Published in final edited form as:

Chem Res Toxicol. 2011 February 18; 24(2): 217–228. doi:10.1021/tx100282b.

Thiol-Activated DNA Damage By α -Bromo-2-cyclopentenone

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Abstract

Some biologically active chemicals are relatively stable in the extracellular environment but, upon entering the cell, undergo biotransformation into reactive intermediates that covalently modify DNA. The diverse chemical reactions involved in the bioactivation of DNA-damaging agents are both fundamentally interesting and of practical importance in medicinal chemistry and toxicology. The work described here examines the bioactivation of α -haloacrylyl-containing molecules. The α -haloacrylyl moiety is found in a variety of cytotoxic natural products including clionastatin B, bromovulone III, discorhabdins A, B, and C, and trichodenone C, in mutagens such as 2-bromoacrolein and 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), and in the anticancer drug candidates brostallicin and PNU-151807. Using α -bromo-2-cyclopentenone (**1**) as a model compound, the activation of α -haloacrylyl-containing molecules by biological thiols was explored. The results indicate that both low molecular weight and peptide thiols readily undergo conjugate addition to **1**. The resulting products are consistent with a mechanism in which initial addition of thiols to **1** is followed by intramolecular displacement of bromide ion to yield a DNA-alkylating episulfonium ion intermediate. The reaction of thiol-activated **1** with DNA produces labile lesions at deoxyguanosine residues. The sequence specificity and salt-dependence of this process is consistent with involvement of an episulfonium ion intermediate. The alkylated guanine residue resulting from the thiol-triggered reaction of **1** with duplex DNA was characterized using mass spectrometry. The results provide new insight regarding the mechanisms by which thiols can bioactivate small molecules and offer a more complete understanding of the molecular mechanisms underlying the biological activity of cytotoxic, mutagenic, and medicinal compounds containing the α -haloacrylyl group.

Introduction

Many DNA-damaging natural products are relatively stable in the extracellular environment but, upon entering cells, undergo biotransformation to reactive intermediates that covalently modify the genetic material (1, 2). For example, DNA damage by mitomycins (3, 4), saframycins (5), and dynemicin (6) is activated by enzymatic reduction of their quinone moieties. Azomycin and 2-carboxyquinoline 1,4-dioxide are activated by enzymatic reduction of nitro and heterocyclic *N*-oxide groups, respectively (7, 8). Bioactivation of

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acylfulvene natural products is carried out by NADPH-dependent alkenal/one reductase (9), while DNA alkylation by pyrrolizidine alkaloids and aflatoxins is triggered by cytochrome P450-mediated monooxygenation reactions (10–12).

Intracellular thiols (13, 14) also have the potential to activate DNA-damaging natural products. For example, DNA strand cleavage by calicheamicin, dynemicin, and neocarzinostatin are triggered by reactions with thiols (6, 15–17). DNA alkylation by acylfulvenes may be triggered by 1,4-addition of thiols (18). Reaction of the *Streptomyces*-derived natural product leinamycin with thiols initiates both DNA alkylation and generation of reactive oxygen species (ROS) (19–27). The natural products varacin, lissoclinotoxin A, and thiarubrin C also generate ROS following reactions with thiols (28–31). In addition, thiol-triggered analogues of mitomycin C have been characterized (32, 33).

The diverse chemical reactions involved in the bioactivation of DNA-damaging agents are both fundamentally interesting and of practical importance in medicinal chemistry and toxicology (1, 2, 34). For example, elucidation of bioactivation processes can reveal new chemical mechanisms by which small organic molecules can deliver reactive intermediates to the interior of cells. The work described here examines thiol-mediated bioactivation of α -haloacrylyl-containing molecules. The α -haloacrylyl moiety is found in a variety of cytotoxic natural products including clonastatin B, bromovulone III, discorhabdins A, B, and C, and trichodenone C, in mutagens such as 2-bromoacrolein and 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), and in the anticancer drug candidates brostallicin and PNU-151807 (Figure 1) (35–46). We employed α -bromo-2-cyclopentenone as a model compound to examine the thiol-mediated bioactivation of α -haloacrylyl-containing molecules. The evidence presented here suggests that 1,4-addition of thiols to the α -haloacrylyl moiety leads to formation of an episulfonium ion that can alkylate guanine residues in duplex DNA (Scheme 1).

Experimental Section

Materials

Materials were purchased from the following suppliers: 3-[*N*-morpholino]propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 1,4-piperazinediethanesulfonic acid (PIPES), ethylenediaminetetraacetic acid (EDTA), *N,N,N',N'*-tetraethylenediamine (TEMED), sigmacote, *L*-lysine, glutathione-*S*-transferase from equine liver and cysteamine were purchased from Sigma Chemical Company; dimethylsulfate, sodium acetate, 2-cyclopenten-1-one and 3-bromo-2-butanone, *N*-methylaniline were purchased from Aldrich Chemical; G-25 Sephadex, glutathione and 2-mercaptoethanol were purchased from Sigma-Aldrich; diethylenetriaminepentaacetic acid (DETAPAC) was purchased from Fluka Chemical Company; piperidine was purchased from Alfa Aesar; xylene cyanol, bromophenol blue, formamide, urea and ammonium persulfate were purchased from United States Biochemical; herring sperm DNA and TRIS-borate-EDTA (TBE) were purchased from Roche Molecular Biochemicals; acrylamide was purchased through Fischer Scientific Inc., T4 polynucleotide kinase (T4-PNK) and bovine serum albumin (BSA) were purchased from New England Biolabs; [γ -³²P]-dATP was purchased from Perkin-Elmer Life Sciences, and oligonucleotides were purchased from Integrated DNA Technologies.

Reaction of **1** with the Peptide Probe γ -ECGHDRKAHYK

The peptide probe, γ -ECGHDRKAHYK (200 μ M), was incubated with **1** (200 μ M) in potassium phosphate (0.1 M, pH 7.4) at 25 °C for 0.5 h. Following incubation, the modified peptide(s) was extracted from the solution employing a solid phase (C8 HLB cartridges, Waters) chromatographic work-up consisting of a series of acetonitrile-0.1% formic acid

mixtures to wash and subsequently elute the peptide. The peptide-containing eluant resulting from an 80% acetonitrile (0.1% formic acid) wash was subjected to electrospray ionization, tandem mass spectrometry (ESI-MS/MS) and liquid chromatography, tandem mass spectrometry (LC/MS/MS) employing an LTQ-Orbitrap XL high-resolution mass spectrometer (Thermoelectron Corp., San Jose, CA). For direct infusion ESI-MS, the solid phase extracted reaction was infused into the mass spectrometer at a flow rate of 10 μ L/min using a stainless steel emitter on a Thermo-Fisher MaxIon source. The heated capillary was maintained at 275 °C. For MS/MS analysis, ions of interest were selected with an isolation window of 2.0 Da. Fragmentation was catalyzed via collision-induced dissociation (CID) and high-energy C-trap dissociation (HCD), with normalized collision energies varying from 10–40%. All spectra were acquired in the full profile mode with a resolution of 100,000 at m/z 400. Each spectrum was generated by signal averaging for approximately 20 s with a maximum injection time of 1000 ms in MS mode and 2000 ms in MS/MS mode. All the data were acquired using external calibration with a mixture of caffeine, MRFA peptide, and Ultramark 1600, dissolved in a acetonitrile:water (50:50, v/v). The multiply-charged, pseudo-molecular ions of the alkylated peptides were subjected to CID in the LTQ and HCD in the multipole collision cell adjacent to the Orbitrap mass analyzer. For LC-MS/MS, the reaction mixtures were separated employing an Agilent 1200 nanoflow LC system (Agilent, Wilmington, DE) utilizing a gradient of 0.1% formic acid and acetonitrile (3–45% acetonitrile over 45 min) and coupled to a C-18 reverse phase column (5 μ , 200 Å, Michrom Bioresources, Inc., USA) and a 15-cm fused silica emitter (75 μ m i.d., PicoTip Emitter™/PicoFrit Self/P, New Objective, USA). The LC eluent was introduced into the LTQ Orbitrap mass spectrometer equipped with a nanoelectrospray ion source (Thermoelectron Corp, Bremen, Germany). Full scan MS spectra (m/z 300–2000 Da) were acquired with a resolution of 60,000 at m/z 400.

Reaction of **1** with 2-Mercaptoethanol

A mixture of **1** (80.5 mg, 0.5 mmol), 2-mercaptoethanol (35 μ L, 0.5 mmol), and triethylamine (70.3 μ L, 0.5 mmol) were mixed in methanol (10 mL) and incubated at 37 °C for 24 h. Following removal of solvent, the major product was purified by column chromatography on silica gel eluted with 40% ethyl acetate in dichloromethane. The compound 2-(2-hydroxyethyl)cyclopent-2-enone (**6c**) was obtained in approximately 40% yield (contaminated with traces of 2-mercaptoethanol and 2-mercaptoethanol disulfide). ¹H NMR (300 MHz, CDCl₃): δ 7.41 (t, J = 3 Hz, 1H), 3.78 (t, J = 6 Hz, 2H), 3.04 (t, J = 6 Hz, 2H), 2.72–2.68 (m, 2H), 2.54–2.051 (m, 2H).

In Vitro Metabolism of **1** in Human Hepatic S9 Fractions and Hepatocytes

Human hepatic S9 fractions—The in vitro metabolism of **1** was investigated using human hepatic S9 fractions (47). A potassium phosphate-buffered reaction (0.1 M, pH 7.4) of **1** (100 μ M), hepatic S9 fractions (5 mg/mL), glutathione (2 mM) and MgCl₂ (3 mM) was initiated by the addition of NADPH (2 mM) and incubated at 37 °C in borosilicate glass test tubes under ambient oxygenation for 50 min. Protein was precipitated by the addition of two volumes of acetonitrile, and the resulting mixture was chilled at 4 °C for 20 min followed by centrifugation. The residues were reconstituted in 85:15 (v/v) water:acetonitrile, a solution reflective of the ensuing LC/MS analysis.

Hepatocytes—Cryopreserved human hepatocytes (1 $\times$ 10⁶ cells/mL) were incubated with **1** (100 μ M) at 37 °C for 4 h under controlled atmospheric conditions (95% air/5% CO₂). Cells were suspended in Krebs-Hensleit buffer that was supplemented with glucose (5 mM) and sodium pyruvate (1 mM). Cells were precipitated and reactions stopped by addition of two volumes of acetonitrile, followed by storage at –20° C. Samples were prepared for LC-MS analysis by decanting the supernatants and drying under a stream of nitrogen. The

residues were reconstituted in water:acetonitrile (85:15, v/v) in preparation for LC/MS/MS analysis. LC/MS/MS characterization was completed employing an Agilent 1100 HPLC system coupled to a Symmetry C₁₈ column (5 μ m, 3.8 $\times$ 150 mm; Waters Corporation, Milford, MA). Solvent A was formic acid (0.1%) and solvent B was acetonitrile (fortified with 0.1% formic acid). The initial mobile phase was 98:2 A:B (v/v) and by linear gradient transitioned to 10:90 A:B over 22 min.

Reaction of **1** with 2-Mercaptoethanol or Glutathione: Qualitative Rate Measurements

A solution containing **1** (0.05 mM), 2-mercaptoethanol (0.5 mM, 1 mM, or 2 mM), HEPES buffer (50 mM, pH 7), DETAPAC (1 mM as a chelator of adventitious trace metals to suppress autooxidation of thiols and the production of radicals that accompanies this process), and acetonitrile (0.06% v/v) was prepared in HPLC grade water, vortex-mixed, and placed in a quartz cuvette (1 cm path length). Absorption spectra were collected at 5 s intervals at room temperature (Figure 3). Alternatively, the reaction of **1** with glutathione was measured using HPLC. A solution containing sodium phosphate buffer (60 μ L of a 500 mM aqueous solution, pH 7), **1** (15 μ L of a 40 mM solution in acetonitrile), and glutathione (150 μ L of a 40 mM aqueous solution) were mixed with an appropriate amount of distilled, deionized water to give a final volume of 600 μ L and the solution agitated using a vortex mixer and incubated at 37 $^{\circ}$ C for 2 min (final concentrations: 50 mM sodium phosphate buffer, pH 7; 1 mM of **1**; 10 mM of glutathione; 2.5% (v/v) acetonitrile; 600 μ L final reaction volume). After incubation, 5 μ L of the reaction mixture was analyzed by HPLC using a reverse phase Supilcosil LC-18-S column (particle size, 5 μ m; 15 cm L $\times$ 2.1 mm ID) at a flow rate of 0.2 mL/min using a gradient of increasing methanol in water. The HPLC elution protocol was continuous gradient of 80–95% methanol over 30 min. The column was then washed with 95% methanol for 10 min, followed by a continuous gradient of 95–80% methanol over 5 min then 80% methanol for 15 min.

Thiol-triggered DNA Alkylation by **1**

The single-stranded 2'-deoxyoligonucleotide 5'-GTT CGT ATA TGG GAG GTC GCA TGT G-3' were labeled using [γ -³²P]dATP and T4 polynucleotide kinase according to standard protocols(48) and were purified on a 20% denaturing polyacrylamide gel (48). The purified single-strand radiolabeled oligonucleotide was annealed with 1.5 equiv of the complementary strand 5'-CAC ATG CGA CCT CCC ATA-3' by heating to 95 $^{\circ}$ C for 5 min in buffer (25 mM MOPS, pH 7, and 100 mM NaCl), followed by slow-cooling to room temperature overnight (~20 h). In a typical alkylation reaction, 5'-³²P labeled oligonucleotide duplex (3 μ L, approximately 50,000 cpm), HEPES buffer (2 μ L of a 500 mM aqueous solution, pH 7), DETAPAC (0.5 μ L of a 40 mM aqueous solution), 2-mercaptoethanol (0.5 μ L, 2.5 μ L, or 5 μ L of a 40 mM aqueous solution), and **1** (0.5 μ L, 2.5 μ L, or 5 μ L of a 40 mM solution in acetonitrile) were mixed with an appropriate amount of distilled, deionized water to give a final volume of 20 μ L and the solution agitated using a vortex mixer (final concentrations: 50 mM HEPES, pH 7; 1 mM DETAPAC; 1 mM, 5 mM, or 10 mM of 2-mercaptoethanol; 1 mM, 5 mM, or 10 mM of **1**).

The reaction was quenched by precipitation of the DNA. To the mixture was added herring sperm carrier DNA (3 μ L of a 1 mg/mL solution), sodium acetate (2 μ L of a 3 M, pH 5 solution), and absolute ethanol (175 μ L); (final concentrations: 15 μ g/mL of carrier DNA, 30 mM of sodium acetate, 87.5% ethanol, final volume 200 μ L). The DNA was precipitated by cooling this mixture on dry ice for 45 min, followed by centrifuging for 45 min at 14,000 rpm in a benchtop microcentrifuge. The supernatant was removed, and the pellet was washed twice with 70% ethanol–water (v/v). The DNA pellet was redissolved in aqueous piperidine (100 μ L of a 0.5 M solution) and incubated at 90 $^{\circ}$ C for 25 min (49). The solution was frozen on dry ice, lyophilized for 1.5 h in a SpeedVac Concentrator at 37 $^{\circ}$ C. The

oligonucleotides were then taken through two cycles of redissolution in 90 μ L of water, freezing, and lyophilization in a SpeedVac Concentrator at 37 $^{\circ}$ C. The dried DNA fragments were dissolved in formamide loading buffer and denatured at 90 $^{\circ}$ C for 4 min then immersed in ice water. An equal number of counts were loaded in each lane of a 20% denaturing polyacrylamide gel (19:1 cross-linking, containing 5 M urea). The gel was electrophoresed at 800 V for approximately 9 h. The DNA fragments in the gel were visualized by phosphorimager analysis (Molecular Imager FX, Imaging Screen-K, cat 170-7841, Bio-Rad, using Quantity One[®] Version 4.2.3, Bio-Rad). Experiments involving other thiol or amine activating agents were carried out in an identical manner. Control reactions examining alkylation by 3-bromo-2-butanone and 2-cyclopentenone were carried out as described above except **1** was replaced with these molecules. Experiments in which the pH was varied (pH 6.1 and 8) employed a buffer consisting of a HEPES (50 mM) and PIPES (50 mM) mixture.

LC/MS/MS Characterization of the Guanine Adducts Generated by Reaction of **1** and Thiol with Duplex DNA

Herring sperm DNA (double-stranded, 5 mM bp) was treated with **1** (5 mM) and 2-mercaptoethanol (5 mM) in HEPES buffer (pH 7, 50 mM) containing DETAPAC (1 mM). The reaction mixture was incubated at 37 $^{\circ}$ C for 72 h. The resulting alkylated DNA was heated at 90 $^{\circ}$ C for 45 min to release alkylated bases. Large DNA fragments were then removed by filtering the solution through a microconcentrator filter (YM-3, Microcon) by centrifugation at 10,000 rpm for 1.5 h. The resulting solution was lyophilized in preparation for LC/MS/MS analysis. The resulting residue was dissolved in aqueous methanol (25% v/v) and a portion (5 μ L) subsequently diluted in 15% aqueous acetonitrile (45 μ L).

LC/MS/MS characterization was completed employing an Agilent 1100 HPLC system coupled to a Symmetry C₁₈ column (5 μ , 3.8 $\times$ 150 mm; Waters Corporation, Milford, MA). Solvent A was formic acid (0.1%) and solvent B was acetonitrile (fortified with 0.1% formic acid). The initial mobile phase was 95:5 A:B (v/v) and by linear gradient transitioned to 20:80 A:B over 20 min. The flow rate was 0.4 mL/min. The HPLC eluent was introduced via electrospray ionization directly into a Finnigan LCQTM Deca XP^{PLUS} ion trap mass spectrometer (Thermoelectron Corporation, San Jose, CA) operated in the positive ionization mode. Ionization was assisted with sheath and auxiliary gas (ultra pure nitrogen) set at 60 and 40 psi, respectively. The electrospray voltage was set at 5 kV with the heated ion transfer capillary set at 300 $^{\circ}$ C and 30 V. Relative collision energies of 32% were used when the ion trap mass spectrometer was operated in the MS/MS mode.

Results

Reaction of α -Bromo-2-cyclopentenone (**1**) with the Cysteine-Containing Probe Peptide γ -ECGHDRKAHYK, with 2-Mercaptoethanol, or with Glutathione in Human Hepatic S9 Fractions

Compound **1** was prepared by the reaction of 2-cyclopentenone with Br₂ in the presence of triethylamine as described previously (50, 51). To examine the reactivity of **1** with a range of nucleophiles, we incubated **1** (200 μ M) in a phosphate-buffered (100 mM, pH 7.4) solution containing a peptide (γ -ECGHDRKAHYK, 200 μ M) bearing a variety of nucleophilic side chains including carboxylate, thiol, alcohol, imidazole, and amine groups. The peptide is an analog of glutathione (γ -Glu-Cys-Gly) bearing a H₂NHis-Asp-Arg-Lys-Ala-His-Tyr-Lys-CO₂H peptide linked to the C-terminal glycine residue (52). Following a 30 min incubation (25 $^{\circ}$ C), the reaction was subjected to solid phase extraction (C18 Sep-Pak[®]) followed by reversed-phase LC-MS/MS analysis employing accurate mass spectroscopy (Supporting Information). Several products arising from the reaction of **1** with

this nucleophilic probe peptide were observed (Scheme 1 and Figure 2). Most notably, a product eluting at 26.8 min produced a $[M+2H]^{2+}$ and a $[M+3H]^{3+}$ at the mass-to-charge ratios (m/z) of 712.3308 and 475.2226, respectively (Table 1), consistent with adduct **6a** arising from addition of the peptide to **1** (net addition of 80 Da to the peptide, Scheme 1). Importantly, the presence of the y_9 and y_{10}^{2+} fragment ions at m/z 1111.5727 and 647.8070 (Figure 2D), suggest that the peptide was linked to **1** via the cysteine residue. This is indicated by a net 40 Da increase in the y_{10}^{2+} fragment ion compared to the same ion seen for the native peptide (607.7940 Da, Figure 2A), while the y_9 ions in both spectra appear at an m/z of 1111.5730 (isobaric). Upon closer examination of the reaction mixture, we observed two additional peptide adducts. Specifically, LC/MS/MS analysis revealed a product eluting at 25.7 min, producing a $[M+2H]^{2+}$ and a $[M+3H]^{3+}$ at m/z of 721.3359 and 481.2255, respectively (Figure 2B), consistent with a structure such as **4a** (Scheme 1). The presence of the y_9 fragment ion of **4a** (m/z 1111.5743 Da) and the corresponding y_{10}^{2+} fragment ion (m/z 656.8138 Da) were again indicative of attachment of the small molecule to the cysteine residue of the probe peptide. Interestingly, another product eluting at 23.3 min was observed, producing a $[M+2H]^{2+}$ and a $[M+3H]^{3+}$ at m/z 761.3193 and 507.8815 Da (Figure 2C), respectively, consistent with a phosphate adduct such as **5a**. Again, both the y_9 ion (m/z 1111.5731 Da) and the y_{10}^{2+} ion (m/z 696.7955 Da) were consistent with the aforementioned attachment of the peptide to **1** via the cysteine thiol group.

The stereochemistry and regiochemistry of the products identified here (**4a–6a**) are not defined by the mass spectrometry experiments. In the case of the sulfide, **6a**, the results of a model reaction lead us to favor a structure in which the sulfur of the attacking thiol is ultimately attached at the α -position adjacent to the carbonyl residue. Specifically, **6c** is observed as a major product of the reaction between **1** and 2-mercaptoethanol in methanol containing 1 equiv of triethylamine at 37 °C. NMR analysis of this product suggests that the sulfide side chain resides at the α -position adjacent to the carbonyl residue. This assignment is supported by the observation that the vinylic proton in the product appears as a triplet ($J = 3$ Hz) at 7.41 ppm. This is in line with NMR data reported for structurally analogous compounds (53).

The reaction of thiols with **1** in neutral solution is quite facile. We monitored the reaction of **1** with 2-mercaptoethanol in HEPES buffer (50 mM, pH 7) containing acetonitrile (0.06% v/v) using UV-vis spectrometry (Figure 3). A rapid decrease in the absorbance associated with the starting material was observed over the course of several minutes. In a separate HPLC assay, we find that more than 90% of **1** is consumed after incubation for 2 min with glutathione (10 equiv, 10 mM) in sodium phosphate buffer (50 mM, pH 7) containing 2.5% acetonitrile (v/v) at 37 °C (Supporting Information). In the absence of thiol, no degradation of **1** is observed within this time frame under the same conditions. These findings are consistent with literature reports showing that conjugate addition of thiols to α,β -unsaturated ketones occurs readily (54–56).

Although the data presented above indicates that compound **1** reacts readily with thiol groups, there are a variety of nucleophiles in the intracellular environment that could also, in principle, react with this compound. Accordingly, we set out to investigate whether the principle intracellular thiol, glutathione, reacts with **1** in human hepatic subcellular fractions (S9) and in human hepatocyte cells. We incubated **1** with human hepatic subcellular fractions (S9) that were fortified with glutathione to probe formation of the corresponding thiol adduct of **1**. Indeed, LC/MS analysis of the reaction supernatant revealed the presence of two isobaric metabolites producing a $[M+H]^+$ at m/z 388 Da (Figure 4A), consistent with addition of glutathione to **1** to yield compounds with the general structure **6b**. The fragmentation spectra of the two products were nearly identical, producing m/z 259 as a major fragment ion, corresponding to the loss of glutamate (–129 Da) from the parent ion

(Figure 4B). The loss of glycine (–75 Da) produced an ion at m/z 313 and represented an additional fingerprint fragment consistent with glutathione conjugation. We observed the same two glutathione conjugates when cryopreserved human hepatocyte suspensions (1×10^6 cells/mL) were incubated with **1** (Supporting Information).

Thiol-Triggered DNA Alkylation by α -Bromo-2-cyclopentenone (**1**)

A number of compounds containing the α -haloacryloyl fragment (Figure 1) are cytotoxic or mutagenic and may derive biological activity through reactions with DNA (41, 42). Therefore, we felt it necessary to examine the potential for **1** to act as a thiol-triggered DNA alkylating agent. Toward this end, the 5'- ^{32}P -labeled oligonucleotide 5'-GTTTCG₈TATATG₇G₆G₅AG₄G₃TCG₂CATG₁TG-3' (where the underlined region is double stranded, and the subscripts represent the numbering of guanine residues in Figures 5–7) was treated with various concentrations of **1** and 2-mercaptoethanol in 50 mM HEPES buffer, pH 7.0, at 37 °C for 24 h. Maxam-Gilbert workup (500 mM piperidine, 90 °C, 25 min)(49) and sequencing gel analysis revealed strand cleavage selectively at guanine residues in the reactions that contained both **1** and thiol (Figure 5). Control reactions involving the incubation of DNA with **1** alone or thiol alone showed little or no strand cleavage upon Maxam-Gilbert workup. A control reaction in which **1** was preincubated with 2-mercaptoethanol (37 °C, 26 h) prior to addition of the DNA also generated little or no DNA damage above background. This indicates that DNA modification is due to a transient intermediate generated in the reaction of **1** with thiol rather than the final reaction products.

DNA alkylation by **1** and 2-mercaptoethanol displays substantial sequence specificity, with approximately 15 times more alkylation seen at the most favored site versus the least favored site in the 2'-deoxyoligonucleotide duplex used in these experiments (Figures 5 and 6).

The initial 1,4-addition of thiols to **1** (Scheme 1) is expected to yield an α -bromoketone intermediate **2** (54). Previous work has shown that α -bromoketones have the potential to alkylate DNA (57–59). Therefore, we examined the ability of a simple α -bromoketone, 3-bromo-2-butanone, to generate labile lesions at guanine residues in the ^{32}P -labeled DNA duplex. Under conditions identical to those used for DNA alkylation by **1**, we find that 3-bromo-2-butanone produces no strand cleavage above background levels (Supporting Information). A separate control experiment shows that 2-cyclopentenone also does not generate any lesions that are labile to Maxam-Gilbert workup. Finally, we observed that 2-bromo-2-cyclohexenone generates thiol-dependent, labile lesions comparable to **1** (data not shown). Overall the structure-activity data is consistent with the involvement of an episulfonium ion in thiol-triggered DNA alkylation by **1**, rather than direct alkylation by **1**, **2**, or **6** (Supporting Information). DNA alkylation by charged alkylators such as episulfonium ions can be inhibited by added salt (24, 60–63). We find that addition of salt decreases the yield of DNA alkylation by **1** and 2-mercaptoethanol. Specifically, addition of 1 M NaClO₄ caused a $27 \pm 3\%$ decrease in overall alkylation yield.

We also examined the ability of glutathione and cysteamine to trigger DNA alkylation by **1**. We find that the use of these different thiols leads to different DNA alkylation yields and different sequence specificities (Figure 6). This observation is consistent with the generation of different intermediates in which the side chain of the attacking thiol has been incorporated into the ultimate DNA-alkylating species. The relative overall yields for thiol-triggered alkylation of duplex DNA by glutathione, cysteamine, and 2-mercaptoethanol are 1:5.7:8 in double-stranded DNA. It is noteworthy that glutathione, the major thiol present in mammalian cells, is a rather weak activator of the DNA alkylation process measured in this particular assay. The yields for the alkylation of single-strand DNA by **1** using glutathione, cysteamine, and 2-mercaptoethanol were 1:1.9:1.7. Thiol-triggered alkylation of single-

stranded DNA by **1** is less efficient than alkylation of duplex DNA. Previous studies have shown that small, positively charged alkylating agents such as aziridinium ions preferentially alkylate duplex DNA over single-stranded DNA (63).

Addition of amines to α -haloacrylyl systems can generate aziridines in organic solvents (64). Aziridines have the potential to alkylate DNA (65, 66). Accordingly, we examined whether amines activate DNA alkylation by **1**. We found that addition of *N*-methylaniline or *L*-lysine did not activate DNA alkylation by **1** under the conditions identical to those employed for the thiol-triggered DNA alkylation by **1** (<1% alkylation yields, see Supporting Information).

The enzyme glutathione *S*-transferase catalyzes the addition of glutathione to various electrophiles (67). We examined whether glutathione *S*-transferase had any effect on the yields of glutathione-triggered DNA alkylation by **1**. We found that addition of equine liver glutathione *S*-transferase had no significant effect on the yield of DNA strand cleavage caused by glutathione and **1** upon Maxam-Gilbert workup (Supporting Information).

We examined the effects of pH on thiol-triggered DNA alkylation by **1** (Figure 7). In the case of 2-mercaptoethanol and cysteamine, alkylation yields were highest at pH 7.0, with lower yields observed at pH values of 6.1 and 8.0. For 2-mercaptoethanol, the relative DNA alkylation yields obtained at pH 6.1, 7.0, and 8.0 are 1.5:2.9:1. For cysteamine, the relative DNA alkylation yields at pH 6.1, 7.0, and 8.0 are 2.6:5.1:1. In the case of glutathione, total alkylation yields are comparable at pH values of 6.1 and 7.0, while the yield of alkylation at pH 8.0 is decreased by a factor of 0.7 (relative to pH 7.0). The basis of the observed pH dependence remains uncertain. There are a number of reaction steps that could be pH sensitive or subject to acid-base catalysis, including addition of thiol to the haloketone and the attack of water, thiol, and DNA on the resulting reactive intermediate.

We employed LC/MS/MS to characterize DNA alkylation products generated by **1** in the presence of 2-mercaptoethanol. Mixed sequence, double-stranded herring sperm DNA was treated with **1** in the presence of 2-mercaptoethanol, followed by thermal workup (90 °C, 45 min) to induce depurination of the anticipated guanine adduct (21, 68). LC/MS/MS analysis revealed two adducts (eluting at 2.47 and 3.22 min) that produced an $[M+H]^+$ at m/z 310 Da (Figure 8A). Formation of these adducts was only observed when **1**, thiol, and DNA were all present in the reaction mixture.

Further MS³ analysis of the major adduct provided information regarding the site at which thiol-activated **1** becomes attached to the guanine residue. A mass transition from 232→215, corresponding to loss of ammonia (−17 Da) is observed for the major adduct (Figure 8C). MS³ mass transitions of 232→204 and 189, corresponding to loss of CO and HNCO are also observed. Collectively, these findings argue against covalent attachment at the N², N1, or O⁶ positions of the guanine residue in this particular adduct.

Discussion

We find that α -bromoacrylyl-containing compounds are thiol-triggered DNA-alkylating agents. Overall, the results of our experiments are explained by a mechanism involving initial 1,4-addition (54) of the thiol group to the α,β -unsaturated ketone followed by intramolecular displacement of the halide by the sulfide group in **2** to produce an episulfonium ion intermediate **3** (Scheme 1) (69–72). Products such as **6** observed in our mass spectroscopy experiments (Figures 2 and 4) are envisioned to arise via an elimination/ring-opening reaction of the episulfonium ion **3** (Scheme 2). Compound **6** is not likely to arise via direct displacement of the bromide because nucleophilic displacement of vinyl halides by thiols typically does not occur under mild conditions unless there is an electron-

accepting substituent in the in 2-position (73), although a mechanism involving 1,4-addition of thiol, followed by displacement of bromine by a second equivalent of thiol and, subsequent elimination of thiol cannot be ruled out. Mass spectrometry does not rigorously define the regiochemistry of thiol attachment in the products **6**, but two lines of evidence lead us to suggest that this product is formed predominantly with the sulfide residue in the α -position relative to the carbonyl (Scheme 2). First, this is the regiochemistry observed for the analogous elimination/ring-opening reaction of a presumed bromonium ion intermediate generated in the reaction of Br₂ with cyclopentenone (50). Second, we find that this regioisomer is the major isolable product arising from a model reaction of 2-mercaptoethanol with **1** (Scheme 2). It remains possible that lower yields of other regioisomer also may be formed in the model reaction. Indeed, mass spectrometric analysis of the products generated in the incubation of **1** with glutathione in human hepatocytes reveals two isobaric metabolites having a mass-to-charge ratio consistent with the structure **6b** (Scheme 1). We suggest that these products may represent two regioisomers of **6b**. Elimination reactions that yield **6** presumably will limit the efficiency with which **1** alkylates nucleic acids and other macromolecules in cells. Products with mass-to-charge ratios corresponding to the structures **4a** and **5a** (Scheme 1) produced in the reaction of **1** with the peptide probe can be envisioned to arise via attack of water and inorganic phosphate buffer ion on the episulfonium ion **3**, though it remains possible that these products are produced by attack on **2**.

When activated by thiols, **1** generates guanine lesions residues in DNA that lead to strand cleavage upon Maxam-Gilbert workup (Figures 5 and 6). It is well known that alkylated guanine residues are labile to Maxam-Gilbert workup (34, 49). Accordingly, this result is consistent with generation of a DNA-alkylating episulfonium ion in the reactions of **1** with thiol because alkylation of guanine residues can be considered a characteristic reaction of episulfonium ions (24, 74). For example, the chemistry seen here is reminiscent of 1,2-dibromoethane. Addition of glutathione or alkylguanine transferase (AGT) to 1,2-dibromoethane yields a 2-bromoethylsulfide intermediate that generates a DNA-alkylating episulfonium ion (55, 56, 62). In fact, the strand cleavage efficiency of **1** is comparable to that of other small, bioactive episulfonium ions (34, 49, 68, 75, 76). We find that addition of NaClO₄ (1 M) caused a 27 ± 3% decrease in the yield of DNA alkylation by **1** and 2-mercaptoethanol. Again, this result is consistent with the involvement of an episulfonium ion intermediate, as it has been shown that the reactions of positively charged alkylating agents, including episulfonium ions, diazonium ions, and aziridinium ions with DNA are inhibited by the addition of salt (24, 60–63). For example, previous work showed that addition of NaClO₄ (0.1 M) caused 18% and 27% decreases in the yields of DNA alkylation by leinamycin and chloroethyl ethylsulfide-derived episulfonium ions, respectively (24). The most favored alkylation site 5'-GGG site (underlined G is alkylated) for thiol-activated **1** (Figure 5) is broadly consistent with the sequence specificity expected for episulfonium ion intermediates (24, 75). For example, previous experiments have shown that 5'-GGG in duplex DNA was a preferred site for alkylation by the episulfonium ion derived from chloroethylethyl sulfide (24).

It is important to note that the observed alkylation at guanine residues could, in principle, be mediated by the α -haloketone intermediate **2**. Indeed, previous work has shown that α -bromoketones have the potential to alkylate DNA (57–59). However, control experiments showed that the α -bromoketone, 3-bromo-2-butanone produces no strand cleavage above background levels under the conditions employed for the assays with **1**. A separate control experiment showed that 2-cyclopentenone also does not generate Maxam-Gilbert labile lesions in DNA. Overall, these structure-activity results are consistent with the notion that thiol activation of **1** generates a DNA-alkylating episulfonium ion (Scheme 1).

Mass spectrometric analysis of the nucleobase lesions released upon thermolysis of duplex DNA treated with **1** and thiol revealed the products with mass-to-charge ratios consistent with the attachment of **3** to guanine (Scheme 1). We speculate that the two products observed in our experiment (Figure 8) represent the regioisomeric guanine adducts **7** and **8** (Scheme 1). The MS/MS spectrum of the major adduct (Figure 8B, upper trace) reveals a strong signal for a fragment ion at m/z 152 Da ($\approx 100\%$ relative abundance), corresponding to the liberation of guanine and relatively weak signals corresponding to the loss 2-mercaptoethanol (m/z 232 Da). This suggests that the major adduct possesses the regiochemistry shown in structure **7** for which the more facile loss of proton in the position α to the carbonyl group leads to elimination of guanine. In the minor adduct, the relative abundance of the fragments at m/z 232 ($\approx 100\%$) and 152 ($\geq 40\%$) are reversed (Figure 8B, lower trace). This is consistent with the proposed structure **8**, in which the elimination reaction involving the acidic α -proton leads to the m/z 232 fragment via loss of 2-mercaptoethanol unit. Episulfonium ions typically react to give *trans* ring-opening products; (77) however it remains possible that the two observed products represent *cis* and *trans* isomers.

The MS³ mass transitions observed for the major adduct argue against covalent attachment of the lesion at the N², N1, or O⁶ positions of this particular guanine adduct that is released upon thermal workup. This mass spectroscopic data along with the gel electrophoresis results and literature precedents are consistent with the N7-attachment proposed in structures **7** and **8** (Scheme 1). First, N7-guanine adducts are known to generate strand cleavage with Maxam-Gilbert workup (68). Second, the N7 assignment is consistent with literature showing that the N7-atom of guanine residues is the predominant alkylation site for all episulfonium ions that have been characterized (21, 24, 68, 74, 75, 78–82), while alkylation at N3-G by an episulfonium ion has not been observed (74). In fact, there are no examples of small alkylating agents that selectively alkylate guanine residues at the N3-position in duplex DNA. Small episulfonium ions typically alkylate DNA at several sites in addition to guanine residues and it is likely that non-labile adducts are formed in our experiments that are invisible to the thermal workup employed in our mass spectrometric analyses and the Maxam-Gilbert workup employed in our gel experiments (74).

An interesting aspect of the proposed mechanism is that the side chain of the activating thiol is incorporated into the putative episulfonium ion reactive intermediate. The data in Figure 6 clearly shows that the properties of the DNA-alkylating intermediate generated by the reaction of **1** with thiol vary depending upon the nature of the thiol employed. This observation is consistent with the involvement of different intermediates in which the side chain of the attacking thiol becomes incorporated into the ultimate DNA-alkylating species. The major thiol present in mammalian cells is glutathione (Scheme 1). On the other hand, some pathogens contain cationic or glycosylated thiols such as N¹-monogluthionylspermidine (*E. coli*), mycothiol (*Actinobacteria*), and trypanothione (*Trypanosoma* and *Leishmania*) (83, 84). Thus, α -haloacrolein derivatives will yield structurally distinct episulfonium ions in different organisms. As a result, α -haloacrolein-containing agents have the potential to display distinct biological activities that are dependent upon the thiol content of different organisms. In this regard it may be significant that glutathione, the major thiol present in mammalian cells, is a rather weak activator of guanine alkylation by **1** (Figure 6), while the cationic thiol cysteamine is a relatively efficient activator.

A number of studies have characterized adducts formed by the direct, in vitro reaction between α -haloacrolein derivatives and DNA (in the *absence* of thiols) (85–88). The products resulting from these direct reactions are distinct from those described here. For example, the reaction of 2-bromoacrolein with 2'-deoxycytidine yields a cyclic, bromine-containing

adduct resulting from addition of the exocyclic N⁴-nitrogen to the carbonyl carbon and 1,4-addition of the endocyclic N³ nitrogen to the olefin (85–88). Rate constants for these thiol-independent DNA alkylation reactions have not been reported. To the best of our knowledge, there are no reports indicating that such “thiol-independent” adducts have been observed in cell culture or in vivo. The interior of cells contains high concentrations (about 1–10 mM) of thiols (13, 14) and we find that the addition of thiols to the α -haloacrylyl moiety is rapid, reaching completion in minutes under physiologically-relevant conditions (Figure 3). The results of our experiments with human hepatic subcellular fractions (S9) provide evidence that human cells metabolize **1** via thiol conjugation pathways, even when other metabolic machinery such as cytochrome P450 enzymes are present. Thus, thiol-dependent adduct formation may compete effectively with thiol-independent adduct formation. Our data indicate that the covalent adducts generated following thiol activation of α -haloacrylyl compounds are quite different from those generated by the direct reaction of these agents with DNA(85–88). Therefore, the potential influence of thiols on the spectrum of DNA adducts should be considered if biomarkers are sought for assessing human exposure to this class of molecules.

Elements of the proposed mechanism (Scheme 1) involving thiol-mediated generation of an episulfonium ion may shed additional light on the molecular mechanisms underlying the bioactivity of medicinally interesting α -haloacrylyl-containing compounds (Figure 1). For example, the anticancer drug candidate brostallicin causes cytotoxic DNA alkylation (41, 42). Furthermore, the cell-killing activity of brostallicin is potentiated by glutathione and glutathione *S*-transferase enzymes (41, 42). It has been proposed that the α -bromoketone unit generated by addition of glutathione to the α -bromoacrylyl fragment is the active DNA alkylating intermediate (a species analogous to compound **2** in Scheme 1) (41, 42). While this is a reasonable suggestion because bromoacetyl moieties tethered to non-covalent DNA binding units are able to alkylate DNA (57–59), our data indicate that an episulfonium ion intermediate also should be considered as a possible alkylating intermediate in thiol-triggered DNA alkylation by the anticancer agent brostallicin.

Understanding the mechanisms by which xenobiotics are converted to reactive species inside cells is important in the fields of medicinal chemistry and chemical toxicology (1, 2, 34, 68, 89–92). The results reported here provide new insight regarding the bioactivation of α -haloacrylyl-containing compounds. These molecules present the cell with a sort of molecular snare, where attempts to detoxify the electrophilic α,β -unsaturated ketone via thiol conjugation actually generate an intermediate of increased rather than decreased reactivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Bioactive compounds containing an α -haloacrylyl group

4a

Figure 2.

Mass spectra of the products obtained from the reaction of **1** with the peptide γ -ECGHDRKAHYK.

Figure 3. Time-dependent change in the UV-vis spectra of the reaction between **1** (50 μ M) and 2-mercaptoethanol (1 mM) in 50 mM of HEPES buffer, pH 7, at 24 $^{\circ}$ C.

Figure 4. Mass spectrometric analysis of products obtained from the reaction of **1** with glutathione in human hepatocytes.

Figure 5.

Thiol-activated DNA damage by **1**. The 5'-³²P-labeled oligonucleotide 5'-GTTCGTATATGGGAGGTCGCATGTG-3' was treated with **1** and 2-mercaptoethanol. Reactions were conducted in HEPES buffer (50 mM), pH 7.0, containing DETAPAC (1 mM) at 37 °C for 23 h 20 min, followed by Maxam-Gilbert workup and analysis using 20% denaturing polyacrylamide gel. Lane G, Maxam-Gilbert G-sequencing reaction. Lanes 1–8 employed duplex DNA, where the underlined region of the oligonucleotide shown above is double stranded: lane 1, duplex with Maxam-Gilbert workup (no **1** or 2-mercaptoethanol); lane 2, duplex treated with **1** (10 mM); lane 3, duplex treated with 2-mercaptoethanol (10 mM); lane 4, duplex treated with **1** (10 mM) and 2-mercaptoethanol (1 mM); lane 5, duplex treated with **1** (10 mM) and 2-mercaptoethanol (5 mM); lane 6, duplex treated with **1** (10 mM) and 2-mercaptoethanol (10 mM); lane 7, duplex treated with **1** (5 mM) and 2-mercaptoethanol (10 mM); lane 8, duplex treated with **1** (1 mM) and 2-mercaptoethanol (10 mM).

Figure 6.

Alkylation of DNA by **1** triggered by different thiols. DNA alkylation reactions were carried out and analyzed using polyacrylamide gel electrophoresis as described in the Legend of Figure 5 and in the Experimental Section. The sequence and numbering of the guanine residues in the oligonucleotide are as follows: 5'-GTTTCG₈TATATG₇G₆G₅AG₄G₃TCG₂CATG₁TG-3'. The bars represent residues G1 to G8 moving left to right. Abbreviations: GSH = glutathione; 2-ME = 2-mercaptoethanol; CA = cysteamine; SS = single strand DNA; DS = double strand DNA.

Figure 7.

Alkylation of duplex DNA by **1** triggered by different thiols at different pH values. DNA alkylation reactions were carried out as described in the Legend of Figure 5 with the exception that the buffers used in the pH 6.1 and 8.0 assays consisted of a HEPES (50 mM) and PIPES (50 mM) mixture. Bar heights represent relative alkylation yields at various G-residues in the oligonucleotide (the sequence and numbering of the guanine residues in the oligonucleotide are as follows: 5'-GTTCG₈TATATG₇G₆G₅AG₄G₃TCG₂CATG₁TG-3'). The bars represent residues G1 to G8 moving left to right. Abbreviations: GSH = glutathione; 2-ME = 2-mercaptoethanol; CA = cysteamine

Figure 8.

Mass spectrometric analysis of guanine adducts resulting from thiol-triggered DNA alkylation by **1**. **A**. LC/MS of the thermally labile adducts released from duplex DNA treated with **1** and 2-mercaptoethanol. **B**. MS/MS analysis of m/z 310 ion of the major (above) and minor (below) adducts eluting at 2.47 min and 3.22 min, in panel A. **C**. MS3 of the m/z 232 ion for the major adduct eluting at 2.47 min in panel A.

Scheme 1.

Scheme 2.

Table 1

Calculated exact mass (calcd) and measured accurate mass (obsvd) for the native and adducted peptides.

Compound	[M+2H] ²⁺ calcd	[M+2H] ²⁺ obsvd	[M+3H] ³⁺ calcd	[M+3H] ³⁺ obscd
ECGHDRKAHYK native peptide	672.3173	672.3170	448.5473	448.5468
4a	721.3356	721.3359	481.2262	481.2255
5a	761.3188	761.3193	507.8817	507.8815
6a	712.3304	712.3308	475.2227	475.2226