contributes to the observed chemoprotection against cisplatin-induced cytotoxicity.

In this study, MSeCys showed the strongest chemoprotection against the cisplatin-induced loss of viability and cisplatin-induced ROS formation. Therefore, the present results indicate that the selenocysteine substituent influences the chemoprotective potential of SeCys-conjugates. Despite the fact that MSeCys also was the best β-lyase substrate, the protective potential does not clearly correlate with the β-lyase activity. This is illustrated by the fact that MPSeCys and CBSeCys are β-eliminated to the same extent, whereas their protective potential differs, indicating that the intrinsic activity of the selenol contributes to the protective potential. The fact that preincubation was essential to obtain a protection against the cisplatin toxicity, may implicate that a direct reaction between cisplatin and the SeCys-conjugates themselves do not contribute to the protective effects. This may be important because these prodrugs are distributed throughout the body, but will only be activated to selenols at β-lyase-expressing sites. The selenols might subsequently bind to cisplatin, thereby preventing its toxicity. Similarly for the combination of sodium selenite and selenodiglutathione with cisplatin, no reaction products were formed based on 195mPt and 75Se radioactivity detection (Baldew et al., 1991). However, methylselenol, a metabolite of sodium selenite formed by glutathione-dependent reduction, was shown to form a complex with cisplatin containing one or more Pt-Se-CH₃ bonds (Baldew et al., 1991). Preincubation of SeCys-conju-
gates with cells containing $\beta$-lyase activity will result in elevated levels of selenols as observed in the present study. However, whether these levels are high enough to form a complex with cisplatin needs further investigation. As an alternative mechanism of cisplatin protection by SeCys-conjugates, selenols may act by cellular responses, such as antioxidant enzyme and phase II enzyme induction, as we recently observed in rat hepatoma cells and hepatocytes (van't Hoen et al., 2002). As reviewed by Talalay et al. (1995), thiol-reactive compounds may induce phase II enzymes by reaction with an unidentified "target" protein that can subsequently react with the antioxidant/electrophilic response element. Because selenols and oxidized derivatives are highly reactive with thiols, phase II enzyme induction might be accomplished by this mechanism, however, this still remains to be established.

In the present study, the complete lack of protection against the cisplatin-induced loss of viability in LLC-PK$_1$ cells by SeCys-conjugates as opposed to the significant protective effect in R1J cells expressing high $\beta$-lyase activity, further indicates the involvement of $\beta$-elimination of SeCys-conjugates in their protective activity. In addition, the involvement of selenols in the protective effects of SeCys-conjugates is supported by the complete inhibitory effect of aminooxyacetic acid. This inhibitor inactivates PLP-dependent enzymes, such as cysteine conjugate $\beta$-lyases, thereby preventing the formation of selenols. Flavin-containing monooxygenases and amino acid oxidases, previously shown to bioactivate SeCys-conjugates into selenenic acids and selenols, respectively, do not significantly contribute to the presently observed cisplatin protection because these enzymes are not inhibited by aminooxyacetic acid (Roosboom et al., 2001a,b).

Whether the presently observed protection by SeCys-conjugates will influence the antitumor activity of cisplatin still has to be delineated. However, the fact that SeCys-conjugates are mainly bioactivated in the kidney and the liver (Commandeur et al., 2000; M. Roosboom, N. P. E. Vermeulen, E. J. Groot, and J. N. M. Commandeur, submitted for publication) and that the observed protective activity is $\beta$-lyase-dependent may imply that the antitumor activity of cisplatin in other organs will remain unaffected. Furthermore, selenite and ebselen have been shown to protect against cisplatin nephrotoxicity without reducing the antitumor activity (Baldew et al., 1989, 1990). In both cases, selenols were suggested as the critical metabolites (Baldew et al., 1990, 1991), as has been shown in this study for SeCys-conjugates. Because SeCys-conjugates were shown to be efficiently bioactivated by human kidney enzymes (Roosboom et al., 2000), the presently observed protection might be clinically relevant to limit the nephrotoxicity of cisplatin and other drugs known to affect the proximal tubule, such as cyclosporin A and gentamicin.

In conclusion, this is the first report on SeCys-conjugates showing chemoprotection. Bioactivation of SeCys-conjugates to selenols seems to be essential for the protective activity against the cytotoxicity of cisplatin. Although only a partial protection was observed against cisplatin-mediated toxicity, more effective chemoprotectors might be developed by modification of the selenocysteine substituent. This is permitted because $\beta$-lyase/GTK has a broad substrate specificity (Commandeur et al., 2000).

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References


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