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N-Biotinyl-S-(1,2-dichlorovinyl)-L-cysteine Sulfoxide as a Potential Model for S-(1,2-Dichlorovinyl)-L-cysteine Sulfoxide: Characterization of Stability and Reactivity with Glutathione and Kidney Proteins In Vitro

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Abstract

S-(1,2-Dichlorovinyl)-L-cysteine sulfoxide (DCVCS) is a reactive and potent nephrotoxic metabolite of the human trichloroethylene metabolite S-(1,2-dichlorovinyl)-L-cysteine (DCVC). Because DCVCS covalent binding to kidney proteins likely plays a role in its nephrotoxicity, in this study biotin-tagged DCVCS, N-Biotinyl-DCVCS (NB-DCVCS), was synthesized and its stability in buffer alone and in the presence of rat blood or plasma was characterized in vitro. In addition, reactivity toward GSH and covalent binding to selected model enzymes and isolated kidney proteins were characterized. The half-lives of NB-DCVCS (39.6 min) and the DCVCS (diastereomer 1: 14.4 min, diastereomer 2: 6 min) in the presence of GSH were comparable. Incubating the model enzymes glutathione reductase and malate dehydrogenase with 10 μ M NB-DCVCS for 3 h at 37°C followed by immunoblotting using anti-biotin antibodies demonstrated that glutathione reductase and malate dehydrogenase were extensively modified by NB-DCVCS. When rat kidney cytosol (6 μ g/ μ L) was incubated with NB-DCVCS (312.5 nM to 5 μ M) for 3 h at 37°C followed by immunoblotting, a concentration-dependent increase in signal with multiple proteins with different molecular weights was observed, suggesting NB-DCVCS binds to multiple kidney proteins with different selectivity. Incubating rat kidney cytosol with DCVCS (10 – 100 μ M) prior to addition of NB-DCVCS (2.5 μ M) reduced the immunoblotting signal, suggesting that NB-DCVCS and DCVCS compete for the same binding sites. A comparison of the stability of NB-DCVCS and DCVCS in rat blood and plasma was determined in vitro and NB-DCVCS exhibited higher stability than DCVCS in both media. Collectively, these results suggest NB-DCVCS shows sufficient stability, reactivity, and selectivity to warrant further investigations into its possible use as a tool for future characterization of the role of covalent modification of renal proteins by DCVCS in nephrotoxicity.

Introduction

Trichloroethylene (TCE), a halogenated hydrocarbon used in industry as a metal degreaser, is a common air and groundwater contaminant that has been classified as “reasonably anticipated to be a human carcinogen” by the National Toxicology Program’s Eleventh Report on Carcinogens.¹ Epidemiological studies have shown an association between TCE

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exposure and the development of renal cancer in humans, and rats exposed to TCE develop kidney tumors.^{2,3}

Metabolism of TCE results in the formation of reactive metabolites believed to be responsible for TCE renal toxicity and carcinogenicity.^{3,4,5} Following GSH conjugation (Figure 1), γ -glutamyl transpeptidase and cysteinyl-glycine dipeptidases present in kidney cells, the luminal membrane of the bile duct epithelium, the bile canalicular membrane of hepatocytes, and the intestinal lumen cleave off the γ -glutamyl and glycine residues respectively, resulting in the formation of the cysteine S-conjugate, S-(1,2-dichlorovinyl)cysteine (DCVC).⁴ DCVC can then be absorbed into the circulation and translocate to the kidney or return to the liver.⁴

There are three major metabolic pathways that can act on DCVC: acetylation by N-acetyl transferases, β -elimination by cysteine conjugate β -lyases, or oxidation by flavin-containing monooxygenase 3 (FMO3).^{6,7} N-Acetyl DCVC has been detected in the blood of workers occupationally exposed to TCE.^{8,9} Although N-acetylation of DCVC is generally believed to make the compound more easily excreted, N-acetyl DCVC can be bioactivated by cytochrome P450 3A1/2 to N-acetyl-S-(1,2-dichlorovinyl)-l-cysteine sulfoxide.¹⁰ β -Elimination of DCVC by β -lyase results in the formation of chloroethioketene and chloroethene, reactive electrophiles that can modify cellular proteins.¹¹ The bioactivation pathway involving FMO3-mediated oxidation of DCVC results in the formation of S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS).⁶

DCVCS was shown to be a more potent nephrotoxicant than DCVC in in vivo studies of rats given DCVC or DCVCS, as evidenced by increased blood urea nitrogen levels and more severe proximal tubule necrosis.¹² In vitro studies have used isolated or primary cultures of proximal tubule cells because the proximal tubule is the major target of DCVC-induced toxicity in the kidney.¹³ Primary cultures of human proximal tubule cells treated with DCVCS exhibited necrosis, apoptosis, mitochondrial dysfunction, and GSH depletion.^{14,15}

DCVCS contains an α,β -unsaturated moiety that allows it to act as a Michael acceptor in addition-elimination reactions with the thiol group of cysteine, N-acetyl cysteine (NAC), and GSH to form adducts.^{11,16–18} DCVCS exists as a pair of diastereomers due to the presence of chiral centers at the sulfur of the sulfoxide moiety and the α -carbon of the cysteine moiety. The configuration of the sulfoxide group likely plays a role in the slight difference in reactivity of the diastereomers towards nucleophiles.¹⁶ If the sulfoxide is aimed anti to the double bond, nucleophilic addition is less hindered. LC/MS analysis of the reaction between NAC and DCVCS detected the presence of three mono-NAC DCVCS adducts and one di-NAC DCVCS crosslink (Figure 2). The mechanism of adduct formation involves Michael addition of NAC to DCVCS followed by elimination of HCl.¹⁶ NMR and HSQC (¹H-detected heteronuclear single quantum correlation) analysis of the monoadducts demonstrated that two are diastereomers with the proton at the terminal vinylic carbon, while the third has a proton adjacent to the sulfoxide moiety. This third monoadduct is a minor product that is a result of a less favorable elimination of HCl via loss of the proton from the terminal vinylic carbon and the chlorine atom from the carbon adjacent to the sulfoxide.

DCVCS-derived hemoglobin adducts were isolated from erythrocytes treated in vitro and Sprague-Dawley rats treated in vivo with DCVCS.¹⁸ More importantly, DCVCS-derived hemoglobin adducts were isolated from rats treated with a low dose (3 or 30 μ mol/kg i.p.) of DCVC daily over 5 days or a single acute dose (460 μ mol/kg i.p.).¹¹ At least four of the five cysteine residues in the α and β chains of hemoglobin were shown to be targeted by DCVCS and can participate in the formation of adducts or crosslinks. This study was significant

because the administered DCVC doses were relevant to the blood concentrations of S-(1,2-dichlorovinyl)glutathione (Figure 1), the precursor of DCVC, in humans exposed to TCE.¹⁹ In addition, this study was the first to demonstrate the detection of DCVCS-derived adducts in the circulation after in vivo bioactivation of DCVC.

Much of the research on DCVCS has focused on the effects on renal function and markers of nephrotoxicity and cytotoxicity, however less is known about which proteins are targeted by DCVCS and how covalent modification of these proteins relates to the mechanism of DCVCS-induced nephrotoxicity. Covalent modification of proteins by reactive electrophiles can result in alterations of critical cellular functions or changes in signal transduction, thereby causing toxicity.^{20–22}

Although comparative mass spectrometry-based analysis has been used to demonstrate the formation of DCVCS-derived hemoglobin adducts in vitro and in vivo, the number of potential protein targets in the kidney makes this approach unfeasible. In addition, modified low abundance proteins that might be relevant to the mechanism of toxicity might be undetectable without some form of enrichment. Chemical proteomics, where drug affinity chromatography is combined with high mass accuracy MS to identify proteins targeted for covalent modification by small molecules, offers a more promising approach²³. Biotin-tagged chemicals have been used to identify human kidney proteins targeted by thiol-reactive Michael acceptors via affinity purification with immobilized streptavidin to enrich for targets followed by either Western blotting with anti-biotin antibodies or MS analysis to identify the modified proteins.^{20,24,25} The development of a biotin-tagged DCVCS probe could be used with these methods to provide insight into which proteins are modified by DCVCS, information that can be used in future studies to investigate whether covalent modification can contribute to DCVCS-induced nephrotoxicity. In addition, a biotin-tagged probe could have uses that extend beyond in vitro studies, such as ELISA-based quantification of adducts in tissues of animals dosed with the probe or immunohistochemical studies to determine tissue and cellular localization. The present study describes the development of such a probe, N-biotinyl-DCVCS (NB-DCVCS), and our efforts to characterize its stability in buffer alone and in the presence of GSH and blood proteins. We also characterized NB-DCVCS reactivity with endogenous thiols, selected model enzymes, and isolated rat kidney cytosolic proteins.

Experimental Procedures

Caution: DCVC and DCVCS are known to be hazardous and should be handled with care. DCVC is a strong, direct-acting mutagen in the Ames test.²⁶

Materials

DCVC and DCVCS were synthesized as previously described.^{7,17} Purity was determined by HPLC to be >95%. N-Hydroxysulfosuccinimidyl biotin (Sulfo-NHS-biotin) and streptavidin-agarose resin were obtained from Thermo Fisher Scientific (Waltham, MA). 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). Glutathione reductase (GRd) from baker's yeast and goat anti-biotin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Porcine heart mitochondrial malate dehydrogenase (MDH) was obtained from Calzyme Laboratories (San Luis Obispo, CA). Donkey anti-goat secondary antibody conjugated to horseradish peroxidase was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Synthesis and Characterization of NB-DCVCS

Biotinylation of DCVCS was performed by adding 21.6 mM DCVCS to 43.2 mM Sulfo-NHS-biotin in phosphate buffer (0.1 M KH_2PO_4 , 0.1 M KCl, 5 mM Na_2EDTA , pH 7.4) to achieve a 2-fold molar excess of biotinylation reagent. The reaction mixture was incubated for 60 min at room temperature. Separation of the reaction mixture was accomplished with a Gilson gradient controlled HPLC system (model 306 pumps) equipped with either a Beckman Ultrasphere ODS 5 μm analytical (4.6 mm $\times$ 25 cm) or semipreparative (10 mm $\times$ 25 cm) column. 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 and 3 mL/min for the semipreparative column. A unique peak not detected in the controls was purified by fraction collection off of semipreparative HPLC using the following method: The initial concentration was 3% B, which was held for 5 min. The gradient was increased to 42.5% B over the next 20 min before returning to 3% B over 10 min. The gradient was held at 3% B for a total run time of 45 min. The isolated product was then lyophilized overnight and purity verified to be > 95% by HPLC.

MS analysis of the isolated product was carried out at the Mass Spectrometry Facility of the University of Wisconsin Biotechnology Center. The product was reconstituted in 50:50 ACN:H₂O ratio before being subjected to LC/MS with electrospray ionization. Mass spectra were obtained by direct infusion of the sample into a 3200 Q Trap mass spectrometer (Applied Biosystems, Foster City, CA) and analyzed with a Q1 MS scan.

Isolation and Characterization of NB-DCVCS-GSH Adducts by LC/MS

NB-DCVCS (0.31 mM) was incubated in phosphate buffer only or with GSH (1.02 mM) in phosphate buffer. These concentrations were chosen to reflect the 3:10 molar ratio previously used to characterize the reactivity of DCVCS towards GSH.¹⁷ The reaction mixture was incubated in a 37°C shaking water bath for 3 h. The reaction mixture was analyzed by HPLC with UV detection set at 220 nm to monitor depletion of NB-DCVCS and 260 nm to monitor for appearance of products. Four new peaks were detected and these were fractionated together and lyophilized to dryness. Purity of these peaks was 96% by HPLC analysis. LC/MS analysis of these peaks 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 $\times$ 50 mm column. The flow rate was 0.25 mL/min and mobile phase A was 0.1% aqueous formic acid and mobile phase B was 0.1 % formic acid in ACN. The following method was used: the initial concentration was 2% B, which was held for 1 min before increasing to 35% B over the next 24 min. The gradient was then increased to 95% B over 3 min before returning 2% B over 2 min and being held at this concentration for a total run time of 42 min. Electrospray ionization was used in the positive mode.

To determine whether the biotin tag on NB-DCVCS affects the reactivity of the compound towards sulfhydryl groups, we compared the half-life of NB-DCVCS in the presence of GSH to that of DCVCS. GSH (1.02 mM) was incubated with NB-DCVCS or DCVCS (0.31 mM) in the phosphate buffer described above in a 37°C shaking water bath. Aliquots (50 μL) were removed every 30 min for 3 h for NB-DCVCS and every 5 min for 30 min for DCVCS, diluted with doubly deionized H₂O (100 μL), and acidified with 6.67% aqueous TFA (6.25 μL). Samples were then analyzed by HPLC with UV detection set to monitor at 220 nm and 260 nm. Half-lives for NB-DCVCS and each DCVCS diastereomer were calculated using first-order elimination kinetics: $C_t = C_0 \times e^{-kt}$ and $t_{1/2} = \ln 2/k$.

Determining Reactivity of NB-DCVCS towards the Model Enzymes GRd and MDH

GRd and mitochondrial MDH were used as model proteins to determine whether NB-DCVCS can covalently modify proteins containing sulfhydryl groups. These proteins were chosen because they contain a number of cysteine residues and are potential targets for modification by DCVCS. GRd is a cytosolic protein involved in protecting cells against oxidative stress and MDH is a mitochondrial protein involved in cellular respiration. Previous studies have demonstrated that GRd activity was inhibited in rat renal proximal tubule cells treated with DCVC²⁷ and MDH activity was inhibited in rat liver mitochondria in the presence of DCVC.²⁸ GRd and MDH were washed four times with phosphate buffer pH 7.4 using centrifugal filters to remove any storage solution components. NB-DCVCS (10 μ M final concentration) was added to the proteins so that final protein concentrations were 0.5 mg/mL. Reactions were incubated for 3 h at 37°C in a shaking water bath and then NB-DCVCS was removed using centrifugal filters to wash samples 3 times, adding 300 μ L phosphate buffer each time. Aliquots of the samples were added to an equal volume of Laemmli sample buffer, loaded onto two precast Criterion 12.5% resolving gels (Bio-Rad, Los Angeles, CA), and separated by SDS-PAGE. One gel was visualized by silver staining. The other gel was transferred to a nitrocellulose membrane. Goat anti-biotin primary antibodies and donkey anti-goat secondary antibodies were used to detect NB-DCVCS bound to proteins. The membrane was incubated with enhanced chemiluminescence (Thermo Fisher Scientific) and transferred to film. Transfer efficiency and loading were checked by Ponceau S staining (Sigma-Aldrich).

Determining Reactivity of NB-DCVCS towards Kidney Cytosolic Proteins

To determine whether NB-DCVCS could bind kidney proteins, we incubated different concentrations of NB-DCVCS with male Sprague Dawley rat kidney cytosol. The cytosolic fraction was chosen as the first subcellular fraction to examine because of the better resolution of cytosolic proteins by SDS-PAGE compared to nuclear, mitochondrial, and microsomal proteins. Kidneys from male Sprague Dawley rats (7–8 weeks) were received frozen on dry-ice by overnight shipping (Pel-freez Biologicals, Rogers, AR). After thawing tissues in cold phosphate buffer pH 7.4, cytosol was prepared by decapsulating kidneys, mincing the tissue into small pieces and adding phosphate buffer pH 7.4 (3 mL buffer per gram tissue). Tissue was then homogenized and centrifuged at 20,000 rpm for 30 min at 4°C. After removing the lipid layer, the supernatant was transferred to ultracentrifuge tubes and centrifuged at 45,500 rpm for 90 min at 4°C. The supernatant was retained as the cytosolic fraction.

Cytosol was washed two times with phosphate buffer using centrifugal filters to remove GSH and other small molecules and protein concentration was determined using the Lowry method.²⁹ Initial immunoblotting experiments with intact cytosol exhibited significant biotin background signal, with bands at approximately 70 kDa and 130 kDa being the most prominent. To reduce the biotin background signal so that NB-DCVCS binding could be better detected, cytosol was incubated with streptavidin-agarose resin to remove biotin-binding proteins as follows. Streptavidin-agarose resin was washed two times with phosphate buffer to equilibrate resin and remove storage solution components. Filtered cytosol (1 mL) was added to achieve a final concentration of 20 μ L resin per mg protein and incubated overnight at 4°C on a rocker. Resin was pelleted by centrifugation at 1000 $\times$ g for 2 min and supernatant was retained. Protein concentration of cleared filtered cytosol was determined by the Lowry method.

Cleared filtered cytosol was added to phosphate buffer or NB-DCVCS to achieve final concentrations of 6 μ g/ μ L cytosolic protein and 0, 0.3125, 0.625, 1.25, 2.5, or 5 μ M NB-DCVCS. Samples were incubated for 3 h at 37°C and then filtered 3 times with centrifugal

filters, adding 300 μ L phosphate buffer before each filtration step, to remove NB-DCVCS. Aliquots of the samples were added to an equal volume of Laemmli sample buffer, separated by SDS-PAGE and immunoblotted as described above.

To determine whether DCVCS and NB-DCVCS target the same proteins, cleared filtered male rat kidney cytosol was incubated with phosphate buffer or 10, 50, 100, or 250 μ M DCVCS for 3 h at 37°C. DCVCS was then removed by washing the samples 3 times with 400 μ L phosphate buffer and centrifugal filtration. NB-DCVCS was added to a final concentration of 2.5 μ M and samples were incubated for 3 h at 37°C before removing NB-DCVCS with the same filtration procedure described above. Aliquots of the samples were added to an equal volume of Laemmli sample buffer and proteins were separated by SDS-PAGE before immunoblotting as described above.

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. Rats were euthanized by CO₂ asphyxiation and blood collected by cardiac puncture and stored in heparinized vacutainer tubes (BD, Franklin Lakes, NJ). Plasma was prepared from half of the collected blood by centrifuging heparinized blood for 10 min at 3000 $\times$ g, decanting the plasma, and adjusting pH to 7.4. Plasma was stored frozen at –20°C until ready to be used. Plasma was thawed and any precipitate was pelleted by centrifugation (3000 $\times$ g, 10 min) and removed before using.

Stability of NB-DCVCS and DCVCS in Isolated Sprague Dawley Rat Whole Blood and Plasma

Stability of NB-DCVCS and DCVCS in rat blood and plasma was assessed to determine whether NB-DCVCS has sufficient stability in these biological compartments to be used for in vivo mechanistic studies. Blood and plasma were equilibrated to 37°C in a water jacketed incubator (5% CO₂, 85% humidity) and DCVCS or NB-DCVCS was added (50 μ M final concentration). Samples were incubated at 37°C and at predetermined timepoints 150 μ L aliquots were removed and immediately put on ice. Aliquots from the incubation of NB-DCVCS and DCVCS in blood was filtered using centrifugal filters and 75 μ L of the filtrate was added to 75 μ L doubly deionized water and 1 μ L 6.67% aqueous TFA. Aliquots from the plasma samples were acidified with 6.67% aqueous TFA to lower the pH 2 to stop further Michael addition-elimination reactions from occurring. Acidification prior to filtration was not possible with the blood samples due to marked hemolysis upon addition of acid. The plasma samples were then filtered with centrifugal filters. Filtrates were analyzed by HPLC and the half-life of NB-DCVCS and DCVCS in both media was calculated using equations for first order elimination kinetics.

Results

Synthesis and Characterization of NB-DCVCS

DCVCS was incubated with a 2-fold molar excess of Sulfo-NHS-biotin for 60 min at room temperature. These experimental conditions were based upon pilot experiments investigating the effect of variation of molar ratios of reactants and the time of incubation on product yield. Disappearance of the DCVCS peak and appearance of a unique peak not present in the controls (minus DCVCS) were detected by HPLC. This peak was purified by fractionation on HPLC and lyophilized before being analyzed by LC/MS. The purity of the isolated peak was >95% by HPLC. The purified compound displayed a molecular ion at m/z 456.1 in the

negative mode, consistent with the loss of one hydrogen atom and the addition of the biotin tag to the amino group of DCVCS (Figure 3).

Isolation and Characterization of NB-DCVCS-GSH Adducts by LC/MS

To ensure that NB-DCVCS retains the ability to react with sulfhydryl groups, NB-DCVCS and GSH were incubated together (3:10 molar ratio) at 37°C and the products of the reaction were characterized. Four new peaks in the reaction mixture were detected by HPLC (Figure 4A) and as the area of these peaks increased, a corresponding decrease in the peak area of NB-DCVCS was observed (Figure 4B). These new peaks were purified as a group from the other reaction mixture components by HPLC fractionation and lyophilized before being analyzed by LC/MS. The lyophilized product was resolved by LC/MS as 4 peaks, and all displayed molecular ions at m/z 729 in the positive mode, consistent with the addition of 1 GSH molecule to NB-DCVCS followed by the loss of HCl (Figure 4C).

NB-DCVCS was consumed quickly in the presence of GSH, with a half-life of 39.6 min (Figure 5). Comparatively, the half-life of the DCVCS diastereomers in the presence of GSH was 14.4 min and 6 min. These results suggest that NB-DCVCS and DCVCS have similar reactivity towards sulfhydryl groups and the presence of the biotin tag does not eliminate the ability of NB-DCVCS to act as a Michael acceptor in reactions with sulfhydryl groups.

Determining Reactivity of NB-DCVCS towards the Model Enzymes GRd and MDH

To determine whether NB-DCVCS can react with model enzymes containing sulfhydryl groups, the compound was incubated with GRd and MDH, two functionally important proteins that are potential targets in DCVCS-induced toxicity. The proteins were incubated with 10 μ M NB-DCVCS for 3 h at 37°C and binding by NB-DCVCS was detected by immunoblotting using antibodies against the biotin tag (Figure 6). Silver staining was used to determine where the proteins migrate on a gel to ensure that bands detected in the immunoblot were the proteins of interest. The major bands detected in the GRd and MDH samples were approximately 50 kDa and 35 kDa, respectively, corresponding to the molecular weight of the monomeric subunits of the proteins. In the immunoblot, bands were detected in the GRd and MDH reactions, suggesting that these proteins could be modified by NB-DCVCS.

Determining Reactivity of NB-DCVCS towards Kidney Cytosolic Proteins

Male Sprague-Dawley rat kidney cytosol was filtered to remove small molecules and cleared of biotin-associated proteins with streptavidin-agarose resin. Filtered and cleared cytosol was incubated with different concentrations of NB-DCVCS (312.5 nM to 5 μ M). Multiple modified proteins were detected and concentration-dependent increases in signal and the number of bands were observed (Figure 7). Proteins modified by NB-DCVCS were detected in samples containing 625 nM NB-DCVCS or greater, but the NB-DCVCS concentrations at which many of the bands were first detected, as well as the fold change in signal with increasing NB-DCVCS concentrations varied. Bands at approximately 25, 35, 37, and 55 kDa appear at low concentrations and might represent targets that are particularly susceptible to modification by NB-DCVCS. Incubating cleared filtered male rat kidney cytosol with 10 μ M DCVCS before incubating with 2.5 μ M NB-DCVCS was sufficient to block most of the NB-DCVCS modification (Figure 8A) and incubating with 100 μ M DCVCS appears to reduce the signal almost completely (Figure 8B).

Stability of NB-DCVCS and DCVCS in Isolated Sprague Dawley Rat Whole Blood and Plasma

NB-DCVCS and DCVCS stability in rat blood and plasma were assessed to determine if NB-DCVCS has sufficient stability in rat blood to be used for in vivo studies. Like DCVCS, NB-DCVCS is stable in phosphate buffer pH 7.4 and under acidic conditions (0.2 M HCl/KCl buffer pH 2) for several hours at 37°C (data not shown). However, when NB-DCVCS (50 μ M) was incubated in either male Sprague-Dawley rat whole blood or plasma and monitored by HPLC (Figure 9), NB-DCVCS had a half-life of 40 min in plasma and 68.5 min in whole blood and was not detectable after 150 min. In contrast, NB-DCVCS was found to be stable in phosphate buffer for greater than 120 min at 37°C. No DCVCS formation was observed as NB-DCVCS disappeared suggesting that enzymatic cleavage at the amide bond of NB-DCVCS is not a major contributor to the loss of the compound. Since the half-life in blood and plasma is comparable to the half-life in the presence of GSH, loss of NB-DCVCS might be due to reaction with thiol groups present in plasma and blood rather than enzymatic cleavage of the amide bond.

Compared to NB-DCVCS, DCVCS was less stable in blood and plasma. DCVCS (50 μ M) incubated in blood was not detected in any of the samples, suggesting that it reacts within the 15 minutes it takes to process the samples for HPLC analysis. Since plasma can be acidified prior to filtration, the effects of the extra time from sample processing was minimized. In plasma, the peak area of the first DCVCS diastereomer was 13.3 % of the original area by 15 minutes and became undetectable at 30 minutes (data not shown). The second DCVCS diastereomer was not detected in any of the samples, consistent with the comparative results when these compounds are incubated with GSH.

Discussion

Determining which proteins are targeted for covalent modification by reactive electrophiles is complicated by a number of factors: the fact that many different proteins are likely to be modified, and that modified proteins are likely to be only a small fraction of the total cellular proteins.³⁰ As a result, any attempts to determine the proteins and amino acids targeted require reliable methods for target enrichment and detection of covalent modification. Accurate affinity chromatography, Western blotting with anti-biotin antibodies, and mass spectrometry-based proteomics enables characterization of the formation of protein adducts and identification of the proteins targeted. The present study describes the synthesis of a novel biotinylated probe derived from DCVCS, a reactive electrophile that is known to be nephrotoxic, and the characterization of its stability in buffer at physiological conditions and in rat blood and plasma in vitro. In addition, its reactivity towards GSH, isolated model enzymes, and kidney cytosolic proteins was characterized. Labeling DCVCS with a biotin tag takes advantage of the strong affinity between biotin and streptavidin to create a tool that could potentially be used for target enrichment and modification detection, similar to studies with other biotin-labeled reactive electrophiles.^{24,25}

NB-DCVCS was synthesized by reacting DCVCS with Sulfo-NHS-biotin, an amine-reactive biotinylation reagent. Isolation of the reaction product by HPLC and characterization by ESI-MS provided evidence that this product was NB-DCVCS. The attachment of the biotin tag to the amino group in DCVCS was not expected to interfere with the ability of the compound to react with GSH since the tag does not disturb the α,β -unsaturated moiety that is necessary for the compound to act as a Michael acceptor. To demonstrate that NB-DCVCS retained the ability to form adducts with nucleophilic residues, NB-DCVCS was incubated with GSH at a 3:10 molar ratio. Incubation of DCVCS with GSH at this same ratio was shown to result in the formation of DCVCS-GSH adducts.¹⁷ When NB-DCVCS and GSH were incubated together, four new products were detected by HPLC, and LC/MS

analysis showed them to be consistent with mono-GSH adducts formed by an addition-elimination reaction between NB-DCVCS and a GSH molecule. The consumption of DCVCS and NB-DCVCS in the presence of GSH was used to compare their relative reactivity to sulfhydryl groups. The half-lives of the compounds were on the same order of magnitude, giving further support that NB-DCVCS is a suitable probe to investigate covalent modification by DCVCS.

GRd and MDH were used as model enzymes to determine whether NB-DCVCS can covalently modify proteins containing sulfhydryl groups. These enzymes were chosen because they are functionally important, exhibit differential cellular localization, contain a number of cysteine residues, have different molecular weights, and are potentially relevant in DCVCS-induced toxicity.^{31,32} Both proteins have been shown to have inhibited activity in the presence of DCVC.^{27,28} GRd is a dimeric protein with 5 cysteines per 53 kDa subunit and 2 of the cysteine residues are involved in disulfide bonds.³¹ MDH is a dimeric protein with 8 cysteine residues per 34 kDa subunit and has no disulfide bonds.³² Immunoblotting demonstrated that GRd and MDH were modified by NB-DCVCS. These results suggest that it is possible to carry out studies involving the detection of covalent modification of specific proteins by NB-DCVCS *in vitro*. This could be useful in determining whether specific proteins contain cysteine residues susceptible to covalent modification and help with screening for potential DCVCS targets. Follow-up studies to determine whether incubation with DCVCS affects protein function would be necessary to further refine the list of potential targets.

Furthermore, this study is the first to demonstrate that NB-DCVCS binds to kidney proteins *in vitro*. Male rat kidney cytosol was filtered to remove small molecules and incubated with streptavidin-agarose resin to clear biotin-associated proteins that otherwise cause high background signal during immunoblotting. Biotin is found at high concentrations in the kidney and is mainly localized in the cytoplasm of renal tubular epithelial cells.³³ Renal epithelial cells play an important role in biotin homeostasis by absorbing biotin from the urine.³⁴ The detected endogenous biotin-associated kidney proteins could also include carboxylases, which contain a biotin prosthetic group.³⁴ Immunoblotting filtered and cleared male rat kidney cytosol that was incubated with NB-DCVCS demonstrated a concentration-dependent increase in biotin signal. Multiple bands of different molecular weights were detected, and many of the bands showed variation in the NB-DCVCS concentration at which they could be detected. This suggests that multiple proteins are targeted for covalent modification and that these proteins exhibit different sensitivity to modification by NB-DCVCS. The bands (including prominent ones at 25, 35, 37, and 55 kDa) that are detectable at low doses might represent proteins that are more susceptible to modification and therefore more relevant to better understanding the mechanism of DCVCS-induced nephrotoxicity.

The proteins targeted and sites of covalent modification have been shown to be selective and distinct for different reactive electrophiles.²⁵ To determine whether DCVCS and NB-DCVCS share protein targets, male rat kidney cytosol was incubated first with DCVCS, which was washed out before incubation with NB-DCVCS. Incubating the cytosolic proteins with 10 – 100 μ M DCVCS first appeared to be sufficient to block most of the signal from incubating with 2.5 μ M NB-DCVCS, suggesting that these compounds do in fact share many protein targets.

While immunoblotting suggests that NB-DCVCS would be a suitable reagent for *in vitro* identification of protein targets in tissue and cell extracts, the stability of NB-DCVCS in rat whole blood and plasma was investigated to determine whether NB-DCVCS could be used for *in vivo* studies as well. Although the biotin-amide bond produced by most commercial biotinylation reagents are not stable in plasma when assessed after *in vitro* incubations, the

amide bond formed upon biotinylation of DCVCS is anticipated to be relatively stable in the absence of nucleophiles.³⁵ Studies have shown that a carboxylate alpha to the biotin-amide bond decreased susceptibility of the biotin link to hydrolysis in human plasma by both enzymatic and non-enzymatic processes, possibly due to steric hindrance.³⁶

NB-DCVCS was found to be similarly stable in both rat whole blood and plasma, although the half-life of NB-DCVCS was slightly higher in whole blood. This was not surprising as other studies have shown that some compounds, such as cisplatin, are more stable in blood than in plasma.³⁷ DCVCS was not detected in these samples, suggesting that cleavage of the amide bond by enzymatic factors such as biotinidase, amidase, or esterase might not constitute a significant source of degradation for NB-DCVCS in blood and plasma. NB-DCVCS half-life in blood and plasma was similar to the half-life in the presence of GSH, suggesting that reactions with thiols in plasma and blood could be primarily responsible for the apparent loss of NB-DCVCS over time. NB-DCVCS is more stable than DCVCS in both blood and plasma, a trend that was also seen when these compounds were incubated with GSH. The fact that DCVCS has a shorter half-life in these media but is still a preferential nephrotoxicant in vivo after intraperitoneal injection suggests that NB-DCVCS has the stability to be able to reach the kidney.

In summary, these experiments demonstrate that NB-DCVCS could be a useful probe for investigating covalent modification of proteins by DCVCS in vitro. NB-DCVCS readily forms adducts with GSH, model enzymes, and isolated kidney proteins. Incubating isolated kidney proteins with DCVCS prior to addition of NB-DCVCS blocks NB-DCVCS binding to proteins suggesting that the compounds share common targets. It is reasonably stable in phosphate buffer, rat plasma, and whole blood, suggesting that it is likely to be useful for in vivo applications. Further studies investigating the disposition and toxicity of NB-DCVCS in a whole organism would need to be carried out to confirm that NB-DCVCS can be used as a model in vivo.

List of Abbreviations

ACN	acetonitrile
DCVC	S-(1,2-dichlorovinyl)-L-cysteine
DCVCS	S-(1,2-dichlorovinyl)-L-cysteine sulfoxide
FMO3	flavin-containing monooxygenase 3
GRd	glutathione reductase
GSH	glutathione
MDH	malate dehydrogenase
NAC	N-acetyl-L-cysteine
NB-DCVCS	N-biotinyl-DCVCS
Sulfo-NHS-biotin	N-hydroxysulfosuccinimidyl biotin
TCE	trichloroethylene or trichloroethene
TFA	trifluoroacetic acid

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Figure 1. Glutathione-dependent metabolism of trichloroethylene (TCE). S-(1,2-dichlorovinyl)glutathione (DCVG), S-(1,2-dichlorovinyl)-L-cysteine (DCVC), S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS), N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (N-AcDCVC), N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (N-AcDCVCS), chlorothioacetone (CTK) and chloroacetone (CK).

Figure 2.

The reaction between DCVCS and NAC results in the formation of 3 monoadducts and one crosslink. DCVCS acts as a Michael acceptor, forming a covalent bond with NAC at the thiol group and losing HCl by trans-elimination. Monoadducts 1 and 2 are a pair of diastereomers.

Figure 3.

Negative ion LC/MS and structure of NB-DCVCS. The major ion at m/z 456 corresponds to the expected molecular weight of NB-DCVCS, and ions at m/z 458.0 and 460.1 are isotopic ions of NB-DCVCS due to the presence of Cl atoms. The ion at m/z 360.2 is likely to be the remaining fragment of NB-DCVCS after the loss of H₂C₂Cl₂ due to the lack of isotopic ions at m/z 362 and 364. The ion at m/z 243.2 is a contaminant that is most likely biotin formed by hydrolysis of the biotinylation reagent.

Figure 4.

(A) Typical chromatogram of NB-DCVCS-GSH adducts 1–4 formed when NB-DCVCS and GSH (3:10 molar ratio) are incubated together for 1 h at pH 7.4, 37°C. (B) Formation of NB-DCVCS-GSH adducts (○) and consumption of NB-DCVCS (●) over time at a reaction ratio of 0.157 mM NB-DCVCS to 0.523 mM GSH (3:10 molar ratio). (C) LC/MS of NB-DCVCS-GSH monoadduct 2.

Figure 5. Stability of 0.31 mM NB-DCVCS (●) and DCVCS diastereomers I (▼) and II (■) in the presence of 1.02 mM GSH (pH 7.4, 37°C).

Figure 6.

10 μ M NB-DCVCS was incubated with glutathione reductase (GRd) and malate dehydrogenase (MDH) for 3 h at 37°C. Reactions containing GRd (A) or MDH (B) were separated by SDS-PAGE and visualized by silver stain or immunoblotted (IB) using anti-biotin antibodies to demonstrate that NB-DCVCS binds to GRd and MDH.

Figure 7.

Immunoblot demonstrating concentration-dependent increase in binding of NB-DCVCS to rat kidney cytosol proteins. Male Sprague Dawley rat kidney cytosol was filtered to remove small molecules and cleared of biotin-associated proteins with streptavidin-agarose resin overnight and incubated with 0 – 5 μM NB-DCVCS for 3 h at 37°C. Samples were run by SDS-PAGE and immunoblotted with a goat anti-biotin antibody.

Figure 8.

(A) 1 min exposure and (B) 4 min exposure of the same immunoblot demonstrating that NB-DCVCS and DCVCS target the same proteins. Male Sprague Dawley rat kidney cytosol was filtered to remove small molecules and cleared of biotin-associated proteins with streptavidin-agarose resin overnight. Pre-cleared cytosol was incubated with 0 – 250 μM DCVCS for 3 h at 37°C. Samples were filtered to remove unbound DCVCS before incubation with 2.5 μM NB-DCVCS for 3 h at 37°C. Samples were run by SDS-PAGE and immunoblotted with a goat anti-biotin antibody.

Figure 9.

Degradation of NB-DCVCS over time at pH 7.4, 37°C in various media: phosphate buffer (●), male Sprague Dawley rat plasma (▼), male Sprague Dawley rat blood (○).