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RESEARCH ARTICLE

Glutathione conjugation of busulfan produces a hydroxyl radical-trapping dehydroalanine metabolite

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Abstract

1. The Phase 2 drug metabolism of busulfan yields a glutathione conjugate that undergoes a β-elimination reaction. The elimination product is an electrophilic metabolite that is a dehydroalanine-containing tripeptide, γ-glutamyldehydroalanylglycine (EdAG). In the process, glutathione lacks thiol-related redox properties and gains a radical scavenging dehydroalanine group.
2. EdAG scavenged hydroxyl radical generated in the Fenton reaction in a concentration-dependent manner was monitored by electron paramagnetic resonance (EPR) spectroscopy. The apparent rate of hydroxyl radical scavenging was in the same range as published values for known antioxidants, including *N*-acyl dehydroalanines.
3. A captodatively stabilized carbon-centered radical intermediate was spin trapped in the reaction of EdAG with hydroxyl radical. The proposed structure of a stable product in the Fenton reaction with EdAG was consistent with that of a γ-glutamylserylglycyl dimer.
4. Observation of the hydroxyl trapping properties of EdAG suggests that the busulfan metabolite EdAG may contribute to or mitigate redox-related cytotoxicity associated with the therapeutic use of busulfan, and reaffirms indicators that support a role in free radical biology for dehydroalanine-containing peptides and proteins.

Keywords: Dehydroalanine, busulfan, metabolite, glutathione, hydroxyl radical, carbon-centered radical, EPR

Introduction

In the course of studying the Phase 2 metabolism of the anticancer drug busulfan, we found that the glutathione conjugate of busulfan was converted to a glutathione derivative represented as the dehydroalanine analog, γ-glutamyldehydroalanylglycine (EdAG) (Younis et al. 2008). This unusual dehydroalanine forms spontaneously from the unstable busulfan-glutathione sulfonium ion metabolite by β-elimination of tetrahydrothiophene (Figure 1).

The dehydroalanine moiety differs in reactivity, stability and molecular shape in comparison to natural amino acid residues and is a rarely observed post-translational modification in proteins. The usual sources of dehydroalanine residues result from a β-elimination reaction on cysteine and serine residues. Heat or treatment with alkali can also lead to dehydroalanine formation in proteins and peptides. Insulin when heated, for example, undergoes a β-elimination of cystine groups resulting in formation of dehydroalanine residues (Kim and Kim 2001). In other examples, dehydroalanine formation can be detected as thermally-induced side reactions in sample preparation for mass spectral analysis (Wang et al. 2010). Dehydroalanine residues have been detected in processed foods (Friedman 1999).

In busulfan metabolism, Conjugation with glutathione leads to a derivative of glutathione from which a thiol has been eliminated. Elimination of the thiol group

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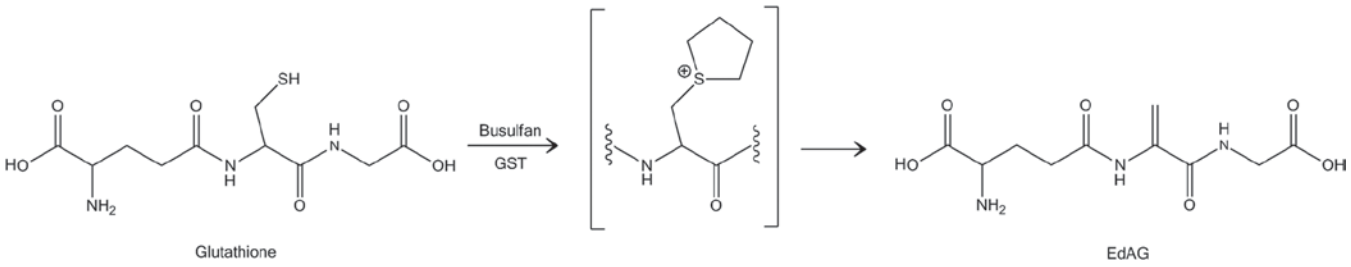

Figure 1. Busulfan metabolism catalyzed by glutathione S-transferase. The intermediate metabolite is an unstable sulfonium ion that undergoes a β-elimination to form tetrahydrothiophene and the dehydroalanine analog of glutathione, γ-glutamyldehydroalanylglycine (EdAG).

from glutathione represents an obvious change in glutathione reactivity by the loss of both thiol redox activity and thiolate nucleophilicity. The nucleophilic character of the glutathione thiol upon transformation to EdAG is replaced by electrophilic reactivity, which is an example of umpolung (polarity inversion) chemistry (Seebach 1979). Consequently, nucleophilic attack on the terminal vinylic carbon of the dehydroalanine residue of EdAG by thiol-containing compounds, such as glutathione and L-cysteine, produce thioether products classified as lanthionines (Younis et al. 2008, Cooper et al. 2011).

Dehydroalanine intermediates and lanthionines are found in the microbial engineering of lantibiotics. Lantibiotics are cyclic peptides formed by intramolecular Michael addition of a nearby cysteine thiol to a dehydroalanine residue (Nagao et al. 2011). The rate of Michael addition of thiols to dehydroalanine amides is facile in aqueous solution (Naidu et al. 2003). The resulting lanthionine thioether linkage is nonreducible in comparison with disulfide linkages (Cooper et al. 2011). In man, lanthionine cross-links have been found in lens (Linetsky & LeGrand 2005) and in central nervous system proteins (Hensley et al. 2010). Dehydroalanine-containing peptides are being incorporated into antibiotic drug discovery strategies (Sit et al. 2011; Rangachari et al. 2009; Chalker & Davis 2010).

In addition to electrophilic reactivity, dehydroalanine residues and *N*-acyl dehydroalanines are known to have radical scavenging properties (Roberfroid & Buc Calderon 1995). *N*-Acylated dehydroalanines add hydroxyl radical to produce a captodatively stabilized α-carbon radical (Sipe et al. 1993). *N*-Acyl dehydroalanine analogs have biological activity in a variety of systems, including enhancing the therapeutic efficacy of anticancer agents (Allemon et al. 1987; Buc-Calderon et al. 1987), protecting against radiation toxicity (Buc-Calderon et al. 1989), inhibiting rat liver peroxidation (Ferreira et al. 2009), evaluating the effects on tumor development in mouse skin (Vo et al. 1991), decreasing ROS formed during microsomal metabolism of nitrofurantoin (Buc-Calderon and Roberfroid, 1990), and protecting against oxidative stress (Buc-Calderon et al. 1990).

As a dehydroalanine-containing tripeptide, EdAG reactivity is an accessible model for understanding the properties and reactivity of dehydroalanine proteins. Here, we report an EPR approach to the characterization of the radical scavenging reactivity of EdAG.

Materials and methods

Chemicals

Glutathione, Tris buffer (pH 7.4) phosphate buffered saline (pH 7.4) 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), α-(4-pyridyl *N*-oxide)-*N*-*tert*-butylnitrone (POBN), ferrous sulfate ($FeSO_4$), hydrogen peroxide (H_2O_2), and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Optima-grade isopropanol and methanol were purchased from Fisher Scientific (Pittsburgh, PA). EdAG was synthesized as previously described (Younis et al. 2008).

Electron paramagnetic resonance spin trapping experiments

Formation of EdAG captodative radical

Hydroxyl radical scavenging activity by EdAG was monitored by electron paramagnetic resonance (EPR) using a Bruker EMX spectrometer (Billerica, MA). Hydroxyl radical was generated by the Fenton reaction consisting of H_2O_2 (0.1–2 mM) and $FeSO_4$ (0.01–2 mM) in water. The reaction mixture also contained either DMPO (100 mM) or POBN (20 mM) as spin traps, and EdAG (0–50 mM) diluted with phosphate buffered saline, pH 7.4, to a final volume of 300 µL. Hydrogen peroxide was added last to initiate the formation of $^{\bullet}$OH and then the reaction was immediately transferred to a flat-cell for EPR analysis. The short-lived hydroxyl radical was rapidly trapped by DMPO or POBN to obtain the DMPO-$^{\bullet}$OH or POBN-$^{\bullet}$OH spin adduct, respectively, that is observed as a spin-trapped signal in the EPR. A decrease in signal intensity resulting from addition of radical scavenging compounds that compete for hydroxyl radical has provided a basis for estimating a rate constant of radical scavenging by EdAG (k_E) in comparison with the rate of hydroxyl radical capture by DMPO (k_D) (Leonard et al. 2003; Shi et al. 2000). Experiments were performed in triplicate under ambient air at room temperature, and the average values used to determine k_E from the equation: $V/v - 1 = k_E[EdAG]/k_D[DMPO]$, where V and v are the velocities of $^{\bullet}$OH scavenging in the presence and absence of EdAG, respectively, and k_E and k_D represent

the rate constant for hydroxyl radical scavenging by EdAG and DMPO, respectively. The literature value used for k_D was 2.1×10^9 $M^{-1}s^{-1}$ (Mariott et al. 1980).

Simulation of EPR spectra

EPR spectra were simulated using WINSIM, part of the PEST software (NIEHS, http://www.niehs.nih.gov/research/resources/software/tox-pharm/tools/index.cfm, accessed 15 May 2012). Initial estimates of g-values and the hyperfine coupling constants were made based on the known spectra for DMPO-•OH and POBN-•OH radicals and inspection of the spectra for other radical species. Parameters were then optimized based on the residuals.

Characterization of EdAG-hydroxyl radical reaction product by LC-MS/MS

EdAG (5 and 10 mM) in aqueous Fenton reagent solution (2 mM H_2O_2 and $FeSO_4$) was stirred at room temperature for 5 min. Isolation of a stable product was done using a Thermo Hypercarb HPLC column (100 × 2.1 mm I.D., 5 µm, ThermoFisher, Waltham, MA). A gradient of mobile phase A (0.5% formic acid in 90/10, v/v, water/acetonitrile) and mobile phase B (0.1% formic acid in 50/50, v/v, isopropanol/methanol) at 0.2 mL/min was run from 0–5 min using 100% A; from 5–7 min, 0→50% B; from 7–9 min, 50→0% B; and from 9–15 min, 100% A. Electrospray ionization mass spectrometry (ESI-MS) was in the positive ion full scan mode. Tandem mass spectrometry (MS/MS) was performed on a stable product in the reaction mixture (*m/z* 581). Accurate mass measurements of the MH^+ ion (*m/z* 581) and significant product ions (*m/z* 563, 545, and 434) were performed on a Fourier Transform mass spectrometer.

Results

Reaction of EdAG with hydroxyl radical

The reactivity of EdAG with hydroxyl radical was determined by generating •OH with the Fenton reaction in the presence of a spin trap (DMPO or POBN), and EdAG. Trapped •OH was monitored by EPR in the absence of EdAG (Figure 2A), based on hyperfine splitting patterns previously characterized (Buettner et al. 1987). When EdAG (0–50 mM) was added to the Fenton reaction mixture, an obvious decrease in the spin adduct (DMPO-•OH) EPR signal intensity was observed, indicating that EdAG competed with DMPO for scavenging hydroxyl radical (Figure 2B). The spectrum shown in Figure 2B was simulated (Figure 2C). The best fit was obtained when it was fit assuming three species, and could not be adequately fit assuming two species. The parameters found were radical species **1**, a_N = 14.9 G, a_H = 14.8 G, line width = 0.58 G, conc. = 49.7% (DMPO-•OH); radical species **2**, a_N = 15.4 G, a_H = 20.2 G, line width = 0.51 G, conc. = 26.6% (DMPO-carbon-centered radical); and radical species **3**, a_N = 15.9 G, a_H = 22.6 G, line width = 0.82 G, conc. = 23.7% (DMPO-carbon-centered radical). In contrast, no detectable carbon-based radicals were observed in the Fenton reaction with glutathione, which is consistent with literature observations on the reaction of glutathione with ROS (Karoui et al. 1996). Figure 2E displays the EPR spectrum of glutathione thiyl radical trapped with DMPO.

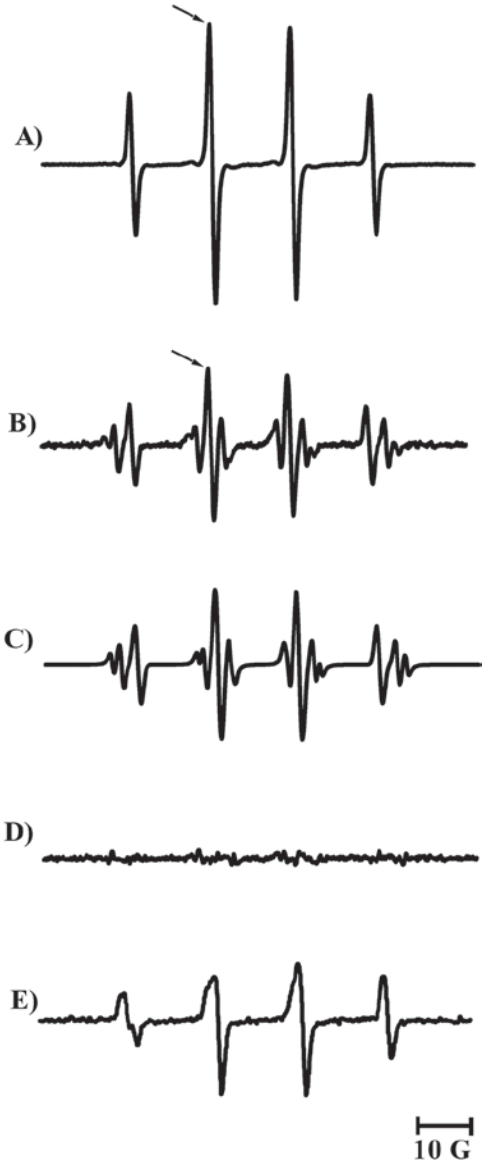

Figure 2. Experimental and simulated EPR spectra of Fenton reaction and DMPO with EdAG. (A) Spectrum of DMPO-•OH spin trap adduct in the absence of EdAG. (B) The presence of 20 mM EdAG caused a decrease in the DMPO-•OH signal quartet, indicating that EdAG was scavenging hydroxyl radical. The arrow indicates at what G-value peak height measurements were taken to determine the rate of scavenging calculations (k_E). (C) Simulation of the experimental EPR spectrum shown in B. The spectrum was fit, assuming three species were present. (D) Residuals from the difference of the experimental (B) and simulated (C) spectra (Corr. Coeff. = 0.994). (E) Glutathione (10 mM) inhibits formation of DMPO-•OH without forming carbon-based radicals.

A stabilized carbon-centered radical was observed during the concentration-dependent reduction in the DMPO-•OH signal by EdAG. The proposed radical intermediate formed, EdAG-•OH (γ-glutamylserylglycine; compound **4**, Figure 3), is suggested to stabilize the free

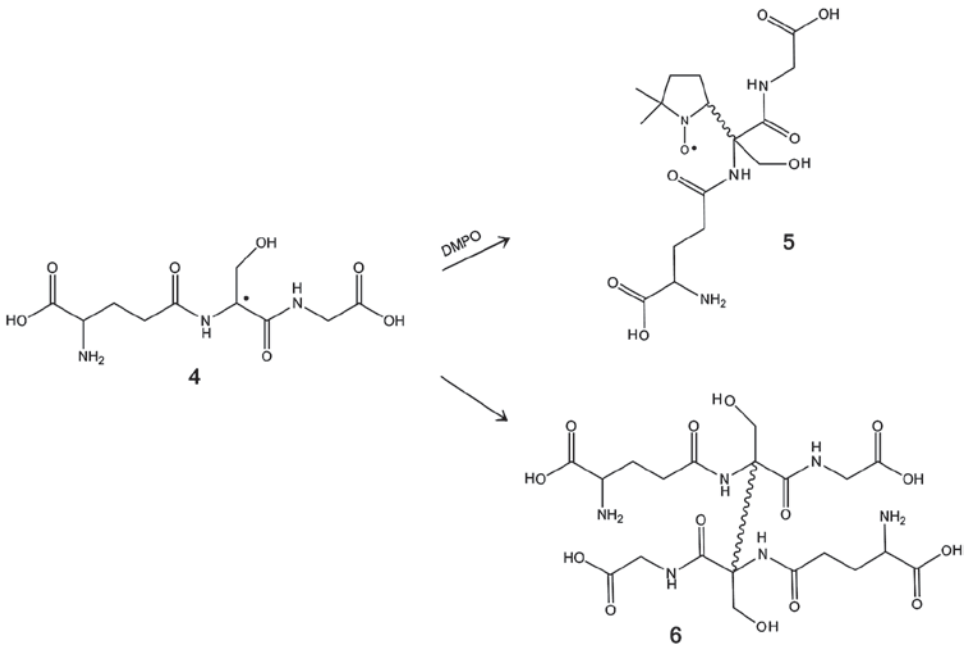

Figure 3. Proposed mechanism of hydroxyl radical scavenging by EdAG. The α,β-conjugated system in EdAG favors conjugate addition on the β-carbon by hydroxyl radical. A captodative mechanism stabilizes the radical on the α-carbon as a short-lived intermediate (EdAG-•OH, **4**), followed by either dimerization (**6**), or trapped with DMPO (**5**).

radical on the α-carbon of the resulting serine residue. At this location, the radical is unlikely to attack the alkene of another EdAG molecule. EPR simulations characterized hyperfine splitting patterns for DMPO-•OH (a_N = 14.9 a_H = 14.8 gauss) and suggested two additional radical species based on simulations. Both of the addition radicals gave hyperfine coupling constants where $a_N < a_H$, consistent with DMPO-carbon-centered radical adducts (Buc-Calderon et al. 1990). Furthermore, the ratio of these two adducts were close to unity. Thus, the simulation results indicated that the two carbon-centered radicals present may be diastereomers of the same compound, suggested to be compound **5** (Figure 3). In a recombination reaction, dimeric compound **6** is formed (Figure 3).

A second spin trap, POBN, was used to further characterize the hydroxyl radical scavenging properties of EdAG and to provide supporting information regarding the assignment of diastereomers of the spin-trapped radicals adducted to radical **4**. When EdAG was introduced to the Fenton reaction mixture containing POBN, an obvious decrease in the POBN-•OH signal was observed and other radical species were detected (Figure 4). In comparison to the spectrum in Figure 4A that depicts the presence of POBN-•OH in the absence of EdAG, Figure 4B exhibits the existence of an additional radical species. The data were simulated assuming either two or three species present. For the two species simulation, the best fit parameters gave: radical species **7**, a_N = 15.0 G, a_H = 1.68 G, line width = 0.35 G, conc. = 34% (POBN-•OH); radical species **8**, a_N = 15.5 G, a_H = 2.75 G, line width = 0.90 G, conc. = 65% (POBN-carbon-centered radical). Fitting with three species gave calculated radical species **9**, a_N = 15.0 G, a_H = 1.68, line width = 0.34 (POBN-OH, 27%); **10**, a_N = 15.5 G, a_H = 3.0, line width = 0.77 (POBN-carbon-centered radical, 48%), and **11**, a_N = 15.2 G, a_H = 1.65, line width = 0.52 (25%). The values for the latter species, **10** and **11**, are not consistent with a POBN-carbon centered radical adduct, indicating that inclusion of three species would be over-fitting the data. Therefore, the two species fit is most likely reflective of the spin adducts trapped with POBN. As there is only nominal dispersion in hyperfine coupling constants for POBN-carbon centered radicals, this is not unexpected. However, EPR simulations indicated that only one carbon-centered radical species was present, not two as the case when DMPO was the spin trap. Therefore, the results indicate that both spin traps form adducts with radical **4** at a chiral center. However, POBN produces only one carbon-centered radical species detectable by EPR, whereas DMPO produces distinguishable diastereomers from the same carbon-centered radical species.

Rate of hydroxyl radical trapping by EdAG

A reaction rate constant of EdAG-hydroxyl radical scavenging (k_E) was determined from the peak height ratio of the signals marked with arrows (Figures 2A and 2B). The calculated second order rate constant was calculated to be 8.41×10^9 M^{-1} s^{-1}.

Characterization of Fenton reaction with EdAG by LC-MS/MS

Figure 3 depicts the proposed mechanism of EdAG reaction with hydroxyl radical to form a postulated γ-glutamylserylglycine radical **4** that is either trapped by DMPO to form diastereomeric carbon-based radicals **5** or two γ-glutamylserylglycine radicals condensed

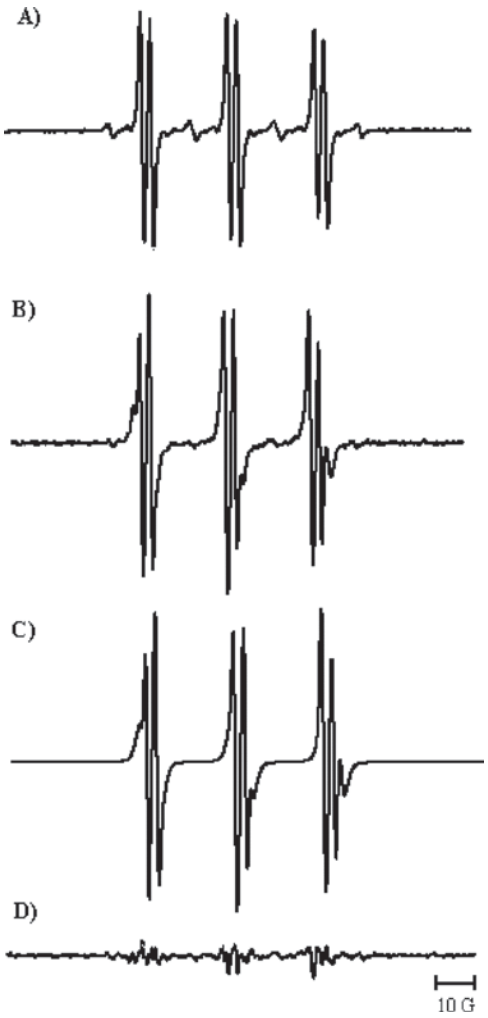

Figure 4. Experimental and simulated EPR spectra of Fenton reaction and POBN with EdAG. The POBN-•OH signal in the (A) absence of EdAG, and (B) presence of 2 mM EdAG. (C) Simulation of the experimental spectrum shown in (B), assuming two simulated POBN carbon-centered radicals are present. (D) Residuals between spectrum (B) and (C).

to form the dimer **6**. The dimer was identified as a reaction product of EdAG with a Fenton mixture and the structure assign based on mass spectral fragmentation patterns. Tandem mass spectrometry (MS/MS) of the molecular species, *m/z* 581, produced a fragmentation pattern consistent with a dimer of hydroxylated EdAG (di-γ-glutamylserylglycine, **6**). Assignment of the MS/MS fragmentation ions were loss of water (*m/z* 563), two water molecules (*m/z* 545), and pyroglutamic acid plus water (*m/z* 434). The assignments are consistent with the proposed fragmentation pathway outlined in Figure 5. Accurate mass determinations performed on a high-resolution mass spectrometer verified the calculated empirical formulas of the $[MH]^+$ ion and MS/MS product ions $[M - H_2O]^+$, $[M - 2H_2O]^+$, and $[M - H_2O$ and pyroglutamic acid$]^+$. Alternative isobaric structures of **6** cannot be ruled out by analysis of the observed MS/MS fragmentation pathway. For example, the product resulting from a 1,2-addition of hydroxyl radical would have the same elemental composition and mass spectral fragments as does compound **6**. The 1,2-addition product was considered to be unlikely because the resulting primary carbon-based radical would not be captodatively stabilized (Carmichael et al. 1985), and the 1,4-addition would be favored.

Discussion

EdAG is a substituted dehydroalanine derived from the desulfuration of glutathione that reacts with and scavenges hydroxyl radical. Structurally and mechanistically similar to *N*-acyl dehydroalanine radical scavenger analogs (Buc-Calderon et al. 1990; Sipe et al. 1993), oxidation of EdAG follows a path involving a stabilized carbon-centered radical intermediate.

Radicals formed at the α-carbon of amino acid derivatives and peptides are stabilized by an adjacent electron-withdrawing carbonyl and an electron-donating amide nitrogen (Hopkinson 2009; Viehe et al. 1985). This push-pull radical stabilization is known as the captodative effect (Viehe et al. 1979). Captodatively stabilized radicals have been proposed as intermediates in the addition of ROS to *N*-acyl dehydroalanines (Sipe et al. 1993).

EdAG was shown to be an efficient captodatively stabilized scavenger through attenuation of both DMPO-•OH and POBN-•OH EPR signals formed under Fenton reaction conditions. Analysis of hyperfine splitting patterns and simulated EPR spectra were consistent with multiple carbon-centered radical species being present in addition to trapped hydroxyl radical, DMPO-•OH. A plausible source of the carbon-centered radical is a Michael addition of hydroxyl radical at the electropositive vinylic carbon of EdAG providing a seryl radical. Two trapped carbon-centered radical species closely related in structure are proposed. The proposed structures of the trapped radicals that fit the EPR data are diastereomers of DMPO-EdAG-•OH (compound **5**, Figure 3). The assignment was based on a comparison with literature spectra of the products of *N*-acyl dehydroalanines with hydroxyl radical (Sipe et al. 1993) and EPR simulations. It is interesting to note that oxidation of cysteine residues in cellular proteins can result in conversion of cysteine to serine (Jeong et al. 2011), presumably by way of a dehydroalanine intermediate.

Mass spectral evidence in support of dimer **6** as a product in the Fenton reaction with EdAG supports the conclusion that a captodative radical intermediate is likely to be formed in the reaction of EdAG with hydroxyl radical (Figure 3). Assignment of the structure of **6** was based on FT-MS/MS accurate mass neutral loss data. Product ions from neutral losses of water and pyroglutamic acid were observed that are consistent with literature spectra of common losses for glutathione-containing compounds when analyzed by ESI-MS/MS (Castro-Perez et al. 2005; Zhu et al. 2007). The simplicity of the MS/MS product ion spectrum of **6** as shown in Figure 5 suggests a symmetrical compound,

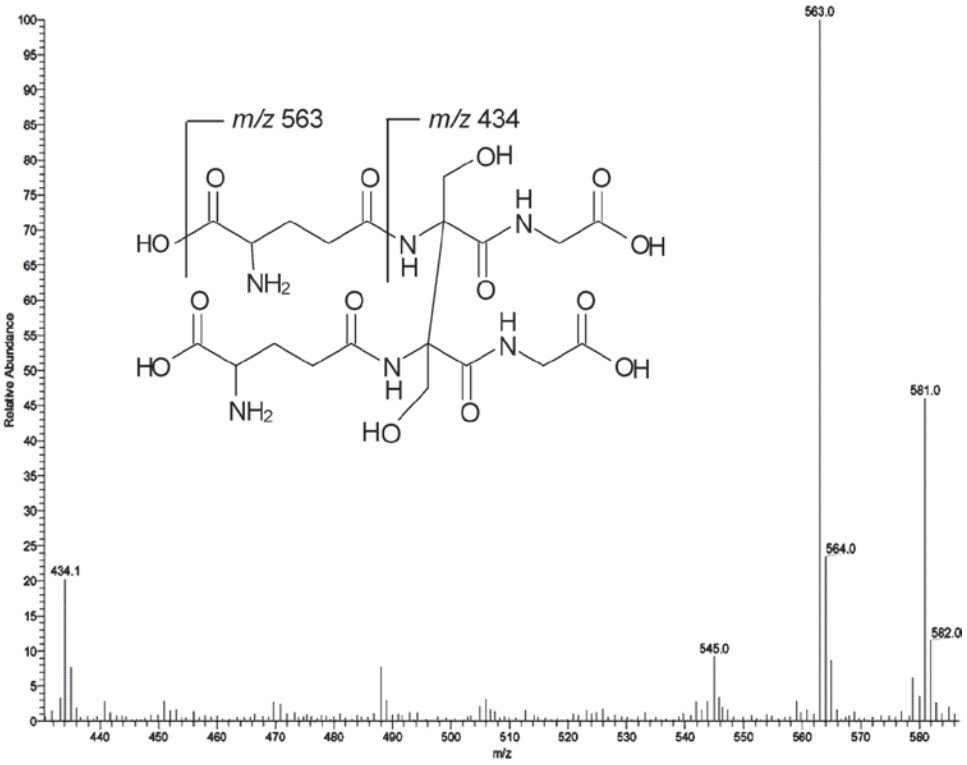

Figure 5. Tandem mass spectrum of an oxidation product in the reaction of EdAG and Fenton reagent. The MS/MS fragmentation pattern was consistent with neutral losses of 18, 36, and 147 Da corresponded to water, two water molecules, and pyroglutamic acid plus water, respectively. The reaction product structure was assigned as the dimer of two molecules of radical species **4** (di-γ-glutamylserylglycine, **6**). The inset depicts the proposed MS/MS fragmentation pattern of **6**.

which is consistent with the assigned structure, although hydroxyl radical attachment on the α carbon producing a methylene radical cannot be ruled out.

A mechanism involving an energetically favorable captodative radical is a plausible explanation for the Michael addition of hydroxyl radical to EdAG to form a carbon-centered radical on the α-carbon of the dehydroalanine moiety. Prominent among protein radicals are α-carbon-centered radical species. Examples are found in enzymes, such as the α-glycyl radical found in class III ribonucleotide reductase (Logan et al. 1999). In another example, Osburn and coworkers proposed a captodative α-carbon centered glycyl radical derived from cysteine methyl ester radical cation (Osburn et al. 2011). Protein radicals have been associated with the biological consequences of oxidative stress (Nauser et al. 2005), although the contribution of radicals produced from dehydroalanine residues have not been directly implicated as participating in the transmission of ROS damage.

Table 1 provides the relative rates of hydroxyl trapping in the Fenton reaction by known antioxidants. The second order rate constant determined for EdAG was found to be about mid-range in comparison with established antioxidants with hydroxyl radical scavenging properties that approach diffusion rates. Antioxidants with faster rates of reaction with hydroxyl radical than that of EdAG include compounds classified as flavonoids (baicalin, epicatechin-3-gallate), quinolines (rebamipide), pyrazolinones (enduravone), thiyl compounds (glutathione), tetrahydroisoquinolines (tetrandrine), hydroquinones (gentisic acid), and phenols (capsaicin). Antioxidants less reactive toward hydroxyl radical include sulfoxides (dimethyl sulfoxide), catechols (chlorogenic acid), salicylates (aminosalicylic

Table 1. Second order rate constants for hydroxyl radical trapping by antioxidants.

Antioxidant	Rate of hydroxyl radical trapping (M^{-1} s^{-1})	References
Baicalin	7.7×10^{11}	Shi et al. 1995
Epicatechin-3-gallate	4.62×10^{11}	Shi et al. 2000
Rebamipide	5.62×10^{10}	Sakurai et al. 2004
Eduravone	3×10^{10}	Abe et al. 2004
Glutathione	1.65×10^{10}	Sakurai et al. 2004
Tetranidrine	1.4×10^{10}	Ye et al. 2000
Gentisic acid	1.1×10^{10}	Joshi et al. 2012
Capsaicin	1.0×10^{10}	Okada et al. 2002
EdAG	8.4×10^{9}	This work
Dimethyl sulfoxide	8.16×10^{9}	Sakurai et al. 2004
Chlorogenic acid	7.73×10^{9}	Zang et al. 2003
Aminosalicylic acid	6.7×10^{9}	Mantena et al. 2008
Perfenidone	5.4×10^{9}	Misra and Rabideau, 2000
AD-5	4.5×10^{9}	Roberfroid and Buc Calderon, 1995
DMPO	2.1×10^{9}	Mariott et al. 1980
Resveratrol	9.45×10^{8}	Leonard et al. 2003
Chlorophyllin	2.7×10^{6}	Kumar et al. 2001

acid), pyridones (perfenidone), dehydroalanines (AD-5), pyrroline *N*-oxides (DMPO), polyphenols (resveratrol), and chlorophyll analogs (chlorophyllin). *In vitro*, EdAG fits the mechanistic classification of radioprotectants as a scavenger of free radicals produced from ionizing radiation. *In vivo* studies would be required to determine the effectiveness of EdAG or protein dehydroalanines as scavengers of biological free radicals, especially in systems that are naturally rich in antioxidants containing high levels of cellular glutathione and protein thiols.

Recognition of radical trapping potential of EdAG provides a basis for a better understanding of the roles of ROS in the toxicology and therapeutic mechanism of busulfan. The hydroxyl trapping properties of EdAG suggests that the busulfan metabolite EdAG may contribute to or mitigate redox-related cytotoxicity associated with the therapeutic use of busulfan, and reaffirms indicators that support a role in free radical biology for dehydroalanine-containing peptides and proteins.

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Declaration of interest

The authors report no conflicts of interest.

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