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Characterization of the chemical reactivity and nephrotoxicity of N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide, a potential reactive metabolite of trichloroethylene

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Abstract

N-Acetyl-S-(1,2-dichlorovinyl)-L-cysteine (NA-DCVC) has been detected in the urine of humans exposed to trichloroethylene and its related sulfoxide, N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (NA-DCVCS), has been detected as hemoglobin adducts in blood of rats dosed with S-(1,2-dichlorovinyl)-L-cysteine (DCVC) or S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS). Because the *in vivo* nephrotoxicity of NA-DCVCS was unknown, in this study, male Sprague-Dawley rats were dosed (i.p.) with 230 μ mol/kg b.w. NA-DCVCS or its potential precursors, DCVCS or NA-DCVC. At 24 h post treatment, rats given NA-DCVC or NA-DCVCS exhibited kidney lesions and effects on renal function distinct from those caused by DCVCS. NA-DCVC and NA-DCVCS primarily affected the cortico-medullary proximal tubules (S₂–S₃ segments) while DCVCS primarily affected the outer cortical proximal tubules (S₁–S₂ segments). When NA-DCVCS or DCVCS was incubated with GSH in phosphate buffer pH 7.4 at 37°C, the corresponding glutathione conjugates were detected, but NA-DCVC was not reactive with GSH. Because NA-DCVCS exhibited a longer half-life than DCVCS and addition of rat liver cytosol enhanced GSH conjugate formation, catalysis of GSH conjugate formation by the liver could explain the lower toxicity of NA-DCVCS in comparison with DCVCS. Collectively, these results provide clear evidence that NA-DCVCS formation could play a significant role in DCVC, NA-DCVC, and trichloroethylene nephrotoxicity. They also suggest a role for hepatic metabolism in the mechanism of NA-DCVC nephrotoxicity.

Keywords

GSH conjugation; N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine; N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide; nephrotoxicity; S-(1,2-dichlorovinyl)-L-cysteine; trichloroethylene

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Introduction

Trichloroethylene (TCE), a common groundwater contaminant, is a known nephrotoxicant (Bruning et al., 1996; Bruning et al., 1998; Bruning et al., 1999; Vermeulen et al., 2012) and suspected renal carcinogen (National Toxicology Program, 2011). Metabolism is required for manifestation of TCE-induced toxicity (Elfarra, 1997). The metabolic pathway that is associated with the formation of nephrotoxic and renal carcinogenic metabolites begins with glutathione (GSH) conjugation, primarily in the liver, followed by translocation via the circulation to the kidney or excretion into the bile. Cleavage of the glycine and γ -glutamate residues to yield the cysteine S-conjugate S-(1,2-dichlorovinyl)-L-cysteine (DCVC) can occur in the kidney, the bile duct epithelium, the intestinal lumen, and the bile canalicular membrane of hepatocytes. DCVC can then reenter the circulation and return to the liver. The nephrotoxicity induced by DCVC is well documented in rats and primary cultures of human kidney cells (Terracini and Parker, 1965; Elfarra, 1997) and has been shown to be mediated through a number of metabolic pathways that result in the formation of reactive electrophiles (Figure 1).

DCVC can be bioactivated by cysteine S-conjugate β -lyases to form chlorothioketene (CTK) and 2-chlorothionoacetyl chloride (2-CTA) (Elfarra et al., 1986; Lash and Anders, 1986; Lock and Reed, 2006; Dekant et al., 1994); two reactive electrophiles (Lash et al., 1986; Elfarra et al., 1987; Chen et al., 1990) that can be hydrolyzed to chloroketene (CK) and 2-chloroacetyl chloride (2-CA). This bioactivation process is widely considered to be primarily responsible for the nephrotoxicity of TCE. An alternative bioactivation pathway has been investigated where DCVC is oxidized by flavin-containing monooxygenase 3 (FMO3) in the liver to form the Michael acceptor S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS) (Krause et al., 2003). Similar to CTK, 2-CTA, CK, and 2-CA, DCVCS is a reactive electrophile that has the ability to modify cellular molecules (Barshteyn and Elfarra, 2009).

DCVC can also be acetylated by N-acetyl transferases in the liver or kidney to form N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (NA-DCVC), a process that is generally considered to be detoxifying, although deacetylation of NA-DCVC to DCVC can occur. NA-DCVC has been detected in the urine of humans for 48 h after a 6 h exposure to TCE (Bernauer et al., 1996). In vitro incubations with rat liver microsomes demonstrated that NA-DCVC can be oxidized in vitro by rat hepatic CYP3A isoforms to form its corresponding sulfoxide, N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (NA-DCVCS) (Werner et al., 1996). NA-DCVC was not oxidized in vitro by male rat kidney microsomes, indicating that NA-DCVCS is likely formed primarily in the liver.

Previous studies involving MS identification of hemoglobin adducts in rats dosed with DCVC or DCVCS detected the presence of both DCVCS-derived monoadducts and crosslinks. In addition, NA-DCVCS-derived mono-hemoglobin adducts and crosslinks were detected, providing evidence that NA-DCVCS can also act as Michael acceptor in addition-elimination reactions with endogenous thiols (Barshteyn and Elfarra, 2008, 2009). Importantly, NA-DCVCS-derived adducts were detected in the circulation of rats that received DCVC at doses relevant to concentrations of the TCE GSH conjugate, S-(1,2-dichlorovinyl)-L-glutathione (DCVG), in the blood of humans exposed to TCE (Lash et al., 1999). NA-DCVCS acting as a crosslinking agent between a hemoglobin molecule and a GSH molecule demonstrated that mixed crosslinks can occur and suggest that the GSH conjugate of NA-DCVCS might be reactive as well. In addition, the detection of NA-DCVCS-derived hemoglobin adducts in rats dosed with DCVCS suggests that an alternative source of NA-DCVCS could be via N-acetylation of DCVCS.

Sulfoxidation of DCVC is considered a bioactivation process since DCVCS has been shown to be a more potent nephrotoxin than DCVC in rats, causing increased blood urea nitrogen (BUN), anuria, more severe proximal tubule necrosis, and renal GSH depletion compared to DCVC (Lash et al., 1994). Similarly, sulfoxidation of NA-DCVC is believed to be a bioactivation process. NA-DCVCS was more cytotoxic than NA-DCVC in isolated rat renal proximal tubule cells. Aminoxyacetic acid (AOAA), a potent inhibitor of cysteine S-conjugate β -lyase that reduces toxicity of DCVC and NA-DCVC, did not affect NA-DCVCS cytotoxicity (Werner et al., 1996), providing support for the hypothesis that the greater cytotoxicity of NA-DCVCS compared to NA-DCVC is likely a result of the ability of NA-DCVCS to act as a Michael acceptor and the associated direct covalent modification of critical cellular macromolecules.

In this study, we sought to test the hypothesis that NA-DCVCS is more nephrotoxic in vivo than NA-DCVC. Although results from in vitro cytotoxicity studies suggest that NA-DCVCS is more toxic than NA-DCVC, in vitro cytotoxicity studies might not accurately reflect in vivo nephrotoxicity because of several potential kinetic and/or pharmacodynamic differences between in vitro and in vivo studies (Blaauboer, 2008; Punt et al., 2010). We also sought to better understand the implications of the ability of NA-DCVCS to act as a Michael acceptor on the potential nephrotoxicity of the compound by characterizing the reactivity of NA-DCVCS toward sulfhydryl-containing molecules, using GSH as a model thiol, and determining the effects of rat liver cytosol which contains multiple forms of GSH S-transferases on the half-life of NA-DCVCS in the presence of GSH.

Materials and Methods

Caution

DCVCS, NA-DCVC, and NA-DCVCS are known to be hazardous and should be handled with care. NA-DCVC is a strong mutagen in the Ames test (Commandeur et al., 1991).

Materials

NA-DCVC and NA-DCVCS were synthesized as previously described (Commandeur and Vermeulen, 1990; Werner et al., 1996). Synthesis of NA-DCVCS was confirmed by MS analysis and purity was determined by HPLC to be >95%. Amicon Ultra-15 and Amicon Ultra-0.5 centrifugal filters with a 3,000 Da nominal molecular weight limit were purchased from Millipore (Billerica, MA). Glucose testing kits were purchased from Cayman Chemical (Ann Arbor, MI) and microprotein and creatinine testing kits were purchased from Pointe Scientific (Canton, MI).

Animals

Male Sprague-Dawley rats (180 – 225 g, Harlan, Madison, WI) were maintained on a 12 h light/dark cycle and feed and water were available ad libitum. All procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison.

Nephrotoxicity of DCVCS, NA-DCVC, and NA-DCVCS

Male Sprague-Dawley rats were injected intraperitoneally with saline or 230 μ mol/kg b.w. DCVCS, NA-DCVC or NA-DCVCS. This dose was chosen because previous studies demonstrated that DCVCS causes unequivocal acute nephrotoxicity at this concentration (Lash et al., 1994) allowing for comparisons of nephrotoxic potential of the three compounds. Intraperitoneal injections were deemed suitable for introducing the compounds into the organism for a couple of reasons. Since the study examines nephrotoxicity of metabolites of TCE and not TCE itself, other routes of exposure (i.e. oral or inhalation) are not relevant. Secondly, since DCVCS was utilized as a positive control for nephrotoxicity,

this route of exposure was used to enable comparisons to nephrotoxicity studies on DCVCS in the literature. After dosing, rats were individually housed overnight in plastic metabolic cages (Nalge Nunc International, Rochester, NY). At 24 h post-injection, rats were euthanized by CO₂ asphyxiation. Blood was collected by cardiac puncture and stored in heparinized vacutainer tubes (BD, Franklin Lakes, NJ). Blood was assayed for serum glucose, BUN, aspartate aminotransferase (AST), and alanine transaminase (ALT) levels by the Clinical Pathology Laboratory at the School of Veterinary Medicine at the University of Wisconsin-Madison.

Urine was retrieved from the metabolic cage collection tube and stored at 4 °C until ready to be assayed. Urine was analyzed using glucose, microprotein, and creatinine testing kits according to the manufacturer's protocol to determine 24 h excretion values for glucose, protein, and creatinine.

These toxicity endpoints were selected because they are well-established markers of nephrotoxicity (BUN, urine glucose, and urine protein) or hepatotoxicity (AST and ALT) and would provide adequate data for this initial characterization of the in vivo liver and kidney toxicity of NA-DCVC and NA-DCVCS.

For histopathology, kidneys were removed and fixed in 10% neutral buffered formalin, then processed routinely into paraffin blocks, sectioned at 5 microns, and sections were stained with hematoxylin and eosin. Histological sections were qualitatively assessed without prior knowledge of the protocol groups to which each section belonged. Because of the differences of lesion severity in various zones, the zones were graded separately. The first zone ('inner zone') is a distinct band-like zone of proximal tubules in the outer stripe of the outer medulla and the adjacent inner cortex along the cortico-medullary junction, along with occasional extension along the tubules in the medullary rays. The second zone ('outer zone') was the remaining cortex outside the inner zone. The grading scheme for lesion severity in both zones was: 0 (0% tubules showing necrosis); 1 (1–25% tubules showing necrosis); 2 (26–50% tubules showing necrosis); 3 (51–75% tubules showing necrosis); 4 (76–100% tubules showing necrosis).

Statistical Analysis

Statistical analysis of the effects on biochemical endpoints of renal function for each treatment group was performed using the Wilcoxon rank sum test (Mstat, <http://www.mcardle.wisc.edu/mstat/>) to compare data from rats dosed with DCVCS, NA-DCVC, or NA-DCVCS against data from saline-treated rats. Results were considered significant if $p < 0.05$.

Characterizing NA-DCVCS reactivity toward GSH

NA-DCVCS was added to GSH in phosphate buffer (0.1 M KH₂PO₄, 0.1 M KCl, and 5 mM Na₂EDTA, pH 7.4) to achieve a 600 µL reaction mixture containing final concentrations of 0.459 mM NA-DCVCS and 1.53 mM GSH. The NA-DCVCS and GSH concentrations were chosen to reflect the 3:10 molar ratio previously used to characterize DCVCS reactivity toward GSH (Sausen and Elfarra, 1991). The reaction mixture was incubated for 30 min in a 37°C shaking water bath and analyzed by HPLC using the following conditions. Analysis of the reaction mixture was accomplished with a Gilson gradient controlled HPLC system (model 306 pumps) equipped with either a Beckman Ultrasphere ODS 5µm analytical (4.6 mm × 25 cm). Mobile phases were 1% acetonitrile (ACN) in 0.1% aqueous trifluoroacetic acid (TFA) on pump A and 75% ACN in 0.1% aqueous TFA on pump B. The flow rate was 1 mL/min for analyses using the analytical column. The HPLC method used began at 5% mobile phase B and increased to 10% B over 8 min. Subsequently, the gradient was raised to

50% mobile phase B over the next 7 min and then to 80% B over 5 min further. The gradient was then lowered to 5% mobile phase B over 2 min and held constant for 5 min. The total run time was 27 min. The reaction was monitored with UV detection set at 220 nm to monitor depletion of the sulfoxides and 260 nm to monitor for appearance of products. Under these conditions, GSH was detectable in the 220 nm trace and had a retention time of 5.1 min. The NA-DCVCS diastereomers had retention times of 10.9 min and 11.3 min for diastereomer I and II respectively (see Results, Figure 4).

To characterize the half-life of NA-DCVCS in the presence of GSH, GSH (1.53 mM) was incubated with NA-DCVCS (0.459 mM) in phosphate buffer in a 37°C shaking water bath. A similar reaction mixture containing DCVCS instead of NA-DCVCS was prepared as a positive control and to compare NA-DCVCS reactivity to DCVCS. Aliquots (50 µL) were removed every 10 min for 60 min for NA-DCVCS and every 5 min for 30 min for DCVCS, diluted with doubly deionized H₂O (100 µL), and acidified with 6.67% aqueous TFA (10 µL). Samples were then analyzed by HPLC using the method described above. Half-lives for the NA-DCVCS and DCVCS diastereomers were calculated using first-order elimination kinetics: $C_t = C_0 \times e^{-kt}$ and $t_{1/2} = \ln 2/k$.

The effect of liver cytosolic enzymes on the reaction between NA-DCVCS and GSH was investigated using a similar method to the one described above. Rat liver cytosol was prepared from livers of male Sprague Dawley rats (180 – 225 g) using a modified version of the method previously used to prepare rat kidney cytosol (Irving et al., 2011). GSH and other small molecules were removed by filtering cytosol (500 µL) with centrifugal filters three times, adding 500 µL phosphate buffer pH 7.4 after the first two filtration steps. After the third filtration step, phosphate buffer pH 7.4 was added to bring volume back to 500 µL. Liver cytosol protein concentration was determined using the Lowry method (Lowry et al., 1951).

GSH (1.53 mM), filtered cytosol (2 mg/mL), and NA-DCVCS or DCVCS (0.459 mM) were incubated at 37°C (reaction volume: 600 µL). Aliquots (50 µL) were removed every 2.5 min for 12.5 min for DCVCS incubations and every 5 min for 20 min for NA-DCVCS incubations. Aliquots were diluted in water and TFA as described above, analyzed by HPLC and half-lives calculated for each NA-DCVCS and DCVCS diastereomer.

Characterizing NA-DCVCS-GSH adducts by LC/MS

NA-DCVCS (3 mM) was incubated with GSH (10 mM) in phosphate buffer pH 7.4 at 37°C for 1 h. At the end of the incubation period, two major products were detectable by HPLC. These products were collected as a fraction by semi-preparative HPLC using the instrument and method described above with a Beckman Ultrasphere ODS 5µm semipreparative (10 mm × 25 cm) column and a flow rate of 3 mL/min. The collected products were subsequently lyophilized to dryness. Purity of the collected fraction was >95% by HPLC. The dried products were dissolved in 50:50 ACN:H₂O and LC/MS analysis of the isolated products was carried out at the Mass Spectrometry Facility of the University of Wisconsin Biotechnology Center. Samples were chromatographically resolved using an Agilent 1200 HPLC (Agilent, Palo Alto, CA) equipped with an Agilent ZORBAX 300 SB-C18 1.8 µm 2.1 mm × 50 mm column and an autosampler held at 5 °C. LC/MS analysis was performed with an Agilent LC/MSD TOF using a 1200 Series LC pump (Agilent, Palo Alto, CA) equipped with a Zorbax SB-C18 1.8 µm 2.1 mm × 50 mm column. The flow rate was 0.25 mL/min and mobile phase A was 2% ACN in 0.1% aqueous formic acid and mobile phase B was 90% ACN in 0.1 % aqueous formic acid. The LC method used began at 2% mobile phase B and was held for 1 min before increasing to 90% B over 40 minutes. Subsequently, the gradient was raised to 95% B over 4 min before returning to 2% B over 1 min and held at 2% B for 15 min. The total run time was 60 min. The mass spectrometer used in

conjunction with chromatographic separation was an Agilent LC/MSD TOF with electrospray ionization used in the positive ion mode. The capillary voltage was 3500 V and the nebulizer was operated with nitrogen at 30 psi, 10 L/min, and 350 °C. The data were processed using Analyst QS 1.1 build:9865 software (Agilent, Palo Alto, CA) to extract parent masses observed between 100 and 3200 atomic mass units.

Results

Nephrotoxicity of NA-DCVC and NA-DCVCS

The *in vivo* nephrotoxicity of NA-DCVCS and NA-DCVC was characterized by dosing rats with saline or 230 $\mu\text{mol/kg}$ b.w. NA-DCVC, NA-DCVCS, or DCVCS (positive control) and assaying a number of biochemical blood and urine endpoints, as well as histopathological analysis of kidney sections. BUN levels in rats dosed with NA-DCVC or NA-DCVCS were raised approximately 3-fold compared to control rats (Figure 2A), clearly indicating adverse effects on renal function. In comparison, rats dosed with DCVCS had elevated BUN levels that were 7-fold higher than saline-treated rats. BUN levels were not altered compared to controls in male rats given DCVC at an equimolar dose to the one used in the current study (Lash et al., 1994). AST and ALT levels in rats dosed with NA-DCVC or NA-DCVCS were not different from saline-treated rats, suggesting that these compounds do not cause hepatotoxicity.

NA-DCVC treatment appeared to have a mildly diuretic effect (0.32 ± 0.11 dL) whereas two out of the four rats dosed with DCVCS were anuric and the other two had reduced urine volume (0.05 ± 0.07 dL) compared to saline-treated rats (0.20 ± 0.04 dL). Urine protein/creatinine ratio was elevated in rats dosed with NA-DCVC and NA-DCVCS (Figure 2B). Urinary excretion of glucose over 24 h was 113-fold and 89-fold greater for NA-DCVC and NA-DCVCS treated rats compared to saline-treated rats (Figure 2C). Glucose excretion in DCVCS treated rats was not different than saline-treated rats but as indicated above, the DCVCS treated rats were either anuric or produced little urine in comparison with the other treatment groups.

Histopathological analysis revealed that treatment with NA-DCVC or NA-DCVCS caused tubular necrosis and degeneration under the experimental conditions used. Tubular necrosis was most often characterized by uniform coagulative necrosis within tubular profiles, with tubular epithelial cells showing granular pale eosinophilic cytoplasm with loss of cytoplasmic detail, karyolysis, occasional fragmentation of cytoplasm, and dissociation of cells from each other and from the basement membrane, which was spared (Figure 3A). Tubular degeneration was characterized by swollen tubular epithelial cells with variably sized clear cytoplasmic vacuoles, occasionally with patchy single cell necrosis, characterized by hypereosinophilic cytoplasm, pyknosis, or karyolysis. Treatment with DCVCS caused similar tubular necrosis and degeneration; however, there were significant regional differences in the tubular necrosis and degeneration observed in rats treated with NA-DCVC or NA-DCVCS compared to those treated with DCVCS (Table 1). In rats treated with NA-DCVC or NA-DCVCS, tubular necrosis and degeneration were usually confined to or most severe in a distinct band-like zone of proximal convoluted tubules in the outer stripe of the outer medulla and the adjacent inner cortex along the cortico-medullary junction, along with occasional extension along the proximal straight tubules in the medullary rays ("inner zone," corresponding to the S₂–S₃ segments of the proximal tubule); remaining cortical tubules ("outer zone," corresponding to the S₁–S₂ segments of the proximal tubule) had more limited degenerative changes (Figure 3B). The selective induction of NA-DCVC-associated lesions in the S₃ segment is supported by the observation that this region was the most sensitive to NA-DCVC-induced lesions in rabbit renal cortical slices (Wolfgang et al., 1989). In rats treated with DCVCS, this pattern was essentially reversed, with the inner zone

showing mostly milder degenerative changes and the remaining cortical proximal convoluted tubules showing extensive coagulative necrosis and tubular degeneration (Figure 3B), findings consistent with results described in previous studies (Lash et al., 1994). In rats treated with DCVCS, there was also extensive tubular proteinosis, with tubules containing homogeneous eosinophilic hyaline protein occasionally with some basophilic stippled mineral, and tubular epithelial cells often containing brightly eosinophilic intracytoplasmic hyaline protein droplets. In rats treated with NA-DCVC and NA-DCVCS, tubules in the distal nephron and collecting tubules also occasionally contained protein or granular eosinophilic casts of cellular debris. Regenerative changes were not seen.

Based on the biochemical endpoints assayed, NA-DCVC and NA-DCVCS appear to have similar nephrotoxicity in male Sprague-Dawley rats, and were less nephrotoxic than DCVCS on an equimolar basis. Histological kidney samples from all of the rats dosed with NA-DCVC and DCVCS exhibited necrosis that scored in the highest category, indicating greater than 75% of proximal tubules in the region affected were necrotic. Two rats treated with NA-DCVCS also scored in this category, while the other two rats received a score of 3, indicating 50 – 75% of proximal tubules were affected (Table 1). The histopathology results suggest that the nephrotoxic potency of these three chemicals is similar at the dose used.

Characterizing NA-DCVCS Reactivity toward GSH

To characterize NA-DCVCS reactivity toward endogenous sulfhydryl containing molecules, NA-DCVCS was incubated with GSH (3:10 molar ratio) at 37°C. The reaction was monitored by HPLC and as the two NA-DCVCS diastereomers (peaks at 10.9 and 11.3 min, Figure 4A) were consumed, two product peaks (peaks 1 and 2, Figure 4A) increased in area (Figure 4B). Under the HPLC conditions described above, these product peaks eluted after GSH (5.1 min).

NA-DCVCS was consumed quickly in the presence of GSH, with half lives of 10.4 min and 22.2 min for diastereomer I and II, respectively (Table 2). In comparison, DCVCS was even more reactive with its two diastereomers having half-lives of 16.7 min and 6.6 min. When these reactions were repeated with filtered rat liver cytosol added into the reaction mixture, the NA-DCVCS diastereomer half-lives were reduced (diastereomer I: 5.4 min; diastereomer II: 2.3 min) and the products were produced earlier and in larger amounts (Figure 5B). Similarly, incubations of DCVCS with GSH in the presence of filtered rat liver cytosol also reduced the half-lives of the two diastereomers (diastereomer I: 1.4 min; diastereomer II: 3.1 min). In the presence of liver cytosol, NA-DCVCS and DCVCS half-life was reduced 10-fold for one diastereomer (NA-DCVCS II and DCVCS I) and 2-fold for the other (NA-DCVCS I and DCVCS II). These results suggest there is diastereomeric selectivity for enzymatic formation of GSH-conjugates. Because of the relatively long half-lives of the NA-DCVCS diastereomers in the absence of rat liver cytosol in comparison with the DCVCS diastereomers, these results suggest that enzymatic catalysis of GSH-conjugate formation could play a significant role in the disposition of NA-DCVCS.

Characterization of NA-DCVCS-GSH adducts by LC/MS

The identification of the products of the reaction between NA-DCVCS and GSH was carried out by isolating the two product peaks formed upon incubation of NA-DCVCS with GSH in phosphate buffer pH 7.4 at 37°C using semi-preparative HPLC and characterizing by LC/MS. Three major peaks were resolved by LC/MS (Figure 6A); the two larger peaks were comprised of ions of 545 Da (Figure 6B, 6C), consistent with the expected m/z of a monoadduct formed by the addition of GSH to NA-DCVCS followed by the subsequent elimination of HCl (Figure 7). The presence of two separate peaks containing this ion is consistent with the HPLC data suggesting that the two peaks represent monoadduct

diastereomers. In similar reactions between DCVCS and N-acetyl cysteine or GSH, diastereomeric monoadducts represented the two largest peaks detected by HPLC. The third peak detected by LC/MS was comprised of an ion of 852 Da (Figure 6D), providing evidence for the formation of a di-GSH adduct, as this is likely a product where a second GSH molecule is added to the monoadduct without further elimination of a second HCl molecule.

Discussion

NA-DCVCS, a reactive sulfoxide derived from TCE, has been hypothesized to play a role in TCE nephrotoxicity. Evidence for the possible formation of NA-DCVCS is based on a number of observations: (1) the precursor mercapturic acid NA-DCVC can be oxidized by CYP3A1/2 to NA-DCVCS in rat liver microsomes in vitro (Werner et al., 1996), (2) CYP3A-mediated sulfoxidation is an established β -lyase independent bioactivating pathway for nephrotoxic mercapturic acids (Sheffels et al., 2004; Birner et al., 1995), and (3) NA-DCVCS-derived hemoglobin adducts have been detected in rats given doses of DCVC relevant to blood concentrations of DCVG in humans exposed to TCE (Barshteyn and Elfarra, 2009). The present study demonstrates that NA-DCVC and NA-DCVCS were nephrotoxic to rats under the in vivo experimental conditions utilized. The two compounds exhibited similar nephrotoxicity under these conditions; these results differ from previous cytotoxicity studies where NA-DCVCS was shown to be more cytotoxic than NA-DCVC in isolated rat kidney epithelial cells (Werner et al., 1996).

There are a number of reasons that might explain the different results between the present in vivo nephrotoxicity study which suggests that NA-DCVC and NA-DCVCS have similar toxicity and previous in vitro cytotoxicity studies that suggest NA-DCVCS is more toxic than NA-DCVC. One possible explanation is that for NA-DCVC, which is likely an indirect acting toxicant, bioactivation reactions by deacetylation to DCVC and/or oxidation to NA-DCVCS (Figure 1) are not efficient in renal cells in vitro compared to in vivo when the reactions can occur in both the liver and the kidney. As a direct acting toxicant, NA-DCVCS presumably has reduced in vivo toxicity as a result of liver detoxification. The shorter half-life of NA-DCVCS in the presence of GSH and rat liver cytosol (Figure 5; Table 2) indicates that the liver GSH S-transferases may play a role in NA-DCVCS fate and toxicity and as a result, demonstrates the importance of characterizing NA-DCVC and NA-DCVCS nephrotoxicity in vivo. The influence of the liver on the metabolic fate of NA-DCVC and NA-DCVCS has not been accounted for in the in vitro cytotoxicity studies.

It is at present unclear why NA-DCVC and NA-DCVCS appeared to have a similar toxic effect on the biochemical and histopathological endpoints measured. One likely possibility is that different detoxication efficiencies for the compounds by the hepatic GSH S-transferases results in similar toxic doses reaching the kidney. Alternatively, NA-DCVC oxidation to NA-DCVCS by CYP3A isoforms might be efficient enough to result in a similar amount of NA-DCVCS reaching the kidney. Further investigations into the translocation of NA-DCVC and NA-DCVCS to the kidney and the metabolism of NA-DCVC could be instructive in gaining a better understanding of the role of these metabolites in TCE-induced nephrotoxicity.

The differences in the nephrotoxicity caused by NA-DCVC, NA-DCVCS, and DCVCS are illustrated by histopathological analysis of kidneys from rats dosed with the compounds. Consistent with previous reports (Lash et al., 1994), DCVCS caused acute proximal tubule necrosis in the cortex, but did not affect the medulla. On the other hand, NA-DCVC and NA-DCVCS appear to cause necrosis in the proximal tubules of the outer stripe of the medulla, the adjacent inner cortex, and the medullary rays. In addition, NA-DCVC and NA-

DCVCS appeared to cause mild degenerative changes in the proximal convoluted tubules of the cortex.

The results of the histopathological analysis of kidneys from rats dosed with NA-DCVC are consistent with the finding that renal uptake of NA-DCVC in the S₂–S₃ segments likely occurs through the basolateral membrane via the organic anion transport system. Blocking the organic anion transport system (which has higher expression in the S₂ segment of the proximal tubule compared to the S₁ and S₃ segments) with probenecid inhibited accumulation of radiolabeled NA-DCVC in isolated rat proximal tubules (Zhang and Stevens, 1989). In addition, the S₃ segment was the most sensitive to NA-DCVC-induced lesions in rabbit renal cortical slices (Wolfgang et al., 1989). Conversely, DCVC is believed to mainly enter proximal tubule cells from the lumen via transepithelial transport after glomerular filtration of the GSH conjugate of TCE, DCVG, and in situ degradation by brush border enzymes (Commandeur et al., 1995). In addition, DCVC may enter the kidney via the basolateral membrane. The organic anion transport system in the basolateral membrane or amino acid transport systems in the basolateral membrane and brush border are potentially involved in renal uptake of DCVC (Commandeur et al., 1995). The transport systems that are involved in DCVCS uptake remain unknown, but further investigations into renal uptake of DCVCS may provide further insight into the difference in regional distribution of lesions induced by DCVCS compared to NA-DCVC and NA-DCVCS.

By comparing the histopathological data from the present study to results from previous reports on tubulotoxicity induced by the precursor compounds TCE and DCVC, we sought to determine whether NA-DCVC or NA-DCVCS might play a role in TCE and DCVC nephrotoxicity. Cytomegaly, karyomegaly, and toxic nephrosis of tubular epithelial cells in the cortico-medullary junction are observed in rats that are chronically exposed to TCE (Lash et al., 2000; Lock and Reed, 2006). DCVC is known to cause necrosis in the proximal tubules of the inner cortex (i.e. the S₃ segment of the proximal tubule) while leaving the medulla spared (Terracini and Parker, 1965; Lash et al., 1994). Interestingly, treatment of rats with an equimolar dose of DCVC to the one used in the present study (230 µmol/kg b.w.) resulted in scattered, patchy foci of tubular necrosis (Lash et al., 1994). In conjunction with the observation that DCVC did not alter BUN levels at this dose (Lash et al., 1994), this suggests that NA-DCVC and NA-DCVCS treatment might have resulted in more severe nephrotoxicity than would be expected with DCVC. Therefore, based on their nephrotoxic potential and apparent similar distribution of renal lesions in rats exposed to TCE, DCVC, NA-DCVC, and NA-DCVCS, it is possible that NA-DCVC and NA-DCVCS could play significant roles in the chronic nephrotoxicity of TCE.

We sought to specifically characterize the reactivity of NA-DCVCS toward cellular thiols using GSH as a model thiol to better understand the implications of the ability of NA-DCVCS to act as a Michael acceptor in reactions with cellular thiols and its nephrotoxicity. Current evidence in the literature for the ability of NA-DCVCS to act as a Michael acceptor is based on the detection of NA-DCVCS-derived hemoglobin adducts in rats dosed with DCVC or DCVCS (Barshteyn and Elfarra, 2008, 2009). In the present study, NA-DCVCS readily reacted with GSH, although the longer half-life of NA-DCVCS compared to DCVCS in the presence of GSH suggests that NA-DCVCS is less reactive toward thiol groups of renal proteins, consistent with biochemical endpoint data from the in vivo experiments. It is possible that the increased stability (i.e. reduced reactivity) of NA-DCVCS could lead to more excretion of this compound in the urine.

In addition to characterizing NA-DCVCS reactivity, the identification by LC/MS of NA-DCVCS-derived GSH adducts produced by these in vitro reactions is significant in that it provides information that could be utilized to identify possible biomarkers of exposure to

TCE. Although the production of the mono-GSH adduct via addition-elimination was expected, the particular di-GSH adduct identified in the present study was not. The di-GSH adduct detected was the product of the addition of two GSH molecules but only elimination of one HCl. This suggests that in addition to the monoadducts and crosslinks of vinyl sulfoxides that have been previously described as possible biomarkers, this particular type of addition-elimination-addition product should also be considered in the characterization and validation of biomarkers of this class of chemicals.

Another potentially important factor investigated in this study is the role of hepatic GSH S-transferases in mediating the detoxification of NA-DCVCS. The addition of filtered rat liver cytosol (which contains multiple forms of GSH S-transferases) to a mixture containing NA-DCVCS and GSH greatly reduces the half-lives of both NA-DCVCS diastereomers and increases product formation (Figure 5; Table 2), suggesting that enzymatic catalysis of GSH conjugate formation could be an important factor in the disposition of NA-DCVCS. Interestingly, the half-lives of the two diastereomers of NA-DCVCS and DCVCS in the presence of GSH are differently affected in the presence of rat liver cytosol. Whereas the half-life of the first NA-DCVCS diastereomer was reduced 1.9-fold, the half-life of the second diastereomer was reduced 9.7-fold. The half-lives of the two DCVCS diastereomers were similarly affected (I: 11.9-fold reduction; II: 1.9-fold reduction), suggesting a possibly shared GSH conjugate pathway for the two compounds. In addition, this suggests that much of the NA-DCVCS formed in the liver likely enters the blood stream in the form of GSH adducts. However, adducts formed by these kind of reactive electrophiles can undergo reverse Michael addition (Chen and Armstrong, 1995; Alary et al., 2003), which might explain the detection of NA-DCVCS and DCVCS bound to hemoglobin (Barshteyn and Elfarra, 2009) and the selective nephrotoxicity of both compounds. In addition, mono-GSH adducts retain the ability to react with another thiol to form a cross-link. Since NA-DCVCS has a longer half-life than DCVCS, it is possible that catalysis of GSH conjugation has more significant implications for NA-DCVCS compared to DCVCS. Whereas DCVCS is very reactive and therefore more likely to react with nearby thiols, the slower reactivity of NA-DCVCS could result in more free NA-DCVCS being directed through GSH conjugation. Thus, it is possible that efficient GSH conjugation and lower reactivity toward thiol-containing cellular proteins could play a role in the observed reduced effects on renal function by NA-DCVCS compared to DCVCS.

One hypothetical mechanism for NA-DCVCS nephrotoxicity involved deacetylation to DCVCS. In isolated rat proximal tubule cells, NA-DCVC was demonstrated to be less cytotoxic than DCVC and it was hypothesized that the reason for the difference between the cytotoxicity of NA-DCVC and DCVC might be slow deacetylation of NA-DCVC to the more cytotoxic DCVC species (Commandeur et al., 1991). However, the present study presents evidence that DCVCS is handled differently by the kidney than NA-DCVC and NA-DCVCS. The difference in localization of necrosis between rats treated with the N-acetylated compounds and DCVCS suggests that NA-DCVCS toxicity is not mediated through deacetylation to DCVCS. Furthermore, NA-DCVCS clearly has the ability to directly react with endogenous sulfhydryl-containing macromolecules, as evidenced by previous detection of NA-DCVCS-derived hemoglobin adducts (Barshteyn and Elfarra, 2009) and the current study demonstrating the formation of GSH adducts. This suggests that NA-DCVCS and/or its corresponding GSH conjugate can be directly involved in covalent modification reactions of critical kidney proteins that could lead to nephrotoxicity.

In summary, NA-DCVC and NA-DCVCS have been found to be nephrotoxic in rats. Differences in effects on urine glucose excretion and localization of tubulotoxicity point to different handling of NA-DCVC and NA-DCVCS by the nephron compared to DCVCS. Administration of NA-DCVC and NA-DCVCS to rats causes necrosis in the outer medulla

and adjacent cortex, while dosing with DCVCS causes cortical necrosis and does not affect the medulla. Exposure of rats to DCVC leads to the development of lesions in the final portion of the proximal tubule in the inner cortex and chronic exposure of rats to TCE causes renal lesions at the inner renal cortex and outer medulla. Therefore, based on the distribution of lesions in the kidneys of rats dosed with DCVC or TCE, the results presented in this manuscript suggest NA-DCVC or NA-DCVCS may play a role in the nephrotoxicity of TCE. Further investigations in NA-DCVC metabolism in vivo, renal uptake of NA-DCVCS, and the association between covalent modification of kidney proteins by NA-DCVCS and nephrotoxicity, would be valuable for fully elucidating the role of NA-DCVC and NA-DCVCS in TCE nephrotoxicity.

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Abbreviations

2-CA	2-chloroacetyl chloride
2-CTA	2-chlorothionoacetyl chloride
ACN	acetonitrile
ALT	alanine transaminase
AST	aspartate aminotransferase
BUN	blood urea nitrogen
CK	chloroketene
CTK	chlorothioketene
CYP	cytochrome P450
DCVC	S-(1,2-dichlorovinyl)-L-cysteine
DCVCS	S-(1,2-dichlorovinyl)-L-cysteine sulfoxide
DCVG	S-(1,2-dichlorovinyl)-L-glutathione
FMO3	flavin-containing monooxygenase 3
GSH	glutathione
NA-DCVC	N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine
NA-DCVCS	N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide
TCE	trichloroethylene
TFA	trifluoroacetic acid

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Highlights

- NA-DCVCS and NA-DCVC toxicity are distinct from DCVCS toxicity.
- NA-DCVCS readily reacts with GSH to form mono- and di-GSH conjugates.
- Liver glutathione S-transferases enhance NA-DCVCS GSH conjugate formation.
- Renal localization of lesions suggests a role for NA-DCVCS in TCE nephrotoxicity.

Figure 1.

Metabolism of the cysteine S-conjugate of trichloroethylene S-(1,2-dichlorovinyl)-L-cysteine (DCVC). N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (NA-DCVC); Chlorothioketene (CTK) and its hydrolysis product chloroketene (CK); 2-chlorothionoacetyl chloride (2-CTA) and its hydrolysis product 2-chloroacetyl chloride (2-CA); N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (NA-DCVCS); flavin-containing monooxygenase 3 (FMO3); S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS).

Figure 2.

Male rats were dosed with saline (S) or 230 $\mu\text{mol/kg}$ b.w. DCVCS (D), NA-DCVC (N) or NA-DCVCS (NS) and sacrificed 24 h after dosing. Blood was analyzed for blood urea nitrogen (BUN) levels (A) and urine was analyzed for protein (B) and glucose (C) levels. Bars and error bars indicate mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$.

Figure 3.

Histopathology of male rats treated with saline or 230 $\mu\text{mol/kg}$ b.w. DCVCS, NA-DCVC or NA-DCVCS. A. Typical areas of renal cortex in rats treated with saline or NA-DCVC depicting tubular necrosis in rats dosed with NA-DCVCS (20 $\times$). Characteristics of tubular necrosis were similar for rats dosed with DCVCS, NA-DCVC, or NA-DCVCS. B. Images depicting regional variation in tubulotoxicity induced in rats dosed with DCVCS, NA-DCVC, or NA-DCVCS (2 $\times$). Treatment with DCVCS resulted in tubule necrosis in the “outer zone” (O) – the outer portion of the cortex (C). Treatment with NA-DCVC or NA-DCVCS resulted in tubular necrosis in the “inner zone” (I) – the inner cortex adjacent to the

medulla (M), the outer stripe of the outer medulla, and the medullary rays. Hematoxylin and eosin stain.

Figure 4.

A. Sample chromatograms of the reaction between NA-DCVCS (0.459 mM) and GSH (1.53 mM) incubated at 37°C for 30 min. Reaction was monitored by HPLC with UV detection set at 220 nm (top panel) and 260 nm (bottom panel). Two product peaks appeared over time, indicated by numerals 1 and 2. B. Typical timecourse of NA-DCVCS consumption and product 1 and 2 accumulation.

Figure 5.

A. Data from a typical timecourse comparing depletion of NA-DCVCS (0.459 mM) in the presence of GSH (1.53 mM) with and without the addition of filtered rat liver cytosol (2 mg/mL). B. Data from a typical timecourse demonstrating that sum of product peak areas over time when NA-DCVCS is incubated in the presence of GSH with and without the addition of filtered rat liver cytosol indicates that products are made earlier and in larger amounts when cytosol is present.

Figure 6.

A. Total ion chromatogram of the LC/MS analysis of the isolated two products formed in the reaction of NA-DCVCS and GSH. B. LC/MS spectra of the first peak (retention time = 2.1 min). An ion of 545 Da corresponds to a mono-GSH NA-DCVCS adduct. C. LC/MS spectra of the second peak (retention time = 2.4 min). An ion of 545 Da corresponds to a mono-GSH NA-DCVCS adduct. D. LC/MS spectra of the third peak (retention time approximately 3.5 min). An ion of 852 Da corresponds to a di-GSH NA-DCVCS adduct that has lost only one HCl molecule.

Figure 7.
Proposed scheme of the formation of GSH conjugates formed when NA-DCVCS is incubated with GSH 3:10 molar ratio) in phosphate buffer pH 7.4 at 37°C.

Table 1

Histopathology lesion severity in kidneys of male rats dosed with saline or 230 $\mu\text{mol/kg}$ b.w. DCVCS, NA-DCVC or NA-DCVCS. Inner zone corresponds to proximal tubules in the outer medulla, adjacent inner cortex, and medullary rays. Outer zone corresponds to proximal convoluted tubules in the remaining cortex outside the inner zone. Scores range from 0 – 4 indicating percent of proximal tubules showing necrosis as described in the methods section.

Treatment	N	Inner Zone Severity	Outer Zone Severity
Saline	3	0 (3 rats)	0 (3 rats)
DCVCS	4	1 (2 rats); 2 (2 rats)	4 (4 rats)
NA-DCVC	6	4 (6 rats)	0 (6 rats)
NA-DCVCS	4	3 (2 rats); 4 (2 rats)	0 (2 rats); 1 (2 rats)

Table 2

Half-lives of NA-DCVCS diastereomers I and II and DCVCS diastereomers I and II in the presence of GSH and filtered rat liver cytosol. Half-lives presented are the means from 2 experiments for all samples but DCVCS + GSH + Cytosol. Calculated half-life from each experiment was within $\pm 25\%$ of the mean.

Analyte	Half-life (min)	
	+GSH	+GSH + Cytosol
NA-DCVCS I	10.4	5.4
NA-DCVCS II	22.2	2.3
DCVCS I	16.7	1.4
DCVCS II	6.6	3.1