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# Sources of 2,5-diaminoimidazolone lesions in DNA damage initiated by hydroxyl radical attack

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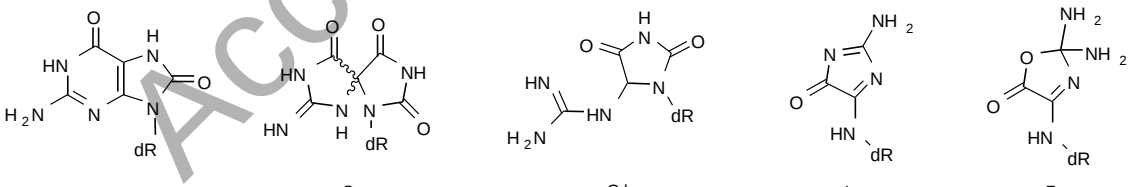
Abstract

The present study reports radiation-chemical yields of 2.5-diaminoimidazolone (Iz) derivatives in X-irradiated phosphate-buffered solutions of guanosine and double-stranded DNA. Various gassing condition (air, $N_20/O_2$ (4:1), $N_2O$, vacuum) were employed to elucidate the contribution of several alternative pathways leading to Iz in reactions initiated by hydroxyl radical attack on guanine. In all systems Iz was identified as the second by abundance guanine degradation product after 8-oxoguanine, formed in 1:5 (guanosine) and 1:3.3 (DNA) ratio to the latter in air-saturated solutions. Experimental data strongly suggest that addition of molecular oxygen to the neutral guanine radical G(-H)$^\bullet$ plays a major in Iz production in oxygenated solutions of double-stranded DNA while in other systems it may compete with recombination of G(-H)$^\bullet$ with superoxide and/or alkyl peroxyl radicals. The production of Iz through hydroxyl radical attack on 8-oxoguanine was also shown to take place although the chemical yield of Iz (ca 6%) in this process is too low to compete with the other pathways. The linearity of Iz accumulation with dose also indicates a negligible contribution of this channel to its yield in all systems.

**Keywords**: hydroxyl radical, guanine, oxidation, diaminoimidazolone, DNA, radiation

## I. Introduction

Oxidative damage to DNA bases has been the subject of active research over the past three decades with the progress in the area extensively reviewed in von Sonntag's book (*1*) and numerous reviews published thereafter (*2-5*). Guanine has long been recognized as the most prone to oxidation DNA base due to its low standard reduction potential of +1.29 V at pH 7 (*6*), and a variety of potentially mutagenic products have been shown to originate from this source. The most well-known of them, 8-oxo-7,8-dihydroguanine (8-oxoG), easily undergoes further oxidation to form a radical cation (*7*) that further leads to a large group of derivatives including, among others, deoxyribosyl-substituted spiroiminohydantoin (Sp) (*8*), guanidinohydantoin (Gh) (*9*), isomerization/oxidation products of the latter (*10*), 2,5-diaminoimidazolone (Iz) and its hydrolysis product 2,2,4-triaminooxazolone (Z) (*11,12*). The latter two products are also formed in a separate oxidation channel that does not involve 8-oxoG as an intermediate (*13-15*).




The relative role of the reaction channels leading to Iz and Z, and their overall significance in oxidative damage to guanine are not well understood. In a quantitative study by Cui et al. (*16*) oxazolone Z has been identified as the second by abundance after 8-oxoG in double-stranded DNA, oxidized with peroxynitrite, singlet oxygen, and γ-radiation, although in much smaller quantities. In other studies, however, Iz has been identified as a major reaction product (*17,18*), and our own preliminary observations also indicated much higher relative yields of this product in X-irradiated solutions of double-stranded DNA. Some of the problems associated with comparing Iz yields from different studies

is the poor understanding of reaction conditions that favor its formation, and the uncertainty regarding the nature of oxidant required in the process. It is generally agreed that the precursor to Iz is a neutral guanine radical G(-H)$^{\bullet}$, which is further oxidized by molecular oxygen (*19-21*), or recombines with either $O_2^{\bullet-}$ (*22-24*), or alkyl peroxyl radicals $RO_2^{\bullet}$ (*25*), or both (*26*). These three forms of oxygen may be present in variable ratios depending on reaction conditions, therefore affecting Iz yields. Second, although oxidation of 8-oxoG has been shown to lead to Iz among other products as mentioned above, the efficiency of this pathway and its contribution to Iz production in course of oxidative DNA damage remain uncertain.

The issues we are addressing in the present publications are i) the absolute and relative yields of Iz in hydroxyl-radical induced damage to the guanine moiety under various gassing conditions; ii) the roles of different forms of reactive oxygen species in its production, and iii) the extent to which further oxidation of 8-oxoG contributes to the production of Iz. Both monomeric guanine derivatives and double stranded DNA were included in our study. The distinctive features of our approach are the use of more direct procedures for quantification of Iz that exclude lengthy intermediate workout, and keeping track of material balance in system whenever it is possible. For the sake of clarity all non-specific derivatives containing guanine, 8-oxoguanine and 2,5-diaminoimidazolone functional groups will generally be abbreviated as G, 8-oxoG, and Iz, respectively in the future discussion. Specific derivatives will be abbreviated as introduced below.

## 2. Materials and Methods

***Materials.*** Commercially available guanosine (Guo) from Sigma and 2´-deoxyguanosine (dGuo) (TCI America) were purified by crystallization from water before use. 8-oxo-2´-deoxyguanosine (8-oxodGuo) was synthesized through copper(II)-catalyzed oxidation of dGuo with hydrogen peroxide in the presence of ascorbic acid as described in the literature (*27*). The product was isolated from the reaction mixture by solid phase extraction using of Strata-X cartridges (Phenomenex) and purified by reverse-phase HPLC on a Luna C18 10 mm × 250 mm column (Phenomenex) washed isocratically with 0.1% acetic acid/7% acetonitrile running phase at a 2 ml/min flow rate. Double-stranded salmon testes DNA (dsDNA, formerly Type III from Sigma) and all other commercially available reagents mentioned in this manuscript were used as received.

***Sample preparation and irradiation***. Room-temperature solutions containing Guo (1 mM), dGuo (2-4 mM), 8-oxodGuo (0.6 mM) or dsDNA (2.3 mM in base pairs) in 50 mM phosphate buffer (pH 6.9) were irradiated by X-rays generated by a Phillips tube with tungsten anode operated at 60 kV and a 20 mA current. The dose rates delivered to the samples were from 4.2 to 9.4 Gy/s depending on the experimental setup, as determined by Fricke dosimetry. Oxygen-containing samples were either air-saturated or bubbled with a pre-made $N_2O/O_2$ (4:1) mixture for 15 min prior to irradiation, and kept equilibrated with the corresponding gas phase during irradiation. Fully degassed samples were prepared in 1.5-ml glass ampoules by means of freeze-pump-thaw procedure with subsequent sealing. Nitrous oxide -saturated samples were prepared the same way, except a controlled amount of $N_2O$ was condensed into the ampoules before the final evacuation and sealing step. The $N_2O$ pressure in the samples prepared this way was close to 1 bar at room temperature.

***Product analysis***.

*Monomeric precursors*. Irradiated solutions of Guo, dGuo and 8-oxodGuo were analyzed immediately after irradiation by reverse-phase HPLC (Shimadzu UFPC) on a Gemini C18 4.6 mm × 250 mm column (Phenomenex) washed with 40 mM ammonium acetate at a 1 ml/min flow rate at 30°C. A linear acetonitrile gradient (0-16% MeCN over 15 min) was applied. 5-Fluorouracil ($\varepsilon_{254}$ = 5500 $M^{-1}cm^{-1}$) was employed both as an external and in some experiments as an internal reference. Quantification of 8-oxoGuo and 8-oxodGuo was performed at 293 nm using the literature value of the extinction coefficient $\varepsilon_{293}$ = 10300 $M^{-1}cm^{-1}$(*28*). The extinction coefficient of free guanine (Gua) was measured directly using the authentic compound ($\varepsilon_{254}$ = 9280 $M^{-1}cm^{-1}$).

The well-known UV spectrum of the Iz chromophore with $\lambda_{max}$ at 320 nm and 253 nm (*14,20,29*) was employed for identification and quantification of all imidazolone derivatives. The extinction coefficient at 253 nm was estimated from relative band intensities in the UV spectra of two conjugates containing this and another chromophore with distinct and well-characterized absorption features. Employed for that purpose were β-(4-hydrophenyl)ethyl and β-(indol-3′-yl)ethyl chromophores, responsible for the absorption of tyrosine and tryptophan in near UV, respectively. The conjugates were synthesized by treatment of 2-amino-5-(2′-deoxyribose-1′-yl)aminoimidazolone (dIz) with tyramine (Tyr) and tryptamine hydrochlorides (0.2 M), both from Sigma, in aqueous sodium acetate (0.2 M) at 50°C for 15-20 min following the procedure described for aliphatic amines (*30,31*). The UV spectrum of the Tyr-Iz conjugate thus obtained, and its reconstruction from the spectra of individual non-interacting chromophores is shown in Figure 1. A similar result was obtained with tryptamine (data shown in the Supplementary Material section). Based on these reconstruction and the literature extinction coefficients of tyrosine and tryptophan ($\varepsilon_{280}$ = 1480 $M^{-1}cm^{-1}$ and 5540 $M^{-1}cm^{-1}$, respectively (*32*)) the averaged value of $\varepsilon_{253}$ = 1.5×10$^4$ $M^{-1}cm^{-1}$ for the Iz chromophore was obtained. We note that this estimate is somewhat lower than the 19900 $M^{-1}cm^{-1}$ value reported previously (*29*).

*dsDNA*. Irradiated solutions of DNA were treated with ethanolamine acetate (0.2 M in final solution) for 30 min at 45°C, followed by precipitation of the DNA with protamine sulfate (ca 220 μg/ml in final mixture) and centrifugation. The supernatant was analyzed as described in the previous section to determine the concentrations of free bases and 2-amino-5-(β-hydroxyethyl)aminoimidazolone (EIz) formed in reaction of DNA-incorporated Iz with ethanolamine with a 60-80% yield (*31*). DNA-incorporated 8-oxoG was quantified as free 8-oxoguanine (8-oxoGua) in the hydrolysates obtained using a standard procedure of formic acid DNA hydrolysis (88% formic acid for 90 min at 150°C under vacuum) following the same protocol as described previously (*30*).

**3. Results**

*Radiation-induced damage to Guo*. A typical chromatogram produced by irradiation of Guo (1 mM) in air-equilibrated phosphate buffer is shown in Figure 2. Three out of four major products are readily identifiable as 2-amino-5-(ribose-1′-yl)aminoimidazolone (rIz), free guanine (Gua) and 8-oxoGuo by their characteristic UV spectra and by comparison with an authentic reference compound (Gua). An unidentified product X has the UV-spectrum identical to that of Guo, and, therefore, likely contains a modified ribose moiety. Dose response curves for Guo loss and Iz accumulation at different gassing conditions are shown in Figure 3, and the corresponding radiation-chemical yields *G* are reported in Table 1. Dose dependencies for Gua and 8-oxoGuo production were also obtained and can be found in the Supplementary Materials section. While the release of Gua in all systems is reasonably linear over the entire dose range, the accumulation of 8-oxoGuo shows a tendency to saturation at higher doses, which is especially pronounced in $N_2O$-containing samples. As a result, only low-dose parts of the dose dependencies, assumed to be linear, were used to determine the yields of 8-oxoGuo in the presence of $N_2O$. The *G*-values thus obtained are also listed in Table 1.

The numbers in the Table clearly identify rIz as the second by abundance product after 8-oxoGuo with the rIz/8-oxoGuo ratio about 0.2 in samples irradiated under air. It may also be concluded that altogether the pathways leading to products listed in the Table account for the majority (from 69 to 95%) of the processes responsible for Guo destruction. Among them the production of rIz accounts for up to 18% of the total Guo loss (under $N_2O/O_2$ mix) that definitely makes this reaction channel worth of paying attention to.

*dsDNA*. A typical chromatogram showing the peaks of EIz and free DNA bases in the supernatant obtained by post-irradiation treatment of phosphate-buffered solutions of DNA irradiated under oxic conditions is shown in Figure 4. An extra peak denoted as Lac belongs to 1-(β-hydroxyethyl)-5-methylene-$\Delta^3$-pyrrolin-2-one formed in reaction of ethanolamine with C4′-oxidized abasic sites (*33*). Preliminary tests showed that the release of EIz is below reliable detection limit under anoxic conditions both in the absence and the presence of $N_2O$, and these systems were excluded from further consideration. A representative set of dose response curves for free base, EIz, and 8-oxoG production in solutions of DNA saturated with air or $N_2O/O_2$ mix are shown in the insert. The *G*-values estimated from initial slopes of the curves are reported in Table 1 (the last two entries). The *G*-value for Iz production in DNA was calculated from the yield of EIz using the scaling factor of 1.7 obtained in the previous publication (*31*). This scaling factor accounts for partial hydrolysis of Iz in parallel with the formation of EIz upon post-irradiation treatment of irradiated DNA with ethanolamine. The data show that the Iz/8-oxoG ratios found in dsDNA (0.3 under air, in particular), are not much different from those measured with Guo as a target. The latter number, however, is roughly 10 times greater than the Z/8-oxoG ratio (0.03) found by Cui et al. (*16*) in calf thymus DNA under similar conditions. Since the formation of Z from Iz is generally considered to be quantitative, the source of such a significant disagreement remains unclear. Other yet unidentified factors, therefore, may be involved.

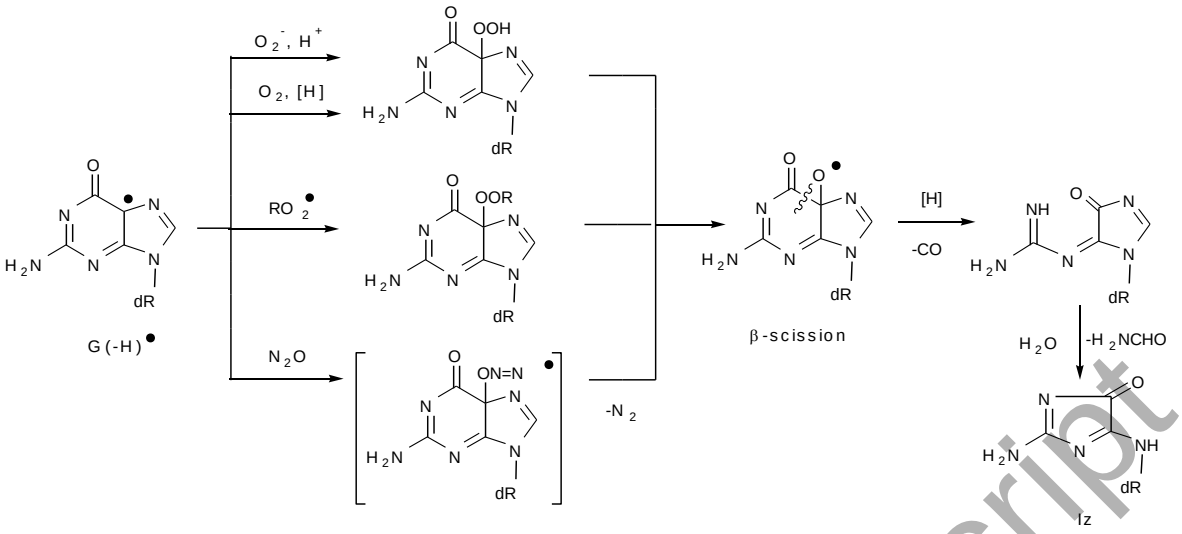
*Imidazolone yield in reaction of hydroxyl radicals with 8-oxodGuo*. Irradiation of 8-oxodGuo in a phosphate-buffered aerated solution results in accumulation of dIz along with free 8-oxoGua and a set of products whose identification was not attempted (Figure 5). Previously characterized deoxyribosyl-substituted Sp (*8*), Gh (*9,10*), and other related derivatives are likely present among those with retention times shorter than 5 min under reverse phase separation conditions.

Dose dependencies for both 8-oxodGuo destruction and dIz accumulation are approximately linear (insert in Figure 5) with the corresponding *G*-values of 138 and 7.8 μmol $J^{-1}$, respectively. These results identify dIz a minor product of 8-oxodGuo destruction by $HO^{\bullet}$, formed with ~ 6% chemical yield. Linearity of the dose response curve for dIz accumulation is also inconsistent with the involvement of a long lived (on the experimental timescale) intermediate precursor to dIz. This makes neither of the above mentioned stable oxidation products suitable as intermediates in hydroxyl radical-induced formation of dIz from 8-oxodGuo.

**4. Discussion**

There is a consensus in the literature that the major outcome of hydroxyl radical attack on guanine (60-70% according to Candeias and Steenken (*34*)) is the neutral guanine radical $G(-H)^{\bullet}$ that formally results from the loss of $N_1$-hydrogen, although different views have been expressed on whether its immediate precursor is a $C_4$-hydroxyl radical adduct (*34*), an ion pair formed by the guanine radical cation and $HO^-$ (*35*), or an $N_2$-aminyl radical (*36,37*). For the purpose of our discussion it is important to note that regardless of the intermediates involved the formation of $G(-H)^{\bullet}$ is not affected by molecular oxygen in sub-millimolar concentrations available in air-saturated solutions (*34*). This fact makes $G(-H)^{\bullet}$ the most abundant precursor to imidazolone derivatives under either oxic or anoxic conditions. Another reaction pathway that accounts roughly for 17% of events, is the $C_8$-adduct formation that further leads to 8-oxoguanine in oxygenated media, which may also form imidazolone upon further oxidation. The non-linear dose response for 8-oxoG accumulation observed in solutions of both Guo and DNA does indicate its further degradation likely in reaction with $HO^{\bullet}$, $G(-H)^{\bullet}$ (*38*), or mobile holes in DNA (*39*). However, the linearity of Iz accumulation in these systems is inconsistent with the involvement of a long-lived intermediate, that makes the participation of 8-oxoG in this process unlikely. In addition, the low chemical yield of dIz in hydroxyl radical-initiated oxidation 8-oxodGuo (ca.6%) measured directly makes this particular pathway even less feasible.

As follows from the data presented in Table 1, the production of Iz is obviously enhanced in the presence of molecular oxygen. However, at least in the case of Guo it also occurs with appreciable efficacy under strictly anoxic conditions if $N_2O$ is present in the system. Electron scavengers are known to increase the yield of radiation-produced hydrogen peroxide in aqueous solutions (*40*), which may act as a source of superoxide under anoxic conditions. To test this possibility an anoxic solution of Guo (1 mM) in 50 mM phosphate containing $H_2O_2$ (0.18 mM) was irradiated and analyzed as other similar systems. This amount of $H_2O_2$ corresponded to its expected concentration in a medium with the scavenging capacity of $2\times10^6$ $s^{-1}$ after a 2.7 kGy dose (*41*). No effect on rIz production was observed, which remained very low regardless of the presence of $H_2O_2$ as shown in the Supplementary Materials section. This fact clearly demonstrates the existence of pathways leading to Iz that do not require the involvement of peroxyl-type intermediates at any step. Direct involvement of $N_2O$ in oxidation of free radical intermediates may, therefore, be considered. The scheme that summarizes hypothetical reaction pathways from the literature (*20-22,24,25*) with some additions and modifications is shown in the Scheme below.




From the top three pathways shown in the Scheme, the recombination of G(-H)$^{\bullet}$ with $O_2^{\bullet-}$ can hardly play a significant role in air-saturated 1 mM solutions of Guo due to the low expected yield of superoxide anion in this system. The rate constant of electron attachment to dGuo ($k$ = 1.7×10$^{10}$ M$^{-1}$s$^{-1}$ (*42*)) is practically the same as to $O_2$ (1.8×10$^{10}$ M$^{-1}$s$^{-1}$ (*43*)) and, therefore, dissolved oxygen could scavenge only about 16% of radiation-produced electrons due to a 5-fold difference in concentrations of the scavengers (1 mM Guo vs 0.2 mM $O_2$ in air-saturated solutions). The guanine radical anion formed in the process undergoes rapid C-protonation to form a neutral radical, which is unreactive towards $O_2$ (*44*) and, therefore, cannot give rise to $O_2^{\bullet-}$. The situation, however, should be different in dilute (2.3 mM in base pairs) solutions of DNA since the effective rate of solvated electron attachment to DNA ($k$ = (1.0÷1.4)×10$^{8}$ M$^{-1}$cm$^{-1}$, DNA concentration in bases (*45,46*)), is two orders of magnitude slower than to Guo. As a result, 84% of the solvated electrons must be scavenged by $O_2$ leading to $O_2^{\bullet-}$. This process, however, should be completely suppressed by $N_2O$ in solutions saturated with $N_2O$:$O_2$ = 4:1 mix, which contains about 22 mM of $N_2O$ under ambient temperature and pressure, and converts solvated electrons into HO$^{\bullet}$ in a diffusion-controlled process ($k$ = 9.1×10$^{9}$ M$^{-1}$s$^{-1}$ (*47*)). Therefore, the fact that no suppression in Iz production takes place in the presence of $N_2O$ (actually the opposite is observed) suggests a negligible role of G(-H)$^{\bullet}$ + $O_2^{\bullet-}$ recombination as a source of Iz in polymeric DNA as well.

Regarding the other two oxygen-dependent pathways, it remains uncertain which one (the recombination of G(-H)$^{\bullet}$ with $RO_2^{\bullet}$ or direct addition of $O_2$ to G(-H)$^{\bullet}$ ) plays the major role in the case of Guo. Substantial yields of free guanine indicate the presence of a significant source of ribose-based radicals R$^{\bullet}$, and, therefore, of $RO_2^{\bullet}$ in oxygenated systems. Both pathways are expected to be enhanced in the presence of $N_2O$ (as observed) due to the simultaneous increase in production of R$^{\bullet}$ and G(-H)$^{\bullet}$. The same can take place (and is also observed experimentally) in solutions of polymeric DNA. There is a reason to believe, however, that recombination of G(-H)$^{\bullet}$ with $RO_2^{\bullet}$ in DNA should be far less efficient than in solutions of Guo. Peroxyl radicals formed in this system are polymeric, therefore, are far less mobile than monomeric Guo-derived species. Based on this logic and the fact that Iz formation in $N_2O$-saturated solutions of DNA still occurs with the efficiency similar to that in Guo may serve as an indication of direct addition of $O_2$ to G(-H)$^{\bullet}$ is a major contributor to Iz production in DNA. A potential problem with this pathway is the relatively low reactivity of G(-H)$^{\bullet}$ towards molecular oxygen ($k \leq 10^6$ M$^{-1}$s$^{-1}$) reported by Candeias and Steenken (*34*) although the possibility of this reaction has not been

ruled out. In particular, it may become competitive in the absence of reducing agents and recombination reactions as in the case of polymeric DNA in inert medium.

The $H_2O_2$-independent formation of rIz in $N_2O$-saturated solutions of Guo under strictly anoxic conditions suggests that $N_2O$ itself can possibly act as an oxidant of G(-H)$^\bullet$ . Nitrous oxide is known to react with metal ions in low oxidation states through oxygen atom transfer with appreciable rates (for reactions with $Zn^+$, $Cd^+$, $Co^+$ and $Ni^+$ see, for example, ref. (*48*)). It reacts in a similar way with the hydrogen atom (*49*) but there is practically no information on reactivity of $N_2O$ towards carbon-based free radicals. A few reported examples include the reaction with photolytically generated phenyl radicals (*50*), and the oxidation of 2′-deoxyuridine-1′-yl radical with the rate constant of $1.77\times10^8$ $M^{-1}s^{-1}$ (*51*). A hypothetical pathway for oxidation of carbon-based radicals by $N_2O$, shown in the scheme, is through addition-elimination, which in the case of G(-H)$^\bullet$ would lead to an alkoxyl radical, identical to the precursor to Iz in the process initiated by recombination of G(-H)$^\bullet$ and $O_2^{\bullet-}$. Alternatively, an iminoxyl-type intermediate R-N=N-O$^\bullet$ has also been considered (*51*). In dsDNA, however, the yield of Iz turned out to be negligible under anoxic conditions even in the presence of $N_2O$. The reason for that could be restricted accessibility of dissolved $N_2O$ to G(-H)$^\bullet$ in double-stranded DNA.

**5. Conclusion**

The present study identifies Iz as the second by abundance (after 8-oxoG) product of hydroxyl radical-induced damage to guanine, although the relative yields were found to be much higher than reported earlier. Besides recombination with superoxide and peroxyl radicals, experimental data strongly suggest the involvement of direct addition of molecular oxygen to G(-H)$^\bullet$ as a source of this end product, especially in double-stranded DNA. There is an indication that nitrous oxide can modify free radical processes in DNA, including those leading to imidazolone formation, not only through scavenging of solvated electrons, but also through direct oxidation of free radical intermediates. Secondary oxidation of 8-oxoguanine by HO$^\bullet$ can only be a minor source of Iz in radiation-induced damage to DNA and its constituents.

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**7. Disclosure of interest**

The authors report no conflict of interest.

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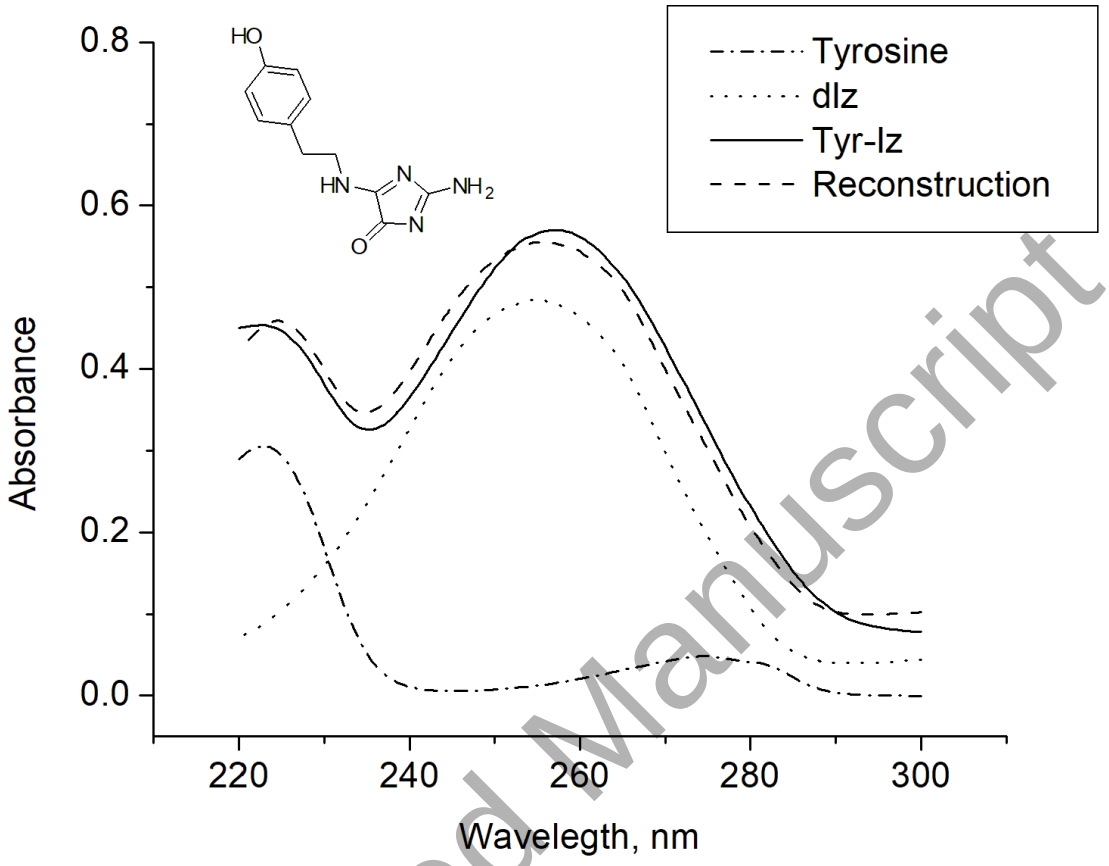
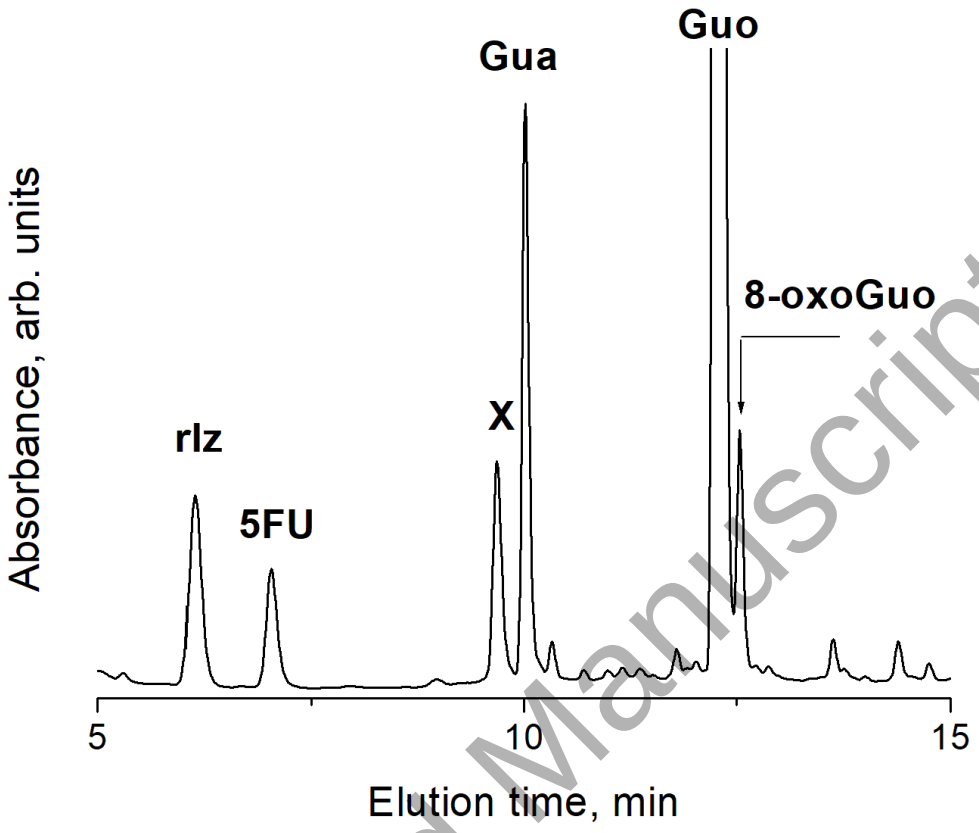
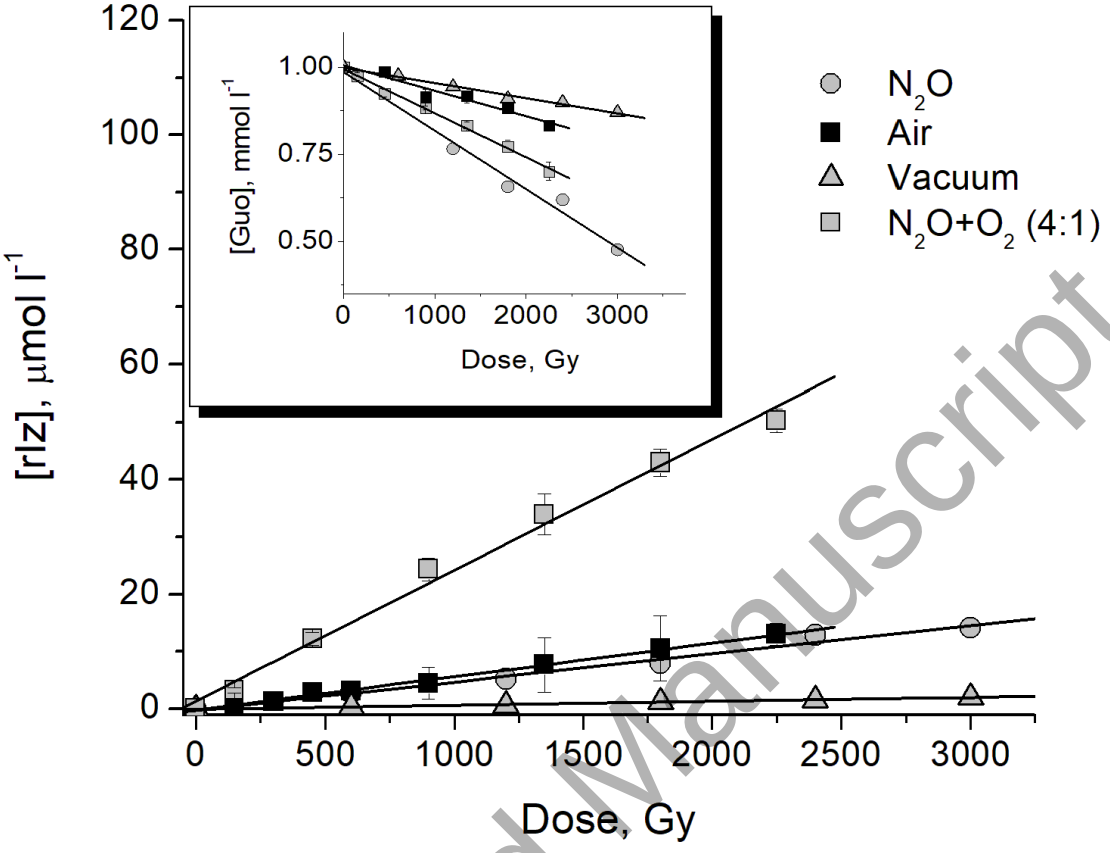
Figure Legends



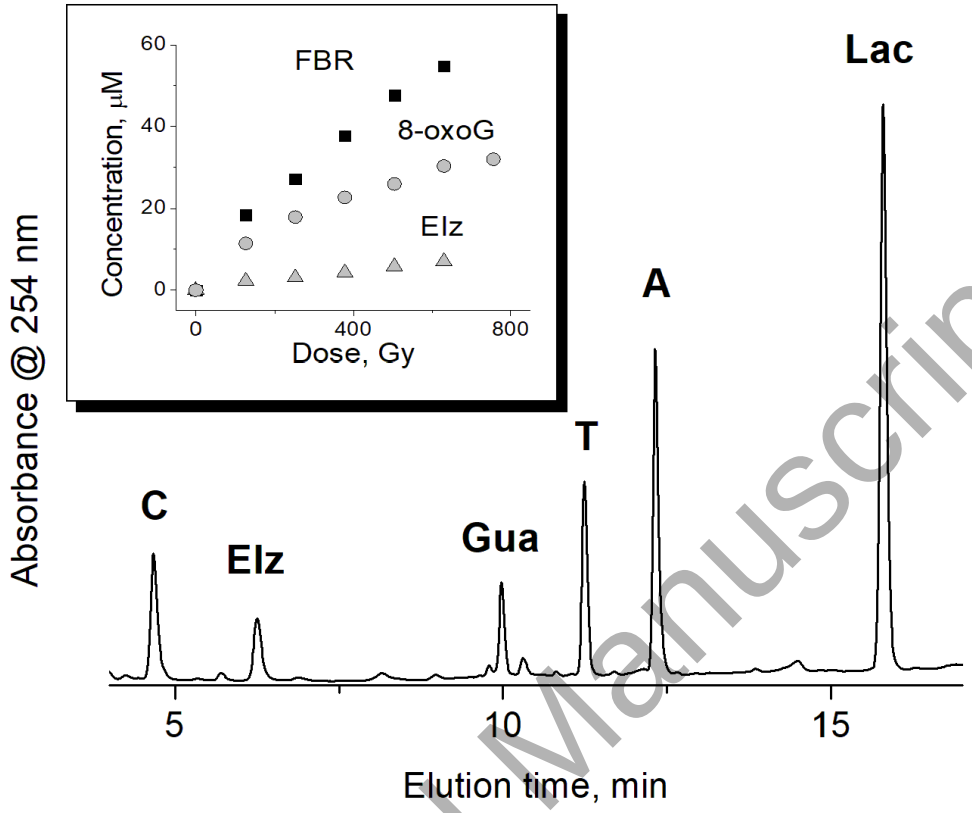

1. UV spectrum of the tyrosine-imidazolone conjugate (Tyr-Iz, structure shown in the Figure) and its reconstruction as a linear combination of spectra of tyrosine and dIz.



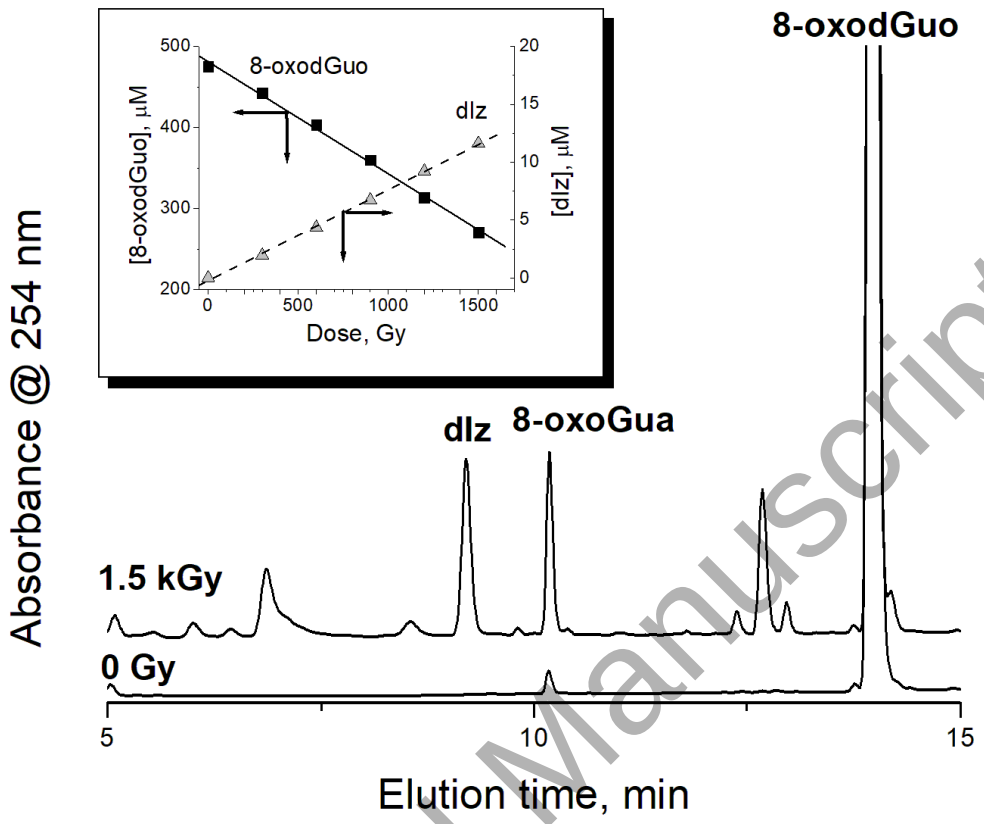

2. Sample chromatogram of Guo (1 mM) X-irradiated (200 Gy) in 50 mM phosphate buffer under air. 5-Fluorouracil (5FU) was added as an internal standard after irradiation.




3. Dose response curves for rIz accumulation and Guo loss (shown in the insert) in phosphate-buffered solutions of Guo under various gassing conditions (specified in the Figure).




4. Sample chromatogram of the supernatant obtained by X-irradiation (200 Gy) of double-stranded DNA (2.3 mM in base pairs) in 50 mM phosphate under air after treatment with ethanolamine (0.2 M) and precipitation of the polymeric component with protamine. Dose response curves for free base release, 8-oxoG and EIz accumulation are shown in the insert.




5. Sample chromatograms of 8-oxodGuo (0.6 mM) X-irradiated in 50 mM phosphate under air. Dose response curves for 8-oxodGuo loss and dIz accumulation are shown in the insert.

Table 1. Radiation-chemical yields *G* of starting material (SM) degradation and product formation in X-irradiated solutions of Guo and double-stranded DNA

| Gassing | *G*, nmol $J^{-1}$ | | | | |
|---|---|---|---|---|---|
| | SM loss | Iz | 8-oxoG | Gua | All products |
| | | | Guo | | |
| Air | 73 | 5.8 | 29 | 26 | 61 |
| $N_2O/O_2$ (4:1) | 126 | 23 | 48 | 49 | 120 |
| $N_2O$ | 168 | 4.9 | 46 | 65 | 116 |
| Vacuum | 58 | 0.7 | 11 | 42 | 54 |
| | | | DNA | | |
| Air | n. d.* | 6.6 | 22 | 55** | 84 |
| $N_2O/O_2$ (4:1) | n. d.* | 18 | 74 | 107** | 92 |

* Total yield of DNA oxidation, not determined

** Free base release