Preventing metal-mediated oxidative DNA damage with selenium compounds†

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Copper and iron are two widely studied transition metals associated with hydroxyl radical (*OH) generation, oxidative damage, and disease development. Because antioxidants ameliorate metal-mediated DNA damage, DNA gel electrophoresis assays were used to quantify the ability of ten selenium-containing compounds to inhibit metal-mediated DNA damage by hydroxyl radical. In the Cu(I)/H₂O₂ system, selenocystine, selenomethionine, and methyl-selenocysteine inhibit DNA damage with IC₅₀ values ranging from 3.34 to 25.1 µM. Four selenium compounds also prevent DNA damage from Fe(II) and H₂O₂. Additional gel electrophoresis experiments indicate that Cu(I) or Fe(II) coordination is responsible for the selenium antioxidant activity. Mass spectrometry studies show that a 1:1 stoichiometry is the most common for iron and copper complexes of the tested compounds, even if no antioxidant activity is observed, suggesting that metal coordination is necessary but not sufficient for selenium antioxidant activity. A majority of the selenium compounds are electroactive, regardless of antioxidant activity, and the glutathione peroxidase activities of the selenium compounds show no correlation to DNA damage inhibition. Thus, metal binding is a primary mechanism of selenium antioxidant activity, and both the chemical functionality of the selenium compound and the metal ion generating damaging hydroxyl radical significantly affect selenium antioxidant behavior.

Introduction

During the course of cellular metabolism, reactive oxygen species (ROS) such as hydroxyl radical (*OH) cause oxidative damage to living organisms. This highly reactive species oxidizes lipids, proteins, and DNA, and contributes to development of cancer, chronic inflammation, and neurodegenerative and cardiovascular diseases. In cells, transition metal ions such as Cu(I) and Fe(II) generate *OH from endogenous hydrogen peroxide (H₂O₂) in the Fenton and Fenton-like reactions (reaction (1)). This metal-mediated *OH formation is the primary cause of cellular DNA damage and death.

Fe(II)/Cu(I) + H₂O₂ → Fe(III)/Cu(II) + *OH + OH⁻ (1)

Copper and iron are essential redox-active metal ions required for normal physiological function. Cells employ multiple pathways to maintain metal homeostasis, but under conditions of oxidative stress, mis-regulation can lead to excessive concentrations of non-protein-bound (labile) metal ions associated with oxidative stress and DNA damage due to *OH generation. Specifically, elevated levels of labile copper and iron have been linked to hemochromatosis, anemia, diabetes, cancer, and amyotrophic lateral sclerosis (ALS), as well as cardiovascular, Wilson’s, Menke’s, and Alzheimer’s diseases. Because antioxidants can ameliorate oxidative damage caused by ROS, there has been an increasing focus on selenium antioxidants to prevent or treat diseases caused by oxidative stress.

Selenium deficiency is associated with many diseases. In areas of China with low soil selenium levels, Keshan disease caused cardiomyopathy in children in the 1970s; incidence of this disease has decreased considerably with selenium supplementation. Selenium deficiency has also been associated with other diseases, including Kashin-Beck, HIV infection, arteriosclerosis, miscarriage, neurological disorders, and cancer.

Selenium in dietary supplements is primarily found as organic selenomethionine and inorganic selenite. Interest regarding selenium supplementation has increased considerably in recent years due to numerous clinical studies indicating the benefits of selenium in disease prevention, especially chemoprevention. The Nutritional Prevention of Cancer (NPC) trial reported that daily selenium supplementation (200 µg day⁻¹) resulted in a 37% decrease in cancer incidence, including prostate cancer. In contrast, the selenium and vitamin E cancer prevention trial (SELECT) found that selenium supplementation (200 µg day⁻¹) did not uniformly protect against...
prostate cancer.\textsuperscript{33} The conflicting results of these studies highlight the need for a greater understanding of selenium antioxidant mechanisms.

In proteins, selenium is incorporated into selenocysteine, an amino acid required for the synthesis of several selenoenzymes, including glutathione peroxidase, iodothyronine deiodinases, and thioredoxin reductases.\textsuperscript{34} Glutathione peroxidase (GPx) is an antioxidant enzyme that protects intracellular components from oxidative stress by catalyzing the reduction of \( \text{H}_2\text{O}_2 \) to water via the selenocysteine-containing active site.\textsuperscript{35} Because of the biological importance of this process, traditional quantification of selenium antioxidant activity has been determined by spectroscopic measurements of \( \text{H}_2\text{O}_2 \) reduction, similar to the GPx mechanism.\textsuperscript{35}

Selenium complexes have been observed with mercury, and this complexation may prevent ROS damage caused by heavy metal toxicity.\textsuperscript{36–38} It has also been proposed that selenium binding protects against lead toxicity.\textsuperscript{39–41} Selenocysteine also coordinates metal ions in enzymes: molybdenum and tungsten in formate dehydrogenases,\textsuperscript{42} and nickel in [NiFeSe] hydrogenase.\textsuperscript{43} Thus, given the role of copper and iron in \( \cdot\text{OH} \) generation, it is important to explore the role of metal coordination in selenium antioxidant behavior.

We previously reported the ability of selenomethionine, selenocysteine, and 2-aminophenyl diselenide to inhibit Cu\textsuperscript{I}-mediated DNA damage.\textsuperscript{44} In the present work, we report the quantification of copper-mediated DNA damage inhibition for seven additional selenium compounds. In addition, we have quantified and compared the ability of all ten selenium compounds to prevent Fe\textsuperscript{II}-mediated DNA damage. Mass spectrometry and cyclic voltammetry studies were also performed to investigate the metal binding and electrochemical activity of selenium antioxidants. Metal coordination is compared with GPx-like activity for prevention of metal-mediated DNA damage in order to determine the primary mechanism for DNA damage inhibition. Determining the chemical properties and mechanisms that affect antioxidant activity of selenium compounds will aid researchers in identifying more potent selenium antioxidants to treat and prevent disease.

**Results and discussion**

**Determining antioxidant activity for selenium compounds**

The selenium compounds in Fig. 1 were examined using gel electrophoresis to determine their ability to inhibit oxidative DNA damage caused by Cu\textsuperscript{I}/\( \text{H}_2\text{O}_2 \) (pH 7) or Fe\textsuperscript{II}/\( \text{H}_2\text{O}_2 \) (pH 6). This DNA damage assay enables quantification and direct comparison of metal-mediated oxidative DNA damage prevention to determine biologically-relevant antioxidant potency for a variety of selenium compounds.

Fig. 2 shows the results of a gel electrophoresis experiment testing methyl-selenocysteine (MeSeCys) for its ability to inhibit oxidative DNA damage caused by Cu\textsuperscript{I}/\( \text{H}_2\text{O}_2 \). Hydrogen peroxide alone does not damage DNA (lane 2); however, combining Cu\textsuperscript{I} with \( \text{H}_2\text{O}_2 \) results in 98\% DNA damage (lane 4). Adding methyl-selenocysteine in increasing concentrations results in significant reduction of oxidative DNA damage, with greater than 95\% DNA damage inhibition at concentrations \( \geq 100 \) \( \mu\text{M} \). A best-fit dose-response curve for methyl-selenocysteine is shown in Fig. 3, and the concentration of methyl-selenocysteine required to inhibit 50\% of copper-mediated oxidative DNA damage (IC\textsubscript{50} value) was calculated to be 8.64 \pm 0.02 \( \mu\text{M} \). The remaining selenium compounds (Fig. 1) were similarly examined for their ability to inhibit copper-mediated DNA damage using the same method (see ESI\textsuperscript{f} for gel images, IC\textsubscript{50} plots, and data tables).

Selenomethionine and selenocysteine have been previously studied, and inhibit \textgreater 90\% of copper-mediated DNA damage at 100 \( \mu\text{M} \) with IC\textsubscript{50} values (Table 1) of 25.10 \pm 0.01 \( \mu\text{M} \) and 3.34 \pm 0.08 \( \mu\text{M} \), respectively.\textsuperscript{44} In contrast, selenocystamine had significant prooxidant activity at low concentrations (1–500 \( \mu\text{M} \)), causing a maximum of 22 \( \pm 5\% \) DNA damage (\( p = 0.02 \)), but preventing 59 \( \pm 3\% \) DNA damage at 1000 \( \mu\text{M} \). Under these conditions, 3,3'-diselenobispropionic acid, 3,3'-selenobispropionic acid, 2-aminophenyl diselenide, 2-carboxyphenyl diselenide, 2-carboxyphenyl selenide, and 4-carboxyphenyl diselenide do not inhibit Cu\textsuperscript{I}-mediated DNA damage.

Of the ten selenium compounds tested, those containing both amine and carboxylate groups (selenocysteine, selenomethionine, and methyl-selenocysteine) significantly inhibit copper-mediated DNA damage. In contrast, selenocystamine, a selenium compound that has amine functionality but lacks carboxylate groups, shows prooxidant activity at low concentrations despite its structural similarities to the three amino acids. Similarly, selenium compounds with only carboxylate groups (3,3'-diselenobispropionic acid and 3,3'-selenobispropionic acid) or aromatic groups (2-aminophenyl diselenide, 2-carboxyphenyl diselenide, and 2-carboxyphenyl selenide) also show no ability to inhibit copper-mediated DNA damage. Thus, the antioxidant ability of selenium compounds is strongly influenced by the functional groups present in the species.

**Inhibition of iron-mediated DNA damage by selenium compounds**

Using a similar plasmid nicking assay, the ability of selenium compounds to prevent DNA damage by Fe\textsuperscript{II}/\( \text{H}_2\text{O}_2 \) was also determined. These gel electrophoresis experiments with iron were conducted at pH 6 rather than pH 7 due to iron insolubility at pH > 6.\textsuperscript{45–47} Four of the ten selenium compounds tested inhibit iron-mediated oxidative DNA damage. Selenocystamine inhibits 79 \( \pm 1\% \) of iron-mediated DNA damage at 250 \( \mu\text{M} \) (Fig. 4). Methyl-selenocysteine, 3,3'-diselenobispropionic acid, and 3,3'-selenobispropionic acid inhibit 60 \( \pm 2\% \), 76 \( \pm 1\% \), and 42 \( \pm 8\% \) of oxidative DNA damage at 1000 \( \mu\text{M} \), respectively. The remaining selenium compounds have no effect on iron-mediated oxidative DNA damage.

IC\textsubscript{50} values for inhibition of iron-mediated DNA damage were calculated for selenocystamine (121.4 \pm 0.3 \( \mu\text{M} \)) and methyl-selenocysteine (378.4 \pm 0.1 \( \mu\text{M} \); Table 2). An IC\textsubscript{50} value of \( \sim 75 \) \( \mu\text{M} \) is estimated for 3,3'-diselenobispropionic acid based on averaging DNA damage inhibition at 50 \( \mu\text{M} \) (42 \( \pm 7\% \)) and 100 \( \mu\text{M} \) (59 \( \pm 6\% \)), since a dose-response curve did not accurately fit the gel data for this compound. In all cases, inhibition of iron-mediated DNA damage occurred at higher concentrations of selenium compound than inhibition of copper-mediated damage. Surprisingly, only...
methyl-selenocysteine inhibited DNA damage from both CuI and FeII; selenocystamine, 3,3'-diselenobispropionic acid, and 3,3'-selenobispropionic acid inhibited only iron-mediated damage.

The striking differences observed for selenium antioxidant prevention of metal-mediated damage by CuI and FeII highlight the critical role of both the metal ion and selenium functionality in selenium antioxidant behavior. Compounds containing both amine and carboxylate substituents demonstrate the greatest antioxidant activity with CuI-mediated damage; however, for inhibition of FeII-mediated damage, both substituents are not required. Selenocystamine, 3,3'-diselenobispropionic acid, and 3,3'-selenobispropionic acid, compounds that inhibit iron-mediated DNA damage, contain either amine or carboxylate substituents. Interestingly, selenocystamine, with only amine substituents, has a lower IC50 value for prevention of iron-mediated damage than methyl-selenocysteine, with both amine and carboxylate substituents. Other selenium compounds with both amine and carboxylate substituents, such as selenocystine and selenomethionine, do not inhibit iron-mediated oxidative damage.

Studies with CuI/H2O2 suggest that the presence of two selenium atoms instead of one may increase antioxidant activity, and a similar trend is observed in the FeII/H2O2 system. The diselenide compounds selenocystamine and 3,3'-diselenobispropionic acid inhibit significantly more DNA damage at lower concentrations than the selenide compounds methyl-selenocysteine and 3,3'-selenobispropionic acid. Thus, functionality of the selenium compounds also plays an important role in selenium antioxidant activity with iron, but determination of antioxidant efficacy based on chemical features of these compounds is not straightforward and requires further investigation.

The role of metal coordination in antioxidant activity

Previous gel electrophoresis experiments with [Cu(bipy)]2+ (bipy = 2,2'-bipyridine) instead of CuI as the copper source, established that prevention of CuI-mediated DNA damage by selenocystine and selenomethionine is due to CuI coordination.44 The bipyridyl ligands fully coordinate copper in [Cu(bipy)2]+; thus, DNA damage inhibition should not be observed if copper coordination is responsible for selenium antioxidant activity. In DNA damage assays with [Cu(bipy)]2+/H2O2, selenocystine, selenomethionine, and methyl-selenocysteine show no inhibition of oxidative DNA damage even at high concentrations (Supplementary Information). Because prior coordination of the copper eliminates antioxidant activity for these selenium compounds, our gel studies clearly indicate that their antioxidant behavior is due to copper coordination.

UV-vis spectroscopy was also performed to determine whether CuI coordination occurs upon addition of the selenium compounds. Most of the selenium compounds that prevent DNA damage in the gel studies with CuI show absorption bands around 226 nm in the UV-vis spectra (Fig. S7, ESI†), whereas these absorbances are not observed for the selenium compounds that had no antioxidant activity with CuI/H2O2. Selenocystamine showed no UV absorption band in the presence of [Cu(bipy)]2+. Copper-selenium charge transfer bands commonly occur between λmax = 241–260 nm.48,49 Similar UV-vis bands were not observed for selenocystine, selenomethionine and methyl-selenocysteine in the presence of [Cu(bipy)]2+.

Gel electrophoresis experiments with [Fe(EDTA)]2– were also conducted to determine whether the antioxidant activity of methyl-selenocysteine, selenocystamine, 3,3'-diselenobis-propionic

![Fig. 1](image1.png) Selenium compounds tested for DNA damage inhibition.

![Fig. 2](image2.png) Agarose gel showing a reduction in oxidative DNA damage with increasing methyl-selenocysteine concentration. Lanes: (MW) 1 kb ladder; (1) plasmid DNA; (2) DNA + H2O2; (3) DNA + Se + H2O2; (4) DNA + CuII/ascorbate + H2O2; (5–11) same as lane 4 with increasing [MeSeCys]: 0.1, 1, 5, 10, 50, 100, and 1000 μM, respectively.

![Fig. 3](image3.png) A best-fit dose-response curve for methyl-selenocysteine prevention of DNA damage by CuI; the concentration of methyl-selenocysteine (MeSeCys) required to inhibit 50% of copper-mediated DNA damage (IC50) is 8.64 ± 0.02 μM.

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Table 1 | IC$_{50}$ values, $\lambda_{\text{max}}$, and mass spectrometry data for the tested selenium compounds with Cu$^+$, as well as GPx activity measurements

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cu$^+$ IC$_{50}$ (µM)$^a$</th>
<th>Cu$^+$ $\lambda_{\text{max}}$ (nm)</th>
<th>m/z (Da)</th>
<th>Cu$^+$ : Se compound</th>
<th>Relative GPx Activity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenocystine</td>
<td>3.34 ± 0.08$^c$</td>
<td>240$^c$</td>
<td>397.8, 731.6</td>
<td>1:1, 1:2</td>
<td>4.5</td>
</tr>
<tr>
<td>Methyl-selenocysteine</td>
<td>8.64 ± 0.02</td>
<td>241</td>
<td>247.8, 427.8, 598.1</td>
<td>1:1, 1:2, 1:3</td>
<td>0.7</td>
</tr>
<tr>
<td>Selenomethionine</td>
<td>25.10 ± 0.01$^c$</td>
<td>226$^c$</td>
<td>261.9, 456.9, 650.9</td>
<td>1:1, 1:2, 1:3</td>
<td>0.6</td>
</tr>
<tr>
<td>Selenocystine</td>
<td>59 ± 3% (1000 µM)$^c$</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>8.8</td>
</tr>
<tr>
<td>2-Aminophenyl diselenide</td>
<td>—</td>
<td>—</td>
<td>466.9</td>
<td>1:1</td>
<td>~0</td>
</tr>
<tr>
<td>2-Carboxyphenyl diselenide</td>
<td>—</td>
<td>—</td>
<td>383.9</td>
<td>1:1$^d$</td>
<td>~0</td>
</tr>
<tr>
<td>2-Carboxyphenyl selenide</td>
<td>—</td>
<td>—</td>
<td>466.9</td>
<td>1:1</td>
<td>~0</td>
</tr>
<tr>
<td>4-Carboxyphenyl diselenide</td>
<td>—</td>
<td>—</td>
<td>370.6, 673.4</td>
<td>1:1, 1:2</td>
<td>~0</td>
</tr>
<tr>
<td>3,3'-Diselenobispropionic acid</td>
<td>—</td>
<td>—</td>
<td>290.7, 515.6</td>
<td>1:1, 1:2</td>
<td>~0</td>
</tr>
</tbody>
</table>

$^a$ IC$_{50}$ is defined as the concentration at which the compound inhibited 50% of DNA damage. $^b$ GPx relative activity values are reported relative to ebelen in methanol. $^c$ Values from ref. 44. $^d$ ESI mass spectrometry voltage of 5500 V.

Fig. 4 Agarose gel showing reduction in iron-mediated oxidative DNA damage with selenocystamine. Lanes: MW) 1 kb ladder; (1) plasmid DNA; (2) DNA + H$_2$O$_2$; (3) DNA + Se + H$_2$O$_2$; (4) DNA + Fe$^{II}$ + H$_2$O$_2$; (5) same as lane 4 with increasing [SeCysta]; 1, 10, 100, 500, and 1000 µM, respectively.

Table 2 | IC$_{50}$ values, and mass spectrometry data for the tested selenium compounds with Fe$^{III}$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fe$^{III}$ IC$_{50}$ (µM)$^a$</th>
<th>m/z (Da)</th>
<th>Fe$^{III}$ : Se compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,3'-Diselenobispropionic acid</td>
<td>~75$^b$</td>
<td>360.6, 664.4</td>
<td>1:1, 1:2</td>
</tr>
<tr>
<td>Selenocystine</td>
<td>121.4 ± 0.3</td>
<td>415.1</td>
<td>1:3</td>
</tr>
<tr>
<td>Methyl-selenocysteine</td>
<td>378.4 ± 0.1</td>
<td>239.1, 420.8, 601.8</td>
<td>1:1, 1:2, 1:3</td>
</tr>
<tr>
<td>3,3'-Selenobispropionic acid</td>
<td>42 ± 8% inhibition (1000 µM)</td>
<td>288.7, 506.6</td>
<td>1:1, 1:2</td>
</tr>
<tr>
<td>Selenocystine</td>
<td>390.8, 724.7, 1058.2</td>
<td>1:1, 1:2, 1:3</td>
<td></td>
</tr>
<tr>
<td>2-Aminophenyl diselenide</td>
<td>—</td>
<td>404.8</td>
<td>1:1$^b$</td>
</tr>
<tr>
<td>2-Carboxyphenyl diselenide</td>
<td>—</td>
<td>465.7</td>
<td>1:1$^b$</td>
</tr>
<tr>
<td>2-Carboxyphenyl selenide</td>
<td>—</td>
<td>376.9, 696.7</td>
<td>1:1, 1:2$^c$</td>
</tr>
<tr>
<td>4-Carboxyphenyl diselenide</td>
<td>—</td>
<td>455.1</td>
<td>1:1</td>
</tr>
<tr>
<td>Selenomethionine</td>
<td>—</td>
<td>448.9, 643.9</td>
<td>1:2, 1:3</td>
</tr>
</tbody>
</table>

$^a$ IC$_{50}$ is defined as the concentration at which the compound inhibited 50% of DNA damage. $^b$ Estimated value. $^c$ ESI mass spectrometry voltage of 5500 V.
range (~1–10 μM), selenium antioxidant activity may be much more pronounced for copper-mediated oxidative damage than for iron in cells. In addition, the antioxidant activity of these compounds is more complex than previously reported, since the activity is affected by the properties of the selenium compounds and the functional groups present. It is clear that the precise chemical mechanism(s) for the observed selenium activity requires further study. However, this work suggests that it may be possible to select selenium antioxidants for prevention of oxidative damage by specific metal ions, since the observed antioxidant activity of the selenium compounds is greatly affected by the metal ion causing the DNA damage.

**Glutathione peroxidase activity of selenium compounds**

GPx activity measurement is a typical method used to determine selenium antioxidant activity, and a great deal of research has focused on the development of more potent GPx mimics, including small organoselenium compounds. The selenium drug ebselen (2-phenyl-1,2-benzisoselazol-3(2H)-one) protects against ischemic brain injury in human clinical trials, and prevents neurotoxicity in Parkinson’s disease, a disease where ROS are considered the primary cause of pathogenesis.

The mechanism for this protective ability is thought to be reducing oxidative damage by H2O2 in a manner similar to that of the GPx enzyme, and ebselen is typically used as the standard relative to which all selenium GPx measurements are compared.

Although GPx activity is commonly used to assess antioxidant behavior of selenium compounds, these measurements are not necessarily biologically relevant. GPx activity measurements are known to be greatly affected by the experimental method used, and Sarma et al. have demonstrated that an undesired thiol exchange reaction can artificially decrease GPx activity measurements for selenium compounds. In addition, H2O2 is a relatively non-reactive oxygen species in the absence of metal ions. Hydroxyl radical, in contrast, is one of the most highly reactive and damaging radical species. Therefore, directly preventing hydroxyl radical formation or release at the metal center would directly prevent more oxidative damage than scavenging H2O2.69

To determine how this metal-binding mechanism for selenium antioxidant activity compares to GPx activity of selenium compounds, we measured their ability to reduce H2O2 in the presence of benzenethiol according to the method reported by Mareque et al. Selenomethionine, 2-aminophenyl diselenide, and selenocystine have been previously reported to have GPx activities of 0.6, 6.4, and 4.5 compared to ebselen in methanol, respectively. Selenocystamine and methyl-selenocysteine have GPx activities of 8.8 and 0.7 compared to ebselen, whereas the remaining selenium compounds do not demonstrate significant GPx activity under these conditions. Mishra and coworkers reported that selenocystamine has a much higher GPx activity (~22 times higher) than 3,3′-diselenobispropionic acid, a trend that we also observed (Table 1). In addition, Yasuda et al. reported that selenocysteine and selenocystamine have similar GPx activity for decomposition of t-butyl hydroperoxide.

If GPx activity were the primary mechanism for DNA damage inhibition, these results suggest that selenocystamine, 2-aminophenyl diselenide, and selenocysteine should have the highest antioxidant activity, whereas the remaining selenium compounds should be poor antioxidants due to their lower GPx activities. Our DNA damage assays directly contradict this hypothesis. Methyl-selenocysteine has a low GPx activity and prevents both copper- and iron-mediated DNA damage. Selenocystamine has a high GPx activity, but inhibits only iron-mediated DNA damage. Other selenium compounds, including 3,3′-diselenobispropionic acid and 3,3′-selenobispropionic acid show very little or no GPx activity; however, these compounds demonstrate significant antioxidant activity with FeII. Thus, it is apparent that the majority of metal-mediated DNA damage inhibition is due to a mechanism distinct from GPx activity. Based on our DNA damage assay results, metal-antioxidant binding is the primary mechanism for the observed selenium antioxidant activity, although radical scavenging is likely a secondary mechanism for FeII-mediated damage.

**Electrochemistry of selenium compounds**

To determine whether there is a correlation between selenium antioxidant activity and electrochemistry of the selenium compounds (Fig. 2), we conducted cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments in aqueous buffer at pH 7. Only three of the ten compounds have measurable Ec/2 values using a glassy carbon working electrode: selenocystamine (~329 mV), 2-carboxyphenyl diselenide (535 mV), and 2-aminophenyl diselenide (with two redox potentials at 308 mV and ~429 mV; Table 3). In contrast, selenomethionine, 2-carboxyphenyl selenide, 3,3′-diselenobispropionic acid, and selenocysteine show only a single oxidation wave, whereas 3,3′-selenobispropionic acid, 4-carboxyphenyl diselenide, and

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ep1/2 (mV)</th>
<th>Ep1/2 (mV)</th>
<th>ΔE (mV)</th>
<th>Ec/2 (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenomethionine</td>
<td>693</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenocysteine</td>
<td>–115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl-selenocysteine</td>
<td>–974</td>
<td>–429</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenocystamine</td>
<td>–131</td>
<td>–526</td>
<td>394</td>
<td>–429</td>
</tr>
<tr>
<td>2-Aminophenyl diselenide</td>
<td>593, 120</td>
<td>24, –978</td>
<td>569, 1099</td>
<td>308, –429</td>
</tr>
<tr>
<td>2-Carboxyphenyl diselenide</td>
<td>501</td>
<td>569</td>
<td>67</td>
<td>535</td>
</tr>
<tr>
<td>2-Carboxyphenyl selenide</td>
<td>832</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Carboxyphenyl diselenide</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,3′-Diselenobispropionic acid</td>
<td>907</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,3′-Selenobispropionic acid</td>
<td>–</td>
<td></td>
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</tr>
</tbody>
</table>
bispropionic acid also inhibits FeII-mediated DNA damage, but chemical studies of selenium antioxidants have been performed trials at pH 3.9 using a gold working electrode. Many electrochemical studies of selenium antioxidants have been performed with either mercury or gold electrodes; however, both mercury and gold can form complexes with selenium compounds. As a result, observed electrochemical behavior may be a result of the selenium compound interacting with the metal electrode. Our electrochemical measurements using a glassy carbon electrode are reflective of the electrochemical behavior of the uncoordinated selenium compounds. Because *OH generation and neutralization are one-electron redox processes, and because antioxidant activity may be correlated to the redox activity of the selenium compound alone, it is vital to determine the electrochemistry of the uncoordinated selenium compounds.

Selenocysteine, an antioxidant with both amine and carboxylate functionalities, has a single irreversible anodic wave at −115 mV, whereas the amine-functionalized selenocystamine exhibits quasi-reversible behavior with a similar redox potential of −329 mV. In contrast, the similar amino acids selenomethionine and methyl-selenocysteine show very different electrochemical behavior: selenocysteine has a single anodic wave at 693 mV, whereas methyl-selenocysteine exhibited no electrochemical behavior. These electrochemical differences are somewhat surprising, since selenomethionine and methyl-selenocysteine differ only by one methylene group, and one might expect more similar electrochemistry.

The results of our DNA damage prevention assays do not correlate with the observed electrochemistry of the selenium compounds. Methyl-selenocysteine inhibits both CuI- and FeIII-mediated DNA damage, but shows no electrochemical activity, whereas selenomethionine inhibits CuI-mediated DNA damage and has only an oxidation potential. Similarly, 3,3'-diselenobispropionic acid undergoes only oxidation and inhibits FeIII-mediated DNA damage, whereas 3,3'-selenobispropionic acid also inhibits FeIII-mediated DNA damage, but has no observable electrochemical behavior. Both 2-carboxyphenyl selenide and 2-carboxyphenyl diselenide also differ by a single selenium atom, but 2-carboxyphenyl diselenide undergoes both oxidation and reduction, whereas 2-carboxyphenyl selenide exhibits only an oxidation potential. Despite their high oxidation potentials, neither 2-carboxyphenyl diselenide or 2-carboxyphenyl selenide inhibit CuI- or FeIII-mediated DNA damage. Since no correlation is observed between oxidation potential of the selenium compound and DNA damage prevention, radical scavenging is likely not a major mechanism for the observed antioxidant activity.

Mass spectrometry evidence for metal coordination to selenium antioxidants

Electrospray ionization mass spectrometry (ESI-MS) was used to examine the stoichiometry of CuI or FeIII coordination to the selenium compounds in Fig. 2. Most selenium compounds form 1:1 and 1:2 (Cu : ligand) complexes with CuI, except for 2-amino-phenyl diselenide (forms only a 1:1 complex) and selenocystamine (shows no coordination). Interestingly both selenomethionine and methyl-selenocysteine also form complexes with CuI in a 1:3 ratio.

Because CuI typically adopts a tetrahedral coordination geometry, it is probable that many of these selenium compounds bind CuI through the selenium atom, as well as through the amine nitrogen and/or carboxylate oxygen atom(s). Since the majority of selenium compounds show metal coordination regardless of antioxidant activity, these results suggest that metal coordination is necessary but not sufficient for prevention of copper-mediated DNA damage.

Similar mass spectrometry studies with FeIII reveal iron binding to all of tested selenium compounds. Both 1:1 and 1:2 stoichiometry (Fe : ligand) is observed for all compounds except 4-carboxyphenyl diselenide (only a 1:1 ratio) and selenocystamine (only a 1:3 ratio). A 1:3 coordination ratio was also observed for selenomethionine, selenocystine, and methyl-selenocysteine. Again, all the selenium compounds coordinate FeIII, regardless of antioxidant activity, suggesting that iron coordination is necessary but not sufficient for antioxidant activity. Metal-antioxidant binding may prevent DNA damage by altering the redox potential of the metal ion and preventing H2O2 reduction to *OH or CuIII and FeIII reduction by cellular reductants. Alternatively, the metal-bound selenium antioxidant may be poised to efficiently scavenge *OH immediately upon formation at the metal center before it can be released.

A comparison of selenium and sulfur antioxidant activity

The antioxidant properties of sulfur compounds have also been extensively investigated and are commonly compared to those of selenium antioxidants. Sulfur antioxidants have chemopreventive effects against certain types of cancer and are of great interest for the treatment and prevention of diseases. Of the ten sulfur compounds tested, oxidized and reduced glutathione, cystine, cysteine, methylcysteine, and methionine prevented copper-mediated DNA damage with IC50 values between 3–12 μM. 2-Aminophenyl disulfide, 3-carboxypropyl disulfide, and cystamine showed no antioxidant activity with CuI/H2O2, whereas 2-carboxyphenyl disulfide showed prooxidant activity at 25 μM.

We have reported the ability of sulfur compounds to inhibit iron- and copper-mediated DNA damage using the same DNA damage assays. Of the ten sulfur compounds tested, oxidized and reduced glutathione, cystine, cysteine, methylcysteine, and methionine prevented copper-mediated DNA damage with IC50 values between 3–12 μM. 2-Aminophenyl disulfide, 3-carboxypropyl disulfide, and cystamine showed no antioxidant activity with CuI/H2O2, whereas 2-carboxyphenyl disulfide showed prooxidant activity at 25 μM.

Methyl-selenocysteine exhibit no electrochemical activity between −1000 mV and 1000 mV (CV data are provided in the Supplementary Information).

Bai et al. observed two anodic waves for selenocysteine at 810 mV and ~1200 mV; as well as two anodic waves for selenomethionine at ~640 mV and 1100 mV. This discrepancy is likely due to the difference in both the pH and choice of working electrode; Bai and coworkers reported these potentials at pH 3.9 using a gold working electrode. Many electrochemical studies of selenium antioxidants have been performed with either mercury or gold electrodes; however, both mercury and gold can form complexes with selenium compounds. As a result, observed electrochemical behavior may be a result of the selenium compound interacting with the metal electrode. Our electrochemical measurements using a glassy carbon electrode are reflective of the electrochemical behavior of the uncoordinated selenium compounds. Because *OH generation and neutralization are one-electron redox processes, and because antioxidant activity may be correlated to the redox activity of the selenium compound alone, it is vital to determine the electrochemistry of the uncoordinated selenium compounds.

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A comparison of selenium and sulfur antioxidant activity

The antioxidant properties of sulfur compounds have also been extensively investigated and are commonly compared to those of selenium antioxidants. Sulfur antioxidants have chemopreventive effects against certain types of cancer and are of great interest for the treatment and prevention of diseases. Sulfur compounds also protect cells against oxidative damage. However, selenium compounds are generally more effective antioxidants than sulfur compounds. Kumar et al. showed that selenium compounds protected cells and DNA more effectively from peroxynitrite damage than analogous sulfur compounds. Similarly, Ip et al. compared the efficacy of selenium and sulfur compounds for chemoprevention in rats and reported that the concentration of selenium compound (selenocystamine, methyl-selenocysteine, and selenobetaine) required for comparable antitumorogenic efficacy was 500- to 750-fold lower than that of analogous sulfur compounds (cystamine, S-methylcysteine, and sulfobetaine).

We have reported the ability of sulfur compounds to inhibit iron- and copper-mediated DNA damage using the same DNA damage assays. Of the ten sulfur compounds tested, oxidized and reduced glutathione, cystine, cysteine, methylcysteine, and methionine prevented copper-mediated DNA damage with IC50 values between 3–12 μM. 2-Aminophenyl disulfide, 3-carboxypropyl disulfide, and cystamine showed no antioxidant activity with CuI/H2O2, whereas 2-carboxyphenyl disulfide showed prooxidant activity at 25 μM.
Comparing selenium and sulfur antioxidant activity using Cu(1)H₂O₂ mediated DNA damage inhibition, selenocystine and cystine have the lowest IC₅₀ values (3.34 ± 0.08 µM and 3.34 ± 0.07 µM, respectively). Similarly, selenomethionine and methionine have IC₅₀ values of 25.10 ± 0.01 µM and 11.20 ± 0.01 µM respectively, and methyl-selenocysteine and methyl-cysteine have IC₅₀ values of 8.64 ± 0.02 µM and 8.90 ± 0.01 µM, respectively.⁵³,⁷⁵ Both 2,2'-dithioisalicylic acid and 2-carboxyphenyl diselenide have no effect on Cu(1)-mediated DNA damage; similar results were also observed for 2-aminophenyl disulfide and 2-aminophenyl diselenide.⁵³,⁷⁵ Our results indicate that selenium compounds prevent Cu(1)-mediated DNA damage similarly to their sulfur analogs.

With Fe(III) and H₂O₂, however, sulfur compounds do not demonstrate similar antioxidant activity compared to their selenium counterparts. Only 3-carboxypropyl disulfide and glutathione inhibit iron-mediated DNA damage at very high concentrations; methyl-cysteine, and cystamine do not.⁷⁵ Thus, selenium compounds are significantly more potent antioxidants in preventing iron-mediated DNA damage compared to their sulfur analogs.

Based on our results, neither glutathione peroxidase measurements nor electrophoretical activity show a correlation to prevention of metal-mediated DNA damage by selenium compounds, although reactive oxygen species scavenging may occur at high concentrations. Instead, our work demonstrates that the biochemical mechanisms for selenium antioxidant activity are much more complex than previously reported. Prevention of metal-mediated DNA damage by selenium compounds occurs primarily through a metal-binding mechanism, but metal binding alone is not sufficient for antioxidant activity. Both the functional groups on the selenium compound and the identity of the metal ion also play significant roles in selenium antioxidant activity.

Experimental section

Materials

Water was deionized using a Nano Pure DIamond Ultrapure H₂O system (Barnstead International). H₂O₂ (30%) and CuSO₄ were purchased from Fisher. NaCl (99,999% to avoid trace metal contamination), selenocystamine and selenocystine were purchased from Sigma-Aldrich; ethanol (99.5%), 3-(N-morpholino)propanesulfonic acid (MOPS), selenomethionine, 2-aminophenyl diselenide, and methyl-selenocysteine were obtained from Acros. 2,2'-Bipyridine and FeSO₄ were purchased from Alfa Aesar. 2-Carboxyphenyl selenide, 2-carboxyphenyl diselenide, and 4-carboxyphenyl diselenide were purchased from Focus. EDTA (disodium salt) and ascorbic acid were obtained from J. T. Baker; 2-(N-morpholino)-ethanesulfonic acid (MES) buffer was purchased from Calbiochem. All UV-vis spectra and GPx measurements were obtained using a Shimadzu UV-3101 PC spectrophotometer. Electrochemical experiments were conducted on CH Electrochemical Analyzer (CH Instruments, Inc.). Prior to use, all selenium compounds were stored under nitrogen in a desiccator at 15 °C to prevent oxidation.

Purification of plasmid DNA

Plasmid DNA (pBluescript SK) was purified from E. coli DH1 using a Qiaprep Spin Miniprep kit (Qiagen, Chatsworth, CA). Plasmid DNA was eluted in Tris-EDTA buffer and dialyzed for 24 h at 4 °C against 130 mM NaCl. Absorbance ratios of A₂₅₀/A₂₆₀ ≤ 0.95 and A₂₆₀/A₂₈₀ ≥ 1.8 were determined for DNA used in all experiments.

DNA damage experiments

CuSO₄ (6 µM), the indicated concentrations of selenium compound, ethanol (10 mM), deionized H₂O, MOPS (10 mM), NaCl (130 mM), and ascorbic acid (1.25 equiv; 7.5 µM, to reduce Cu(II) to Cu(I)) were combined at pH 7 and allowed to stand for 5 min at room temperature. Plasmid (pBSSK, 0.1 pmol in 130 mM NaCl) was added to the reaction mixture and the solution was again allowed to stand for an additional 5 min. The *OH formation was then initiated by the addition of H₂O₂ (50 µM). After 30 min, EDTA (50µM) was added to quench the reaction. For Cu(bipy)²⁺ gel electrophoresis studies, Cu(bipy)²⁺ (50 µM) was used in place of CuSO₄, and the concentration of ascorbic acid was increased to 62.5 µM. For FeSO₄ DNA damage experiments, FeSO₄ (2 µM) and MES (10 mM, pH 6) were substituted for the CuSO₄, ascorbic acid, and MOPS. For [Fe(EDTA)]²⁻ DNA damage experiments, [Fe(EDTA)]²⁻ (400 µM) was substituted for FeSO₄. All concentrations indicated are the final concentrations in a 10 µL volume.

Damaged and undamaged forms of plasmid DNA were separated using 1% agarose gel electrophoresis in TAE buffer (140 V for 30 min). The gels were stained with ethidium bromide and imaged under UV light, where the amount of damaged (nicked) DNA versus undamaged (supercoiled) DNA was determined using UViProMW (Jencons Scientific Inc., 2003). Due to less efficient staining by ethidium bromide, supercoiled DNA band intensities were multiplied by 1.24 prior to comparison.⁸⁷,⁸⁸ Additionally, DNA band intensities in each lane were normalized so that addition of supercoiled and nicked DNA equaled 100%. At the concentration used in the copper gels, ascorbic acid shows no effect on DNA damage. Studies with only Cu(II)/ascorbate or Fe(II), DNA, and the selenium compounds also showed no DNA damage. MES and MOPS buffers were treated with chelating resin before use and have no significant interactions with metal ions.⁸⁹

Calculation of DNA damage inhibition

DNA damage inhibition was calculated using the I-% N/% B*100, where % N is the percentage of nicked DNA in the selenium-containing lanes (lanes 6–9) and % B is the percentage of nicked DNA in the Cu(II)/H₂O₂ or Fe(II)/H₂O₂ lane (lane 5). Both % N and % B were normalized for residual nicking (lane 2) prior to calculation, and results are the average of at least three trials with standard deviations indicated by error bars. Additionally, for all selenium DNA damage inhibition studies, no linear DNA was observed. Statistical significance was determined by calculating p values at 95% confidence (p < 0.05 indicates significance) as described by Perkowski et al.;⁹⁰ tables listing these values can be found in the ESI†.
**IC\textsubscript{50} determination**

The plots of percent inhibition of DNA damage \textit{versus} log concentration of selenium compound (\(\mu\text{M}\)) were fit to a variable-slope sigmoidal dose-response curve using SigmaPlot (v. 9.01, Systat Software, Inc.). IC\textsubscript{50} value errors were calculated from error propagation of the gel electrophoresis measurements.

**UV-vis measurements**

CuSO\textsubscript{4} (58 \(\mu\text{M}\)), ascorbic acid (72.5 \(\mu\text{M}\)) and selenium compound (116 \(\mu\text{M}\)) were combined; the pH was adjusted to 7 using MOPS buffer (10 mM). Similarly, compounds demonstrating antioxidant activity with Cu\textsuperscript{I} were tested with Cu(bipy)\textsubscript{2}\textsuperscript{+}, where Cu(bipy)\textsubscript{2}\textsuperscript{+} (29 \(\mu\text{M}\)), ascorbic acid (36.25 \(\mu\text{M}\)), and the selenium compound (58 \(\mu\text{M}\)) were combined to pH 7 using MOPS (10 mM). For Fe\textsuperscript{II} experiments, FeSO\textsubscript{4} (300 \(\mu\text{M}\)) and selenium compound (600 \(\mu\text{M}\)) were combined in MES buffer (10 mM; pH 6). All concentrations indicated are the final concentrations in a 3 mL reaction volume.

**Glutathione peroxidase measurements (GPx)**

Glutathione peroxidase measurements were conducted according to the reported procedure by Mareque \textit{et al.}\textsuperscript{65} The tested selenium compound in methanol (selenomethionine, selenocystamine, methyl-selenocysteine, 2-aminophenyl diselenide, 2-carboxyphenyl diselenide, 2-carboxyphenyl selenide, 4-carboxyphenyl diselenide, 3,3'-diselenobispropionic acid, 3,3'-selenobispropionic acid), water (selenomethionine, selenocysteine) or DMSO (ebselen, 2-aminophenyl diselenide) (100 \(\mu\text{M}\)) was added to \(\text{H}_2\text{O}_2\) (3.75 \(\mu\text{M}\)) in methanol and the formation of PhSSPh (ebselen, 2-aminophenyl diselenide) (100 \(\mu\text{M}\)) was added to \(\text{H}_2\text{O}_2\) (3.75 \(\mu\text{M}\)) in methanol and the formation of PhSSPh from PhSH (10 mM) was monitored spectroscopically at 305 nm for 25 min at 25 \(\text{\degree}\text{C}\) (300 scans). This catalytic reaction was initiated by the addition of the \(\text{H}_2\text{O}_2\) to the solution of PhSH. Initial rates were determined by fitting the linear portion of the curve; rates were determined from the slope of best-fit line. All rates were determined as the average of at least three trials and are reported relative to ebselen in methanol. For compounds that were not soluble in methanol, GPx measurements were compared to those of another compound in the same solvent and methanol. The relative GPx measurement in methanol was then calculated based on this comparison.

**Mass spectroscopy measurements**

Electrospray ionization (ESI) mass spectra were obtained using the QSTAR XL Hybrid MS/MS System (Applied Biosystems), with direct injection of the sample (flow rate = 0.05 mL min\textsuperscript{-1}) into the Turbo Ionspray ionization source. Samples were run under positive mode, with an ionspray voltage of 3000 V (except as otherwise indicated) in time-of-flight scan mode (error \pm 2 \text{m}\text{z}). Mass spectrometry samples for a 1 : 3 metal to ligand ratio were prepared by combining CuSO\textsubscript{4} (75 \(\mu\text{M}\)) and ascorbic acid (94 \(\mu\text{M}\)) in methanol–water, and allowed to stand for 3 min at room temperature. The selenium compound (225 \(\mu\text{M}\)) was added to the solution to obtain a final volume of 1 mL, and allowed to stand for 5 min at room temperature. A similar procedure was followed for studies with Fe\textsuperscript{II}, combining FeSO\textsubscript{4} (75 \(\mu\text{M}\)) and the selenium compound (225 \(\mu\text{M}\)) for a 1 : 3 metal to ligand ratio. All concentrations indicated are the final concentrations in a 1 mL volume. All reported \textit{m}/\textit{z} peak envelopes matched theoretical peak envelopes for the assigned complexes.

**Electrochemical measurements**

Cyclic voltammetry samples were prepared by dissolving the selenium compounds (300 \(\mu\text{M}\)) in MOPS buffer (10 mM, pH 7) with KNO\textsubscript{3} (10 mM) as a supporting electrolyte. Solutions were degassed for 5 min with \(\text{N}_2\) before each experiment. All CV experiments were conducted at 100 mV s\textsuperscript{-1}. For differential pulse voltammetry experiments, a pulse amplitude of 0.080 V, a pulse width of 0.100, a sample width of 0.045, and a pulse period of 0.200 were used. Samples of each selenium compound were cycled between \(-1000\) mV and 1000 mV (2-aminophenyl diselenide was cycled between \(-1200\) mV and 800 mV) using a glassy carbon working electrode, a Pt counter electrode, and a Ag/AgCl (197 mV vs. NHE) reference electrode. Electrochemical data for all compounds are given in the ESI\textsuperscript{+}.

**Conclusions**

The selenium compounds selenocysteine, selenomethionine and methyl-selenocysteine effectively prevent copper-mediated DNA damage with IC\textsubscript{50} values of 3-26 \(\mu\text{M}\), 3,3'-Diselenobispropionic acid, 3,3'-selenobispropionic acid, selenocystamine, 2-aminophenyl diselenide, 2-carboxyphenyl diselenide, 2-carboxyphenyl selenide, and 4-carboxyphenyl diselenide show no antioxidant activity under these conditions. For these selenium compounds, antioxidant activity primarily occurs by a copper-binding mechanism, not by GPx-like activity or radical scavenging. The observed antioxidant activity of selenium compounds with Cu\textsuperscript{I} occurs at biological concentrations (\(\sim 1–10\) \(\mu\text{M}\)) of selenium.\textsuperscript{53,54}

In contrast, inhibition of iron-mediated DNA damage was observed for four selenium compounds (methyl-selenocysteine, selenocystamine, 3,3'-diselenobispropionic acid, and 3,3'-selenobispropionic acid) at high concentrations; methyl-selenocysteine was the only compound found to inhibit both copper- and iron-mediated DNA damage. Metal binding also plays a significant role in the observed antioxidant activity with Fe\textsuperscript{II}, but a radical scavenging mechanism is also likely at high concentrations. Overall, selenium compounds were more effective at inhibiting iron-mediated DNA damage than their sulfur analogs.

Electrochemical studies show no clear correlation between selenium antioxidant activity and oxidation potentials of the selenium compounds. Additionally, mass spectrometry studies of Cu\textsuperscript{I} and Fe\textsuperscript{II} coordination to selenium compounds indicate that while metal coordination is necessary for antioxidant activity, it is not sufficient. Both the identity of the metal ion and the functional groups on the selenium compounds play a very significant role in antioxidant behavior. Because reactive oxygen species are implicated in cellular damage and disease, further investigating this novel metal-binding antioxidant mechanism and the effects of selenium speciation on antioxidant activity are vital to understanding the biological antioxidant activity of selenium.
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References


