Oxidised guanidinohydantoin (Gh^{ox}) and spiroiminodihydantoin (Sp) are major products of iron- and copper-mediated 8-oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydro-2'-deoxyguanosine oxidation

Blánaid White,[a] Maricar C.Tarun,[b] Nicholas Gathergood,[a] James F. Rusling,[b, d] and Malcolm R. Smyth[a,]*

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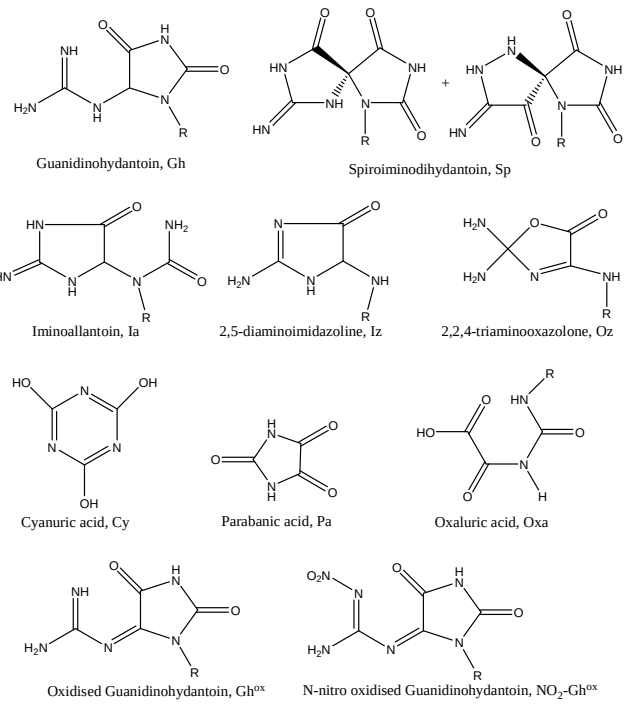
8-Oxo-7,8-dihydroguanine (8-oxoGua), an important biomarker of DNA damage in oxidatively generated stress, is highly reactive towards further oxidation. Much work has been carried out to investigate the oxidation products of 8-oxoGua by one electron oxidants, singlet oxygen, and peroxynitrite. This report details for the first time, the iron- and copper-mediated Fenton oxidation of 8-oxoGua and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo). Oxidised guanidinohydantoin (Gh^{ox}) was detected as the major product of oxidation of 8-oxoGua with iron or copper and hydrogen peroxide, both at pH 7 and pH 11. Oxaluric acid was identified as a final product of 8-oxoGua oxidation. 8-oxodGuo was subjected to oxidation under the same conditions as 8-oxoGua, however, dGh^{ox} was not generated. Instead, spiroiminodihydantoin (Sp) was detected as the major product for both iron and copper mediated oxidation at pH 7. It was proposed that the oxidation of 8-oxoGua was initiated by its one electron oxidation by the metal species, which leads to the reactive intermediate 8-oxoGua$^{\bullet+}$, which readily undergoes further oxidation. The product of 8-oxoGua and 8-oxodGuo oxidation was determined by the 2'-deoxyribose moiety of the 8-oxodGuo, not whether copper or iron was the metal involved in the oxidation.

Introduction

Human DNA is estimated to undergo over 10,000 oxidatively generated hits per day (~ $9*10^4$ hits/cell/day), based on the measurement of damaged DNA bases in human urine.[1] Ubiquitous enzymatic DNA repair processes are operative, but despite these, significant steady-state levels of oxidatively generated damage in mammalian DNA have been recorded, with the background range of 8-oxo-7,8-dihydroGuanine (8-oxoGua) currently estimated (by ESCODD) as likely being from 0.3 to 4.2 8-OxoGua per 10^6 Guanine.[2] Steady state levels of oxidatively generated damage are one or more orders of magnitude higher than those of non-oxidatively generated nucleobase adducts.[3] This result of so called "oxidatively generated stress" has been implicated in mutagenesis, disease and aging.[4-6] One of the primary oxidatively generated stress biomarkers to have emerged in recent years is 8-oxoGua, a primary product of Guanine oxidation.[7]

It is now generally accepted that 8-oxoGua may not be the final product of Guanine oxidation. It has a lower oxidation potential than any of the unmodified DNA bases, including Guanine itself,[8] and has been identified as a "hotspot" for one-electron and singlet oxygen oxidation.[9] The exact nature of Guanine, and indeed 8-oxoGua, oxidation appears to depend on the nature of the nucleobase reactant (*e.g.*, dG, singe-stranded oligomer, double-stranded oligomer) and reaction conditions (pH, temperature), as well as the nature of the oxidant. Table 1 summarises published work on the oxidation products of 8-oxodGuo. Scheme 1 shows their structures.



Scheme 1 Oxidation products of 8-oxodGuo.

Singlet oxygen ($^1O_2$) mediated oxidation of 8-oxodGuo was found to lead to the generation of Cyanuric acid (Cy), Spiroiminodihydantoin (Sp) and Imidazolone (Iz), which degraded to 2,2,4-triaminoOxazolone (Oz).[10] Subsequent studies also detected parabanic acid (Pa), as a precursor to oxaluric acid (Oxa).[11] During this investigation Cy was not

* Malcolm.Smyth@DCU.ie

Table 1 Oxidation products of 8-oxo-7,8-dihydroguanine

Substrate[a]	Oxidant	Experimental conditions[b]	Products [ref][c]
8-oxodGuo	Ir (IV) - one electron oxidation	pH 4, 22 °C	50% Gh, 40% Ia[18]
8-oxodGuo		pH 7, 22 °C	90% Sp[18]
ss-oligomer		pH 7, 4 °C	95% Gh/Ia[18]
ss-oligomer		pH 7, 22 °C	55%Gh/Ia,45%Sp[18]
ss-oligomer		pH 7, 50 °C	95% Sp[18]
ds-oligomer		pH 7, 22 °C	95% Sp/Ia[18]
8-oxodGuo	Cr(V)-Salen complex	pH 7, room temp.	Gh, Sp[19]
ds-oligomer		pH 7, room temp.	Sp[19]
8-oxodGuo	$^1O_2$	-78°C using TPPsensitizer; irradiated for 30 min using 300-W xenon lamp	Sp (when warmed to room temperature) [44]
8-oxodGuo	$^1O_2$	Endoperoxide $DHPNO_2$ source of $^1O_2$	Iz, Oz, Sp[12]
ss-oligomer	$^1O_2$	Endoperoxide $DHPNO_2$ as source of $^1O_2$	Oxaluric acid, via Gh^{ox} [11]
8-oxodGuo	$^1O_2$	Photoirradiation and Methylene Blue as source of $^1O_2$	50% Cy[10] 35% Iz/Oz, 10% Sp
8-oxodGuo	$^1O_2$	Photoirradiation and riboflavin/ anthraquinone	5 – 10% Iz (= 50% of 8-oxoGua formed) [15]
8-oxodGuo	$ONOO^-$	High $ONOO^-$ fluxes	Gh^{ox},NO_2-Gh^{ox} [22]
		Limiting $ONOO^-$ fluxes, high pH	Sp[22]
		Limiting $ONOO^-$ fluxes, low pH	Gh[22]
8-oxodGuo	$ONOO^-$	$ONOO^-$ in presence of thiol (glutathione or cysteine)	Sp[45]

[a] ss-, ds-oligomer = single-stranded and double-stranded oligomers containing 8-oxo-7,8-dihydroguanine; d(GpT) = 2'-deoxyguanylyl(3'→5')-thymidine; [b] TPP = 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine; $DHPNO_2$ = N,N'-di(2,3-dihydroxypropyl)-1,4-naphtalenedipropanamide; [c] Gh = guanidinohydantoin; Sp = spiroiminodihydantoin; Ia = iminoallantoin; Iz = imidazolone; Oz = 2,2,4-triaminooxazolone; Gh^{ox} = oxidised guanidinohydantoin (dehydroguanidinohydantoin); Cy = cyanuric acid; NO_2-Gh^{ox} = N-nitro dehydroguanidinohydantoin

proposed as an oxidation product. Sp, Iz and Oz were detected on oxidation of 8-oxodGuo by chemically induced $^1O_2$,[12] with Oxa detected on oxidation of single stranded oligomers containing 8-oxodGuo.[11] Hydroxyl radical (•OH) oxidation of dG was found to generate Oz under reducing conditions,[13] however, for •OH oxidation induced by iron (II) ions, further oxidation of 8-oxoGua was expected to be prevented.[14] Photoirradiation produced Iz as the major product of DNA oxidation.[15] One electron oxidation using the selective oxidant Na_2IrCl_6 also generated Guanidinohydantoin (Gh)[16] and Sp.[17] The formation of Gh or Sp was found to be dependant on both the reaction temperature and pH.[18] Chromate-induced damage also resulted in the formation of both Gh and Sp, however, as with Na_2IrCl_6, Gh was generated in DNA while Sp was produced from dG oxidation.[19] Both Gh and Sp were formed via a 5-OH-8-oxoGua intermediate.[20] The use of the metalloporphyrin Mn-TMPyP/$KHSO_5$ leads to an oxidised Gh^{ox}, not Gh, as the main oxidatively generated product of Guanine oxidation, along with Iz and Oxa.[21] Peroxynitrite oxidation was also found to generate an oxidised form of Gh, oxidised Guanidinohydantoin (Gh^{ox}) in addition to both Gh and Sp.[22]

The 8-oxoGua oxidation products have been shown to be highly mutagenic. G usually binds to C during DNA replication; however, when G is oxidised to Iz, Iz can base pair with G. This results in a G·C→C·G transversion mutation.[15] Iz slowly hydrolyses and rearranges to form Oz, which during replication gave rise to predominantly G·C→T·A transversion mutations, and to a much lesser extent G·C→C·G transversion mutations, and also blocked DNA synthesis.[23] Sp resulted in a 2:1 preference for insertion of A instead of G opposite it during replication, which resulted in G·C→T·A and G·C→C·G transversion mutations. Gh caused G·C→C·G transversion mutations. With mutation frequencies of approx. 100%, both Sp and Gh demonstrated a more dramatic effect than 8-oxoGua, which had a mutation frequency of ~ 3%. Sp was shown to be a much stronger blocker of DNA synthesis than Gh.[24] Oxa leads to G·C→C·G and G·C→T·A transversion mutations during replication, and also blocks DNA synthesis.[25] The oxidised products of 8-oxoGua oxidation were all at least one order of magnitude more mutagenic than their precursor. Further oxidation of 8-oxoGua within DNA may therefore serve to increase the mutagenicity effects of oxidatively generated stress. Thus, it is important that final products of oxidatively generated attack on DNA are fully elucidated.

Various ROS have been reported from Fe(II) or Cu(II) reaction with hydrogen peroxide in the Fenton reaction.[26-28] However, there have been few reports concerning the oxidation products of 8-oxoGua by metal-mediated Fenton reactions.[29] The exact nature of the ROS involved in the oxidation of DNA and DNA bases by such reactions has been the subject of much debate and research.[30-33] Transition metal-mediated reactions may contribute substantially to H_2O_2-mediated damage to DNA.[34,35] Such DNA damage may involve Fenton chemistry generated by Fe(II) associated with DNA.[36,37] H_2O_2 is ubiquitous in cells; therefore to minimise the risk of Fenton reactions occurring, intracellular levels of Fe(II) are very tightly regulated.[38] There is, however, a physiological demand for easily accessible iron that can be

incorporated into a very wide range of iron-containing proteins.[39] This is accommodated by a low molecular weight pool of weakly chelated iron that passes rapidly through the cell, called a labile iron pool (LIP). The iron for the LIP is delivered from a variety of sources, both extracellular and intracellular. The availability of both iron and H_2O_2 in human cells means that the Fenton reaction, and therefore the generation of ·OH, is possible *in vivo*. Along with iron, copper is one of the most important transition metals *in vivo*, and is a constituent of a number of important enzymes.[40] The adult human body contains approx. 80 μg of copper, with the total blood concentration of copper about 16 μM.[41] Copper can become available *in vivo* for Fenton reactions to occur, as it is present in blood plasma as metalloproteins and as a number of transport and storage complexes. Copper also exists, however, in the cell nucleus where it may be involved in the condensation of DNA-histone fibres into higher order chromatin structures.[42,43] Copper can associate with DNA either by intercalation or by complexation to purine bases, especially to the N7 of G.[27] There is therefore a possibility that endogenous, DNA-associated copper may be able to promote oxidatively generated DNA damage.

The goal of the present work was to investigate what oxidation products were generated from the iron and copper Fenton mediated oxidation of 8-oxoGua and 8-oxodGuo, at both pH 7 and pH 11. The results provide further insights into the conditions which play a role in DNA oxidation processes mediated by the essential transition elements iron and copper. Gh^{ox} was produced from 8-oxoGua oxidation at pH 7 and at pH 11, but Sp produced from 8-oxodGuo at pH 7 under identical conditions. These results indicate dependence of the oxidation product on the exact nature of the nucleobase reactant.

Experimental

Chemicals.

8-OxoGuanine (2-amino-6,8-dihydroxypurine) (8-oxoGua) 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), guanine, and cyanuric acid were purchased from Sigma Aldrich. Parabanic acid was purchased from Fluka. Oxaluric acid was synthesised by hydrolysing parabanic acid. Water was treated with a Hydro Nanopure system to specific resistance > 18 mΩ-cm. All other chemicals were of analytical grade and were used without further purification.

Oxidation of 8-oxoGua.

8-OxoGua does not readily dissolve at pH 7. Therefore samples were prepared at pH 11 (0.1N NaOH) where it dissolved readily and any oxidatively generated products could be detected. For qualitative purposes, incubations were also prepared at pH 7 by serial dilution of concentrated solution in 50 mM ammonium acetate buffer, pH 7. The same oxidatively generated products were detected at pH 7 and at pH 11.

2.4 mM 8-oxoGua was incubated with 150 μM iron(II) sulfate ($FeSO_4$) or 150 μM copper(II) sulfate ($CuSO_4$) and 50 mM hydrogen peroxide (H_2O_2) at 37 °C with constant stirring. Duplicate 100 μl aliquots were taken after various incubation times. The reaction was quenched with 1 ml 200 proof cold ethanol. The solution was dried immediately under a stream of nitrogen gas. The dried hydrolysates were stored at 4 °C until further use. Prior to analysis they were redissolved in 1 ml 10 mM NaOH.

Oxidation of 8-oxodGuo.

8-oxodGuo was prepared in 50 mM ammonium acetate at pH 7, to determine whether the same oxidation products were generated from 8-oxoGua and 8-oxodGuo oxidation. 2.4 mM 8-oxoGua was incubated with 150 μM iron(II) sulfate ($FeSO_4$) or 150 μM copper(II) sulfate ($CuSO_4$) and 50 mM hydrogen peroxide (H_2O_2) at 37 °C with constant stirring. Duplicate 100 μl aliquots were taken after various incubation times. The reaction was quenched with 1 ml 200 proof cold ethanol. The solution was dried immediately under a stream of nitrogen gas. The dried hydrolysates were stored at 4 °C until further use. Prior to analysis they were redissolved in 1 ml 10 mM NaOH.

Apparatus.

For 8-oxoGua analysis, the HPLC system consisted of a Varian ProStar 230 solvent delivery module, and a Varian ProStar 310 UV-VIS detector. A Restek Ultra C18 reversed-phase column (250 x 4.6 mm, particle size 5 μm) with 1 cm guard column was used. The eluent comprised of 5% methanol/95% 50 mM ammonium acetate buffer, pH 5.5, run under isocratic conditions at a flow rate of 1 ml min^{-1}. For UV detection of 8-oxoGua, the detector was set to 280 nm, while for the detection of potential further oxidation products of 8-oxoGua oxidation it was set to 214 nm. For Mass Spectrometry, a Bruker Daltonics Esquire 3000 LC-MS (ion trap) was used with a Supelco Supelcosil LC-18 reversed phase column (250 x 2.1 mm, particle size 5 μm), run under a binary gradient consisting of A 10 mM ammonium acetate buffer, pH 5.5 and B 50/50 methanol/water at the rate of 0.2 ml min^{-1}. Full scan spectra were taken at a cone voltage of 15 V using both positive and negative electrospray ionisation (ESI). ESI conditions were optimised using accompanying software.

For 8-oxodGuo analysis, HPLC (Perkin Elmer) separation with diode array detection (DAD) was carried out using an Ultra C18 (Restek) reversed-phase column (250 x 4.6 mm, particle size 5 μm). A binary gradient consisting of 10 mM ammonium acetate pH 5 and 0.1 % acetic acid in methanol was at the rate of 0.7 ml min^{-1}. A micro-splitter valve (Upchurch Scientific) delivered 5% of the flow to the mass spectrometer. Injection volume was 20 μL. Electrospray ionisation mass spectrometry (ESI-MS) employed a Quattro II (Micromass) with an electrospray source. Ion source temperature was 120 °C, with nitrogen as nebulising gas. Spectra were obtained at a low cone voltage (15kV) in the positive ion mode (ES^+).

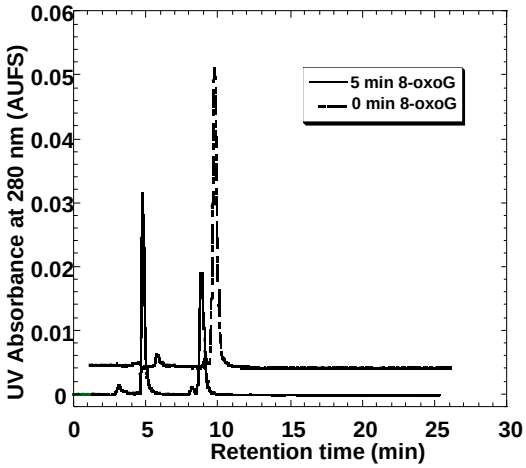


Fig. 1 HPLC separation with UV detection at 280 nm of 2.4 mM 8-oxoGua incubated for 0 min (dashed) and 5 min (solid), showing Product 1 (Gh^{ox}) eluting at 4.6 min, and 8-oxoGua eluting at 8.2 min.

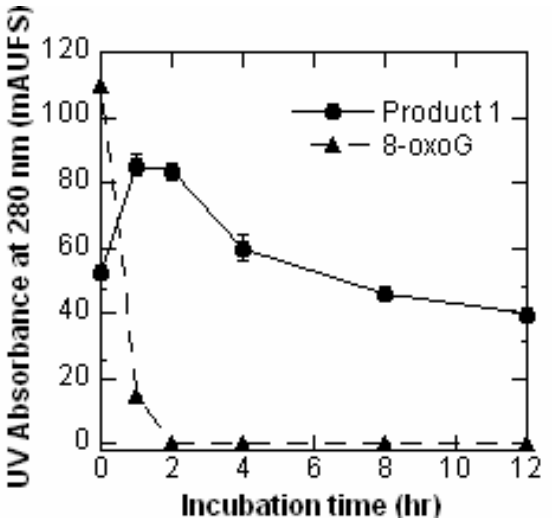


Fig. 3 Concentration of Product 1 (solid) and 8-oxoGua (dashed) for 0 – 8 hr incubation of 8-oxoGua with $FeSO_4$ and H_2O_2.

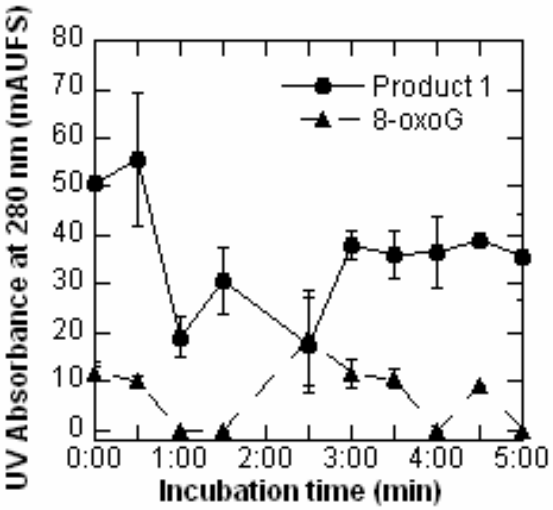


Fig. 2 Concentration of Product 1 (solid) and 8-oxoGua (dashed) for 0 – 5 min incubation of 8-oxoGua with $FeSO_4$ and H_2O_2.

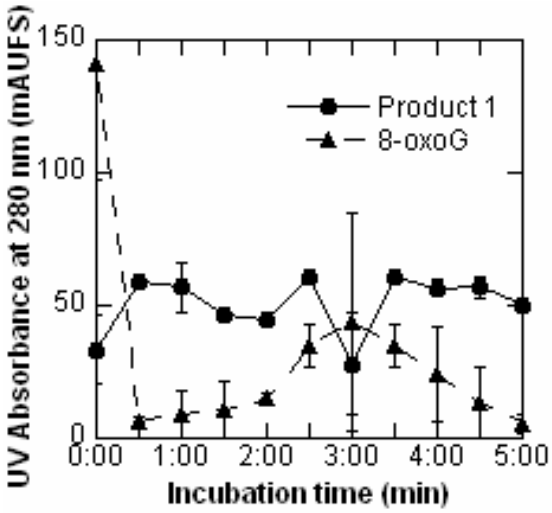


Fig. 4 Concentration of Product 1 (solid) and 8-oxoGua (dashed) for 0 – 5 minutes incubation of 8-oxoGua with $CuSO_4$ and H_2O_2.

Results

Oxidation of 8-oxoGua.

8-OxoGua was incubated with $FeSO_4$ and H_2O_2, and samples were taken every 30 seconds from 0 to 5 min. Fig. 1 shows the UV chromatograms of 8-oxoGua incubated for 0 min and for 5 min with the Fenton reagents. There was a dramatic decrease in the concentration of 8-oxoGua, which was accompanied by a new peak at 4.6 min (Product 1).

Product 1 was immediately generated upon incubation with iron and H_2O_2, and its concentration increased as the concentration of 8-oxoGua decreased. The concentration of 8-oxoGua decreased with increasing incubation time with the Fenton reagents, as previously found.[29] The concentration of Product 1 increased with increasing incubation time, as shown in Fig. 2.

As shown in Fig. 3, Product 1 continued to be generated as the incubation time with $FeSO_4$ and H_2O_2 increased up to 12 hours. After 2 hours, 8-oxoGua was no longer detected. The concentration of Product 1 appeared to be approaching a steady state concentration from 4 to 12 hours incubation with the Fenton reagents.

In the 8-oxoGua incubations with $CuSO_4$ and H_2O_2, Product 1 was again immediately generated and its concentration was observed to increase with increasing incubation time, as with the iron incubations, although this increase was not linear, as shown in Fig. 4. The overall trend of increasing concentration of Product 1 with a corresponding decrease in 8-oxoGua, along with similar magnitudes of Product 1 generated, were observed in both studies.

As with the iron analysis, 8-oxoGua was then incubated with $CuSO_4$ and H_2O_2 for incubation periods of up to 12 hr, to investigate whether Product 1 was the final product of 8-oxoGua oxidation, or would itself be further oxidised and so decrease in magnitude. Product 1 continued to be generated as the incubation time with $CuSO_4$ and H_2O_2 increased up to 12

hours. From 8 to 12 hours, the level of Product 1 appeared to be reaching a steady state. As was observed for the shorter incubation period, the same magnitude of Product 1 was observed in both cases, with no 8-oxoGua detected as the incubation time increased. (data not shown)

HPLC-ESI ion trap MS was used to identify the nature of Product 1 in both the iron and copper mediated Fenton oxidation of 8-oxoGua. For 8-oxoGua oxidised by both metals, analysis in the positive ion mode revealed 2 ions, the protonated molecular ion at *m/z* 156.1 and the sodium adduct ion at *m/z* 178.1. ESI-MSMS of the ion *m/z* 156.1 resulted in fragments of 139.1, 113.1 and 114.1, as illustrated in Fig. 5.

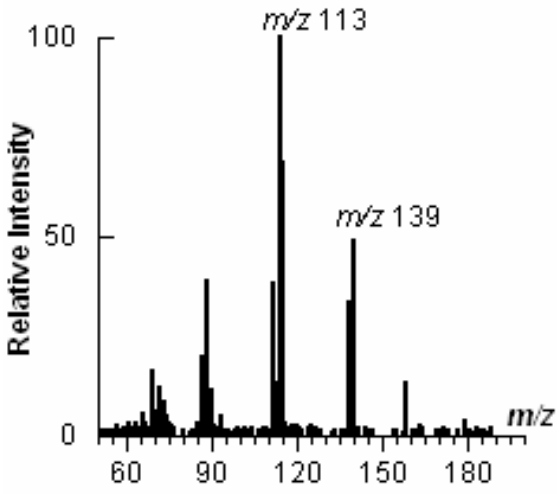
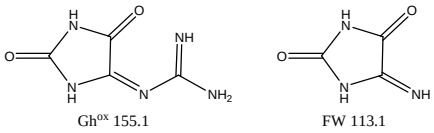


Fig. 5 ESI-MSMS of ion *m/z* 156.1 (Gh^{ox}, which elutes at 4.6 min) showing fragments 139.1, 113.1 and 114.1.

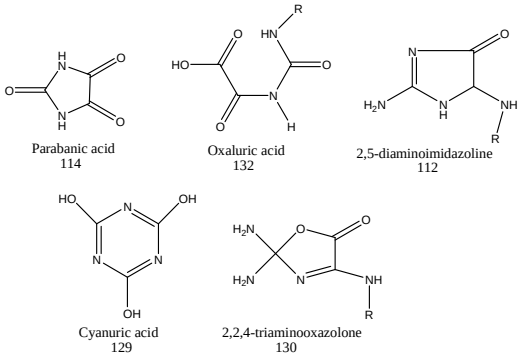
The expected oxidation products of 8-oxoGua were Gh or Sp, which have a molecular weight of 157 and 183 respectively. Product 1, however, was found to have a molecular weight of 155.1, 2 units less than Gh. Product 1 was therefore proposed to be oxidised Guanidinohydantoin, Gh^{ox}, based on previous studies which indicated that this compound, which has been shown to be unusually stable, was a main product of 8-oxoGua oxidation.[11] Gh^{ox} has a molecular weight of 155.1, as shown in Scheme 2. The fragment ion *of m/z* 139.1 represents the loss of NH_3 from Gh^{ox}. The fragment ion of *m/z* 114.1 represents compound of mass 113.1, which is also proposed in Scheme 2.



Scheme 2 Proposed structures of compound 155.1 and for fragment compound 113.1

Further oxidatively generated species.

Gh^{ox}, although quite stable, has been demonstrated to be itself an intermediate in the oxidation mechanism of 8-oxoGua, as it undergoes hydrolysis to parabanic acid and finally to oxaluric acid.[11] Therefore, although it appeared not to be decreasing in concentration with increasing incubation time, which would be expected if it were an intermediate species, incubated samples were analysed by MS for the presence of each of the further oxidatively generated species which have been outlined in literature and are shown in Scheme 3.



Scheme 3 Final oxidation products of 8-oxodGuo oxidation

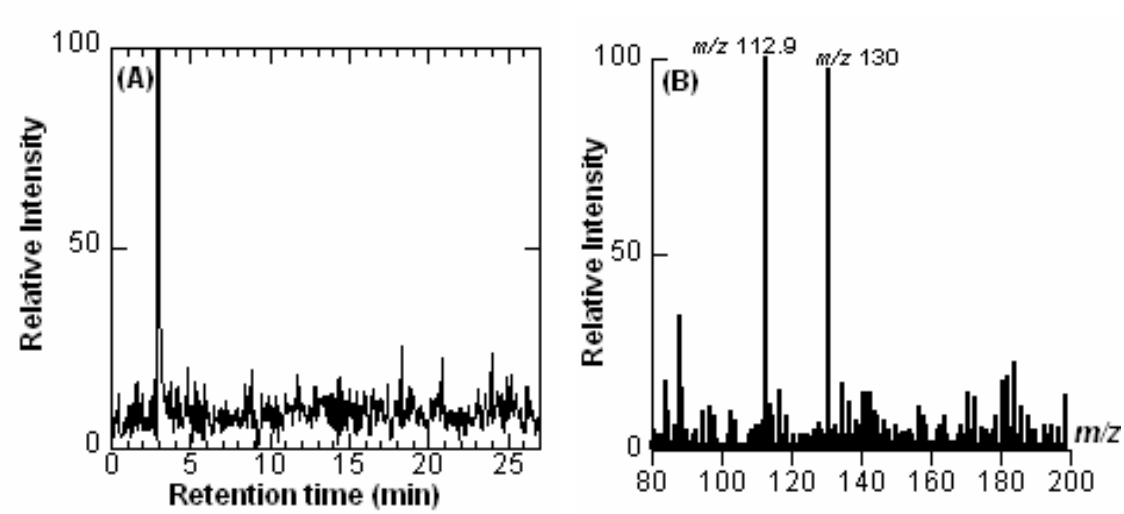


Fig. 6 (A) Extracted Ion Chromatogram of of Oxaluric acid ion generated from 8-oxoGua (*m/z* 131) incubated for 96 hours with $FeSO_4$ and H_2O_2. (B) Mass spectrum of Oxaluric acid.

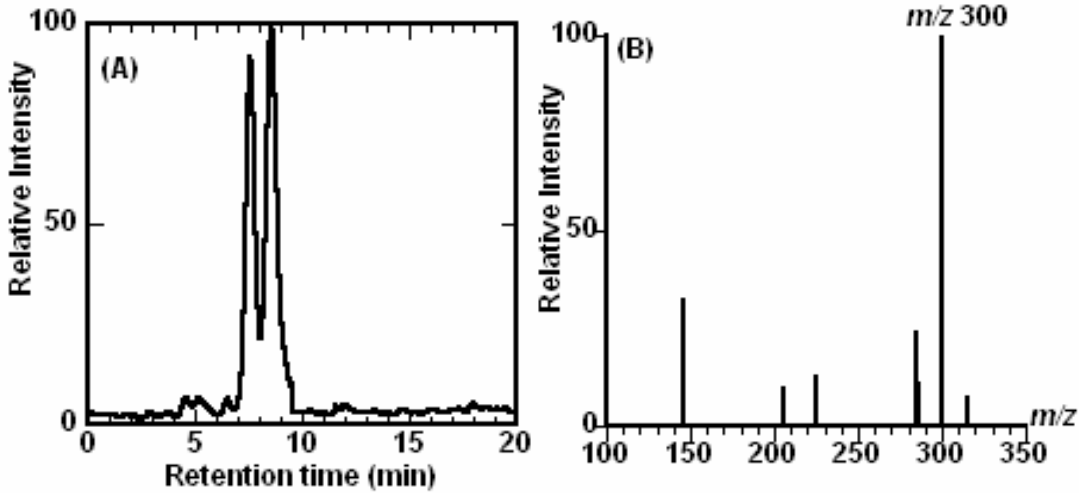


Fig. 7 (A) Extracted ion chromatogram of Sp ion generated on incubation of 8-oxodGuo (*m/z* 300) incubated with $CuSO_4$ and H_2O_2 for 5 min. (B) Mass spectrum of Sp.

8-OxoGua was incubated with either $FeSO_4$ and H_2O_2, $CuSO_4$ and H_2O_2 or water for incubation periods of up to 96 hrs. In the control samples (8-oxoGua incubated in water), no Product 1 was observed, even after 96 hr incubation. This product is therefore solely generated by the Fenton reaction.

The mass spectrometer, operating in the negative mode, was optimised using standard solutions of parabanic, cyanuric and oxaluric acid. Each of the 96 hr incubated samples were analysed for the presence of each of the species in Scheme 3 (Parabanic acid, oxaluric acid, 2,5-diaminoimidazolone, cyanuric acid and 2,2,4-triaminooxazolone). Only oxaluric acid (*m/z* 131) was detected in 8-oxoGua incubated for 96 hours with $FeSO_4$ and H_2O_2. The extracted ion chromatogram (EIC) for ion *m/z* 131 (oxaluric acid) and the corresponding mass spectrum are plotted in Fig. 6. This peak was not present in the control sample; it did, however, appear in samples incubated with $CuSO_4$ and H_2O_2; ion intensities observed were about one third lower than for corresponding iron incubations.

Oxidation of 8-oxodGuo.

The oxidation of 8-oxodGuo was carried out at pH 7, to investigate whether the oxidation product was affected by the presence of the dexoyribose moiety. 8-oxodGuo was immediately consumed, and a new product, Product 2, was immediately generated. MS analysis showed, however, that Product 2 had a *m/z* of 300, corresponding to Spiroiminodihydantoin (Sp). Gh^{ox} was not detected in 8-oxodGuo samples incubated with $FeSO_4$ or $CuSO_4$ and H_2O_2. Fig. 7 shows the extracted mass chromatogram of *m/z* 300 (corresponding to $[Sp + H]^+$) and the corresponding mass spectrum of 8-oxodGuo incubated with $CuSO_4$ and H_2O_2 for 5 min.

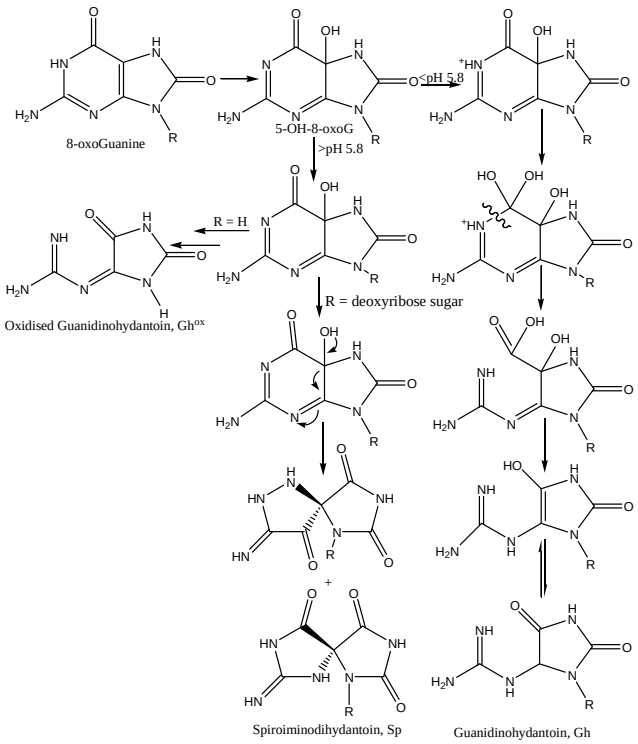
Similar incubations with $FeSO_4$ also gave two peaks corresponding to *m/z* 300, indicating that both diastereoisomeric forms of Sp were generated. The ratio of the two diastereoisomeric forms differed between the copper- and iron-mediated Fenton oxidations of 8-oxodGuo. As with 8-oxoGua, however, Sp was generated immediately on incubation of 8-oxodGuo with the Fenton reagents and similar magnitudes of the species were formed for both metals. Under analogous oxidising conditions at pH 7 therefore, 8-oxoGua oxidised to give Gh^{ox}, while 8-oxodGuo oxidised to produce Sp.

Discussion

Gh^{ox} was generated immediately on incubation of 8-oxoGua with the Fenton reagents, both when copper and iron was the metal in question, at both pH 7 and at pH 11. Oxaluric acid was identified as a final oxidation product for both metal catalysed systems.

When 8-oxodGuo was subjected to iron- and copper-catalysed oxidation, however, the oxidation product was not Gh^{ox}, but Sp. This was in line with previous studies, where its oxidation by one electron oxidants,[18] Cr(V),[19] $^1O_2$[12,44] and peroxynitrites[22,45] all resulted in Sp formation. The 8-oxoGua oxidation product with these oxidants, however, was found to be Gh when an oligonucleotide substrate was oxidised. Recently, a possible reason for the differences in oxidation product during peroxynitrite oxidation of 8-oxodGuo was proposed.[22] It was observed during this oxidation that at low pH, the formation of Gh predominated, but at higher pH, Sp yield increased at the expense of Gh. 5-OH-8-oxodGuo, the common intermediate of Gh and Sp, was found to have a pK_a of 5.8. It was proposed that below pH 5.8, 5-OH-8-oxodGuo was protonated at N7. This caused the amide group to undergo hydrolysis, followed by decarboxylation, leading to formation of Gh. Above pH 5.8, 5-OH-8-oxodGuo was deprotonated, and electrophilicity at C6 was decreased, so that attack by H_2O was not favoured, and 5-OH-8-oxodGuo rearranged to form Sp. The protonation state of 5-OH-8-oxodGuo dictated the nature of the final oxidation product of 8-oxodG oxidation. Within DNA, hydrogen bonding was thought to stabilise 5-OH-8-oxodGuo and activate C6 of 8-oxoGua towards electrophilic attack by H_2O, resulting in the formation of Gh. In the analysis of iron- and copper-mediated Fenton

reaction analysed in this study, Gh^{ox} was formed on oxidation of 8-oxoGua at pH 7, while Sp was formed on oxidation of 8-oxodGuo at pH 7. While this theory explains the formation of Gh at pH 5.5 and Sp at pH 7, it does not explain the formation of Gh^{ox} at pH 7 from 8-oxoGua while Sp is formed from 8-oxodGuo, as shown in Scheme 4. It is evident that the exact nature of the nucleobase also plays a key role in determining the final oxidation product.

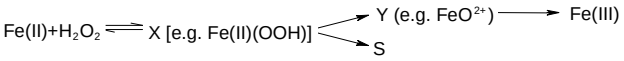


Scheme 4 Proposed mechanism, based on Ref. 22, for dependence of formation of Gh, Sp and Gh^{ox} during iron- and copper- catalysed oxidation on both pH and the nature of the nucleobase.

At pH 7, the iron- and copper-mediated oxidation of 8-oxoGua and 8-oxodGuo yielded the same oxidation products, *i.e.* Gh^{ox} and Oxaluric acid in the case of 8-oxoGua and Sp in the case of 8-oxodGuo, and similar quantities of product were detected for both metals. However, the iron catalysed oxidation of H_2O_2 and the analagous copper catalysed oxidation have been reported to generate different Reactive Oxygen Species (ROS), which have in turn been reported to generate different DNA oxidation products, as discussed in the introduction. The exact nature of the ROS involved in the oxidation of DNA and DNA bases by metal-mediated Fenton reactions, however, have been the subject of much debate and research. Iron and H_2O_2 are reported to generate ·OH via the Fenton reaction according to Reaction 1:[26]

$$H_2O_2 + Fe(II) \xrightarrow{k_1} Fe(III) + HO^- + HO\bullet \quad k_1 = 76\ lmol^{-1}s^{-1} \quad (1)$$

A recent study by Wink *et al.* questioned this generation of ·OH, however.[47] They found that the reaction of Fe(II) and H_2O_2 lead to two intermediates; neither being ·OH. Instead they proposed that the first intermediate (X) was probably a peroxo complex, *e.g.* Fe(II)(OOH), and the second intermediate (Y) an iron(IV) oxo complex, *e.g.* FeO(II). Neither intermediate was found to have the expected reactivity of ·OH. Scheme 5 outlines the mechanism proposed.



Scheme 5 Proposed mechanism for the reaction of Fe(II) and H_2O_2.[47]

A subsequent publication examined the feasibility of the Fenton reaction on thermodynamic grounds.[30] It found that the familiar outer sphere mechanism used to represent the Fenton reaction (Reaction 1) to be thermodynamically extremely unfavourable and therefore unlikely to occur. An inner sphere mechanism where a ferrous peroxide complex was generated was deemed more likely to occur. The iron-oxo complex generated by the Fenton reagents was proposed to behave as a localised or 'bound' ·OH, with preferential interaction of iron with the N7 of guanine within DNA.[48]

The DNA damage induced by Cu(II) and hydrogen peroxide has also been investigated.[31] It was concluded that Cu(II) was the species which bound to DNA but that Cu(I) participated in the DNA damage. Scavenging reactions indicated that ·OH was not the primary oxidatively generated species, but a "bound ·OH", such as a copper-peroxide complex may be involved. In a similar manner $^1O_2$ was ruled out as a primary reactive species, but a "bound $^1O_2$", again a copper-peroxide complex, could cause the damage recorded. A publication by Frelon *et al.*[49] analysed the degradation of DNA bases by copper in the presence of H_2O_2 and again concluded that the ROS species involved was not ·OH. Instead the authors concluded that $^1O_2$ was instead predominantly responsible for the oxidatively generated damage that occurred, with $^1O_2$ being generated *in situ* via a DNA-copper-hydroperoxo complex. Recently, a reaction mechanism for the oxidation caused by the Cu(I)/H_2O_2 system was proposed in the literature.[33] It was proposed that Cu(I) reacted with H_2O_2 via the Fenton reaction to yield ·OH, in contradiction to the studies outlined above, which concluded that Cu(I) with H_2O_2 yielded a copper-hydroperoxo complex. It also proposed, however, that the copper-hydroperoxo complex, Cu(I)•OOH, was an integral part of the reaction mechanism.[33]

A recent quantum chemical study of the interactions between Cu(II) and DNA found that the coordination of Cu(II) to the GC pair within the DNA double helix induced an oxidation of GC and a concurrant reduction of Cu(II) to Cu(I).[50] The ability of Cu(II) to oxidise the GC pair depended on the immediate environment of the GC pair. To investigate whether Cu(II) could coordinate in a similar manner to 8-oxoGua, a concentrated solution of Cu(II) was added to 8-oxoGua. Immediately the solution changed from blue to green and a precipitate was formed. The colour change indicated a reduction of Cu(II) to Cu(I). The concurrant oxidation of 8-oxoGua would lead to 8-oxoGua$^{\bullet+}$, the 8-oxoGua radical cation, which is readily further oxidised. Significant amounts of Gh^{ox} were not observed, however, until H_2O_2 was also

added to the solution. These results indicate, however, that the oxidation of 8-oxoGua to Gh^{ox} was initiated by the Cu(II) induced one electron oxidation of 8-oxoGua to 8-oxoGua$^{\bullet+}$. Therefore one electron oxidation, and not an ROS generated by copper catalysed H_2O_2 decomposition initiated 8-oxoGua oxidation to Gh^{ox}, so that any ROS generated may not have been primarily responsible for 8-oxoGua oxidation. Iron is also reported to complex readily with G.[51] When a concentrated iron solution was incubated with 8-oxoGua (analagous to concentrated copper incubation), an immediate colour change and precipitate were observed. This iron-8-oxoGua complex may possibly have behaved similarly to the copper-8-oxoGua complex, also forming the reactive intermediate 8-oxoGua$^{\bullet+}$. Therefore the same reactive intermediate, 8-oxoGua$^{\bullet+}$, may have been generated by both the Fe(II) and the Cu(II) catalysed Fenton reacction. At pH 7 and pH 11, both iron and copper catalysed oxidation of 8-oxoGua generated Gh^{ox} immediately, with Oxaluric acid identified as an end product of the oxidation. Similar quantities were detected in both cases. At pH 7, both iron and copper catalysed oxidation of 8-oxodGuo leads to Sp, again in similar quantitites. Both iron and copper were observed to complex immediately with 8-oxoGua. This complexation may have lead to the same reactive intermediate, namely 8-oxoGua$^{\bullet+}$, being generated. This may be the reason why the same products were formed, in similar quantities, for both metal catalysed systems. The fact that copper and iron are reported to generate different ROS during the Fenton reaction does not influence the nature of the final oxidation product, as the ROS generated do not initiate 8-oxoGua oxidation.

This hypothesis of one electron oxidation may be tested in a number of ways. One method to investigate the hypothesis that one electron oxidation is the primary oxidaiton method could involve comparing the the observed degradation pattern to that detected by a well known one electron oxidant, such as photoexcited riboflavin. This analysis would serve to further investigate whether one electron oxidation is indeed responsible for the oxidatively generated damage. Whether singlet oxygen ($^1O_2$) or the hydroxyl radical (·OH) was also involved could also be investigated further. $^1O_2$ specifically oxidises the DNA base guanine. Therefore the presence of oxidatively generated thymine products would allow $^1O_2$ to be excluded as the primary oxidation mechanism. These products could indicate that ·OH was involved in oxidation. ·OH reacts via H addition to the 2'-deoxyribose moiety of the nucleoside causing the cleavage of DNA bases in the case of nucleosides, and strand breaks when DNA is involved. The absence of nucleobases and strand breaks could then be used to confirm whether or not ·OH was involved in the oxidation.

The results of 8-oxoGua oxidation carried out during this investigation indicated that both copper and iron generate the same 8-oxoGua oxidation product (Gh^{ox}), and quantitative analysis showed that a similar magnitude of Gh^{ox} was generated by both metal systems. 8-oxodGuo oxidation yielded Sp as the oxidation product of both copper- and iron-mediated oxidation, with a similar magnitude of Sp generated by each system. On the basis of the previous investigations outlined above, it is proposed that the ROS generated by the iron and copper reactions are not responsible for the initiation of oxidatively generated damage to 8-oxoGua. Instead 8-oxoGua oxidation is initiated by metal complexation to 8-oxoGua, which generates the reactive intermediate 8-oxoGua$^{\bullet+}$. This intermediate, upon the addition of H_2O_2, leads to the formation of Gh^{ox} in the case of 8-oxoGua, and Sp in the case of 8-oxodGuo. The difference in end product is not determined by the metal species, or by the ROS generated from the metal catalysed Fenton reaction, but by the presence of the 2'-deoxyribose moiety.

Conclusions

8-OxoGua was oxidised by iron and copper mediated Fenton oxidation at pH 7 and at pH 11, all of which resulted in the immediate formation of oxidised Guanidinohydantoin (Gh^{ox}). Analogous 8-oxodGuo oxidation at pH 7 lead to the formation of Spiroiminodihydantoin (Sp). Oxaluric acid was identified as a minor product of 8-oxoGua oxidation after 96 hr incubation with copper and H_2O_2 and with iron and H_2O_2. It was proposed that both iron and copper could have lead to the formation of the radical cation intermediate 8-oxoGua$^{\bullet+}$. The formation of this common intermediate is a possible cause for the generation of the same products in similar quantities for both metal systems.

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Blánaid White,[a] Maricar C.Tarun,[b] Nicholas Gathergood,[a] James F. Rusling,[b, c] and Malcolm R. Smyth[a,*]

[a] National Centre for Sensor Research (NCSR), School of Chemical Sciences, Dublin City University, Dublin 9, Ireland. Fax: +353 1 836 0830; Tel: +353 1 700 7869; E-mail: Malcolm.Smyth@DCU.ie

[b] Dept. of Chemistry, University of Connecticut, U-60, 55 N. Eagleville Road, Storrs, Connecticut 06269-3060, USA

[c] Department of Pharmacology, University of Connecticut Health Center, Farmington, CT 06032, USA

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