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Involvement of proton transfer in the reductive repair of DNA guanyl radicals by aniline derivatives

Anne Ly, Nancy Q. Tran, Kathrina Sullivan, Simona L. Bandong and Jamie R. Milligan*

Department of Radiology, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA, 92093-0610, USA. E-mail: jmilligan@ucsd.edu; Fax: 858-534-0265; Tel: 858-534-4919

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The most easily oxidized sites in DNA are the guanine bases, and major intermediates produced by the direct effect of ionizing radiation (ionization of the DNA itself) are electron deficient guanine species. By means of a radiation chemical method (γ-irradiation of aqueous thiocyanate), we are able to produce these guanyl radicals in dilute aqueous solutions of plasmid DNA where the direct effect would otherwise be negligible. Stable modified guanine products are formed from these radicals. They can be detected in the plasmid conversion to strand breaks after a post-irradiation incubation with a DNA base excision endonuclease enzyme. If aniline compounds are also present, the yield of modified guanines is strongly attenuated. The mechanism responsible for this effect is electron donation from the aniline compound to the guanyl radical, and it is possible to derive rate constants for this reaction. Aniline compounds bearing electron withdrawing groups (*e.g.*, 4-CF_3) were found to be less reactive than those bearing electron donating groups (*e.g.*, 4-CH_3). At physiological pH values, the reduction of a guanyl radical involves the transfer of a proton as well as of an electron. The mild dependence of the rate constant on the driving force suggests that the electron is not transferred before the proton. Although the source of the proton is unclear, our observations emphasize the importance of an accompanying proton transfer in the reductive repair of oxidative damage to guanine bases which are located in a biologically active double stranded plasmid DNA substrate.

Introduction

Guanine bases are the most easily oxidized sites in nucleic acids.[1,2] Guanine modifications are therefore produced by DNA damaging agents that act by oxidative or electron removal mechanisms. Such agents include ionizing irradiation,[3] photoionization,[4,5] chemical oxidation,[6] and photosensitization.[7] The product of the removal of a single electron from a guanine base is a guanyl radical.[3,8] This species is a strong oxidizing agent: the guanyl radical species derived from guanosine has $E_7 = +1.29$ V (*vs.* NHE).[9] Although long lived by radical standards (lifetime of up to 5 seconds)[10–12] under favorable conditions, guanyl radicals are very reactive with mild reducing agents if any are available.[8,13–17] This back donation by a reducing agent of the missing electron represents the repair of DNA damage that was produced by its removal. Mild reducing agents capable of this reaction include the biological antioxidants ascorbate and glutathione,[18] and their usual physiological concentrations (on the order of 10^{-4} and 10^{-3} mol dm^{-3} respectively)[19] may limit the lifetime of DNA guanyl radicals *in vivo*.

Other common biochemicals are also able to reduce guanyl radicals. The amino acids tyrosine ($E_7 = +0.89$ V)[20,21] and tryptophan ($E_7 = +1.05$ V)[20,21] and their peptide derivatives behave as mild reducing agents, and they are reactive with guanyl radicals located in DNA substrates.[22] We have examined the mechanism of the reaction of DNA guanyl radicals with tyrosine and with tryptophan by using a series of substituted phenols[23] and indoles[24] to alter the driving force of the electron transfer. Because of the acidities of the species concerned, the reaction of phenols and of indoles with guanyl radicals involves the transfer of a proton as well as of an electron. The source of the proton and the timing of its transfer may be a function of the nature of the reducing agent. Phenol radical cations are very strongly acidic (pK_a of the radical cation of phenol, $PhOH^{\bullet+}$ is -2.0)[25] and are excellent proton donors. Indole radical cations are weaker acids (the indole radical cation, $IndNH^{\bullet+}$ has $pK_a = 4.6$)[26] and possibly poorer proton donors than other species present in solution. In the case of double stranded DNA, protons located in the hydrogen bonds between complementary bases would appear to be the most likely alternative sources.[27,28]

It is likely that other classes of compounds with similar reducing properties also exhibit radioprotective behavior, in particular the aromatic amines. Amines and their derivatives are widely distributed in nature.[29] Their reductive behaviour (oxidation by enzymes) plays a significant role in drug metabolism.[30] Their electron transfer reactivity with brominated DNA bases has been examined recently.[31] In electron transfer reactions, anilines are reducing agents comparable in strength to phenols and indoles.[32] However in the proton transfer reactions that are central to DNA redox chemistry,[27] there are differences between them. Unlike phenols or indoles, anilines have the property that the acidity of their radical cations spans the physiological range. The radical cation of aniline itself ($PhNH_2^{\bullet+}$) has $pK_a = 7.1$.[26] Derivatives bearing electron withdrawing groups are significantly more acidic, while those with electron donating groups are less acidic. For example, the pK_a values of the radical cations of the 4-CF_3 and 4-CH_3 derivatives of aniline are 4.8 and 8.5 respectively.[26] So to address the issue of proton transfer under physiological conditions we have employed as reducing agents a series of substituted anilines and measured their reactivity with guanyl radicals located in a plasmid DNA target.

Results and discussion

Ionizing radiation

Our requirement is the efficient generation of guanyl radical species in dilute aqueous solution. Guanyl radicals are major intermediates in the direct effect of ionizing radiation (ionization of the DNA itself). The direct effect makes a significant contribution to the lethality of ionizing radiation to cellular systems, but it is difficult to study with aqueous model systems such as plasmids. This is because in dilute solutions the direct effect is very inefficient with most of the ionizations taking place in the water and not in the target. But by converting the

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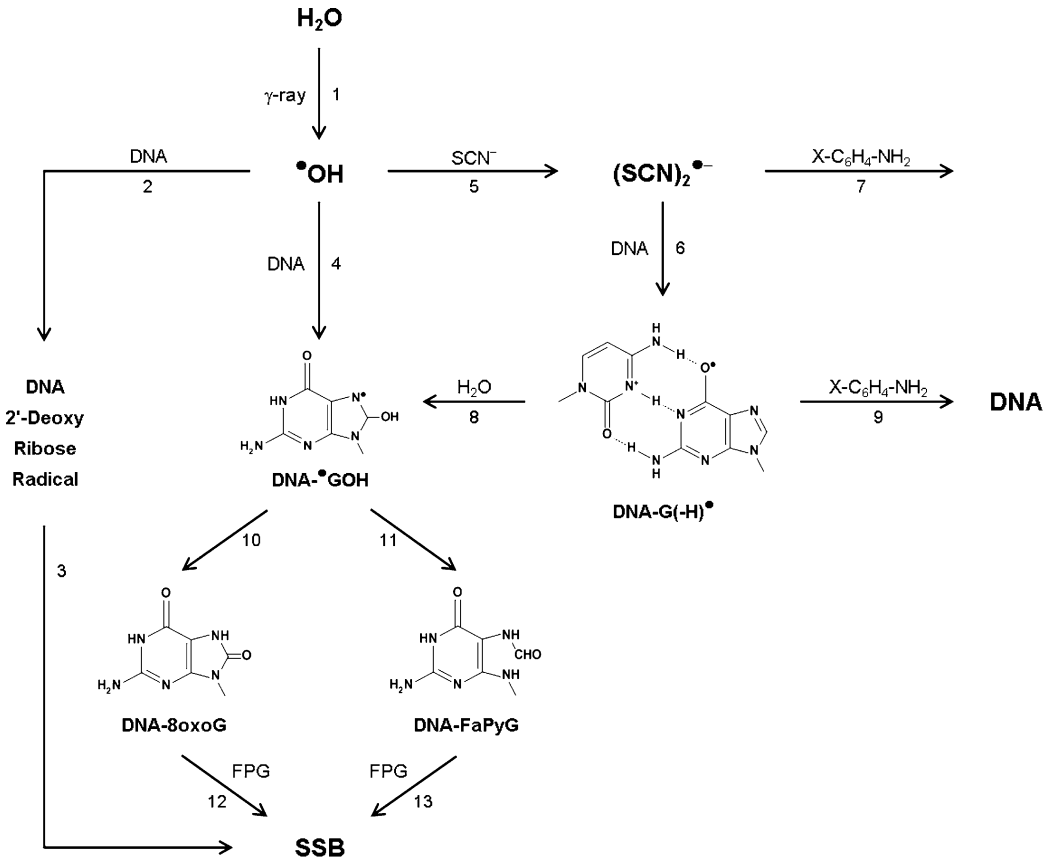

Fig. 1 Reaction scheme summarizing the mechanism of the formation, repair, and subsequent detection of oxidative damage when plasmid DNA is γ-irradiated in the presence of thiocyanate ions and an aniline derivative ($X–C_6H_4–NH_2$).

water derived radicals into a single electron oxidizing agent it is possible to produce guanyl radicals fairly efficiently.[8]

Reaction scheme

We have reported previously on the mechanism of DNA damage by γ-irradiation in the presence of thiocyanate ions.[35] The important reactions are summarized by the scheme in Fig. 1. Radiolysis of water produces the hydroxyl radical •OH (reaction 1). A small fraction (about 1%) of •OH reacts with the 2′-deoxyribose groups to form C-centered radical species (reaction 2) which go on to produce single strand breaks (SSB).[36] Some •OH (a further 1–2%) react by addition to the bases to produce adducts[36,37] such as the 8-hydroxy-7,8-dihydroguan-7-yl radical DNA–•GOH (reaction 4). Adducts of the other bases also form[36] but these have been omitted from Fig. 1. Under the experimental conditions the majority of •OH (*ca.* 98%) react with thiocyanate ions (reaction 5) to produce the dimeric radical anion $(SCN)_2^{\bullet -}$. This species acts as a fairly strong single electron oxidizing agent with $E^0 = +1.33$ V.[38] It is able to remove electrons from guanine bases (reaction 6) but not from any other sites in DNA. The product of electron removal from guanine is a guanyl radical cation DNA–$G^{\bullet +}$. The pK_a of the radical cation of 2′-deoxyguanosine is 3.9,[8] so it is largely deprotonated at pH values higher than this. Guanine radical cations located in double stranded oligonucleotides also deprotonate.[39] The deprotonated conjugate base of the DNA guanine radical cation DNA–$G^{\bullet +}$ is symbolized as DNA–G(–H)•. In double stranded DNA the hydrogen bonded complementary cytosine base is an ideally located proton acceptor.[27] The pK_a of N-3 protonated cytosine CH^+ is 4.3.[6]

In the presence of a reducing agent such as a substituted aniline compound $X–C_6H_4–NH_2$, the DNA guanyl radical may be reduced back to the original guanine (reaction 9).[8,13] This repair reaction competes with the trapping of the guanyl radical by water (reaction 8).[40] The hydroxylated product of this trapping reaction DNA–•GOH is the same as that produced by •OH addition (reaction 4) in the absence of any thiocyanate. Stable products derived from this species are 8-oxo-7,8-dihydroguanine (DNA-8-oxoG) by a further one electron oxidation (reaction 10) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (DNA–FaPy–G) by a one electron reduction (reaction 11).[41,42] These two guanine damage products are stable under the conditions of the irradiation. But a post-irradiation incubation with the *E. coli* base excision endonuclease enzyme FPG converts the sites of both of these modified guanines to strand breaks (reactions 12 and 13).[43] Unmodified guanines remain undisturbed. The purpose of the enzyme incubation is to convert damaged bases to breaks. This is because in a plasmid substrate breaks can be quantified with a high sensitivity.

Under aerobic conditions, but in the absence of a reducing agent, the yield of DNA-8-oxoG is greater than that of DNA–FaPy–G.[39] Since the latter is produced by reduction of its precursor DNA–•GOH, its yield may increase at the expense of that of DNA–FaPy–G if a reducing agent (such as an aniline derivative) is available. Since both DNA-8-oxoG and DNA–FaPy–G are recognized by FPG[43] we are unable to distinguish between these two products. DNA-8-oxoG is susceptible to further oxidation, but this is unlikely under the conditions we have used here because of the relatively low yield of about one 8-oxoG per plasmid (see Experimental section).

In principle the aniline compound is also capable of reacting with the oxidizing agent $(SCN)_2^{\bullet -}$. In practice the reaction conditions are chosen so that the major reaction of $(SCN)_2^{\bullet -}$ is with the plasmid. Evidence in favor of this is: (1) the attenuation in DNA damage produced by different reducing agents does not correlate with their reactivity with $(SCN)_2^{\bullet -}$;[14] (2) equal attenuations are observed for a single reducing agent

with different single oxidizing agents ($(SCN)_2^{\bullet-}$, $Br_2^{\bullet-}$, $SeO_3^{\bullet-}$, $Tl^{II}OH^+$) arguing in favor of a common intermediate; [14,18,44] (3) for the cationic oxidizing agent $Tl^{III}OH^+$ ionic strength effects suggest that the reducing agent reacts with a negatively charged species,[18] such as the polyanionic plasmid.

As summarized in the reaction scheme, SSBs are formed by three different routes: (1) $^{\bullet}OH$ reaction with the 2′-deoxyribose groups (reactions 1 to 3) produces SSBs in the absence of any FPG incubation; (2) $^{\bullet}OH$ addition to guanine bases (reaction 4) produces modified guanine bases (reactions 10, 11) which form SSB only after FPG incubation (reactions 12, 13); (3) $(SCN)_2^{\bullet-}$ oxidation of guanine bases (reaction 6) also produces SSB only after FPG incubation (reactions 8, 10–13). The second and third routes are distinguishable because the third is attenuated by the presence of mild reducing agents while the second is not affected. Under the reaction conditions we have employed here, the relative yields of these three pathways in the absence of any added reducing agents are *ca.* 1 : 1 : 100. If a reducing agent (here we have used aniline compounds) is present in excess, the yield of the third route is attenuated so extensively that it becomes negligible with respect to the second route.[24]

Strand break yields

By making use of a plasmid as the guanine containing target, it is possible to detect single strand break (SSB) formation and to quantify their yields. The introduction of one or more SSBs into the plasmid converts it from the supercoiled to the relaxed (also called open circle) conformation. These two conformers are easily separated by gel electrophoresis, and the proportions (more formally the mole fractions) of each can be estimated from the fluorescence of bound ethidium. The increase in the SSB yield when FPG incubation is included after irradiation but before electrophoresis provides an estimate of the yield of modified guanine bases.

Examples of typical yield dose plots are reproduced in Fig. 2. The γ-irradiation was followed by an incubation in the presence or absence of FPG. This incubation substantially increases the loss of the supercoiled (free of SSBs) form of the plasmid, but increasing concentrations of the aniline compound 4-aminoacetophenone (aniline bearing a 4-$COCH_3$ substituent) attenuate this increase. The radiation chemical yield (called a *G* value) for SSB formation can be calculated from the slopes of semi logarithmic yield dose plots such as Fig. 2. Details may be found in the Experimental section.

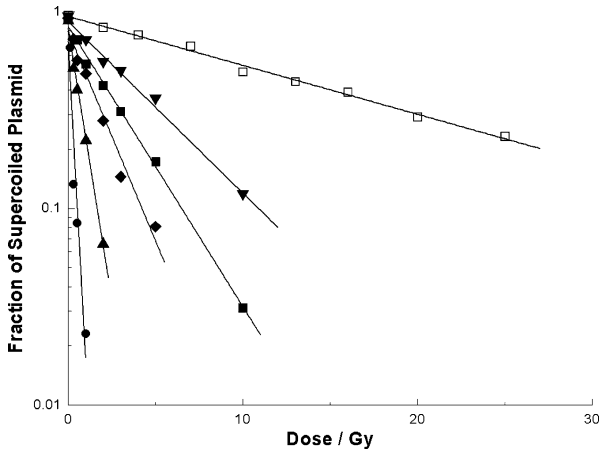

Fig. 2 Effect of radiation dose on the fraction of supercoiled plasmid (see Experimental section). Aliquots of a solution containing the aniline compound 4-aminoacetophenone at a concentrations of 5×10^{-8} mol dm^{-3} (●), 2×10^{-7} mol dm^{-3} (▲), 5×10^{-7} mol dm^{-3} (◆), 1×10^{-6} mol dm^{-3} (□■), or 2×10^{-6} mol dm^{-3} (▼) were γ-irradiated. After irradiation, the solutions were incubated with (●▲◆■▼) or without (□) FPG. The mole fraction of the plasmid remaining in the supercoiled form was then determined by gel electrophoresis. This fraction is plotted against the radiation dose. The six data sets are each fitted with least mean square straight lines of the form $y = ce^{-mx}$. From the slopes *m* of these fitted straight lines, the D_0 doses and SSB yields for the six irradiation conditions are: (●) 0.270 Gy, 1.38×10^{-2} μmol J^{-1}; (▲) 0.787 Gy, 4.73×10^{-3} μmol J^{-1}; (◆) 1.70 Gy, 2.19×10^{-3} μmol J^{-1}; (■) 3.05 Gy, 1.22×10^{-4} μmol J^{-1}; (▼) 7.49 Gy, 2.19×10^{-4} μmol J^{-1}; (□) 17.5 Gy, 2.13×10^{-4} μmol J^{-1}.

Attenuation of breaks by anilines

The three routes by which SSBs are formed respond in different ways to the presence of 4-aminoacetophenone during γ-irradiation. We used additional concentrations of 4-aminoacetophenone from 5×10^{-8} to 1×10^{-4} mol dm^{-3} (not all shown in Fig. 2). The concentration dependence of the derived *G*(SSB) values both with and without FPG incubation is shown in Fig. 3. In the absence of FPG, the SSB yield remains constant at *ca.* 2×10^{-4} μmol J^{-1}. As shown in Fig. 2, FPG incubation in general increases the SSB yield. At the higher concentrations of 4-aminoacetophenone (10^{-5} to 10^{-4} mol dm^{-3}), FPG incubation modestly increases the SSB yield to 4×10^{-4} μmol J^{-1} (a 2 fold increase). But for lower concentrations of the aniline derivative there is a concentration dependent effect on *G*(SSB). The value of *G*(SSB) increases substantially up to about 1.5×10^{-2} μmol J^{-1} (a 30 fold increase) as the 4-aminoacetophenone concentration decreases to 5×10^{-8} mol dm^{-3}.

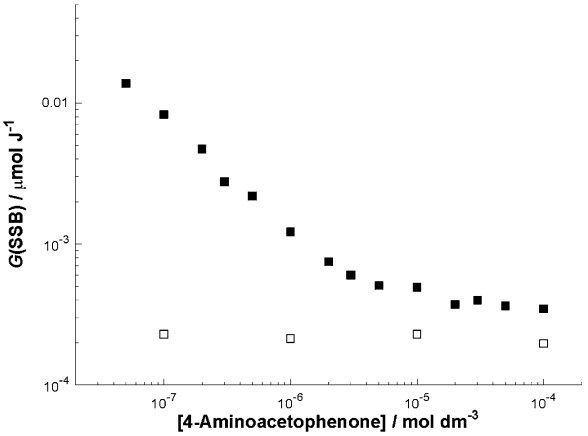

Fig. 3 Effect of 4-aminoacetophenone on the yield of strand breaks after incubation with or without the enzyme FPG. Using the method shown in Fig. 2, SSB yields were determined over a wide range of concentrations of the aniline derivative. After irradiation, but before assay for breaks by electrophoresis, the plasmid was incubated under one of two conditions: in the absence of FPG (□) or in the presence of 3 μg ml^{-1} FPG (■).

Kinetics of DNA repair

The yield of products resulting from reaction of $(SCN)_2^{\bullet-}$ that are recognized by FPG (symbolized as *G*(FPG)) can be estimated by subtracting the residual constant yield of FPG sensitive sites observed at the highest concentrations of 4-aminoacetophenone (2×10^{-5} to 1×10^{-4} mol dm^{-3}). This residual yield is about 4×10^{-4} μmol J^{-1} (Fig. 3). Fig. 4 shows a plot of the reciprocal of *G*(FPG) against the 4-aminoacetophenone concentration, according to competition kinetics. The data in Fig. 4 are described by eqn. (1). Here *G*(FPG) and G_0(FPG) represent the yields of FPG sensitive sites in the presence and absence respectively of the aniline compound, k_8 and k_9 represent the rate constants of reactions 8 and 9 respectively, and [AAP] represents the concentration of 4-aminoacetophenone. From a comparison of eqn. (1) and Fig. 4, it is possible to quantify the competition between the trapping and the repair of a DNA guanyl radical. Eqn. (1) implies that $G(FPG)^{-1}$ should be a linear function of the concentration of the aniline derivative; and also that the value of $k_9 = k_8 m/c$, where *m* and *c* are respectively the slope and intercept of the

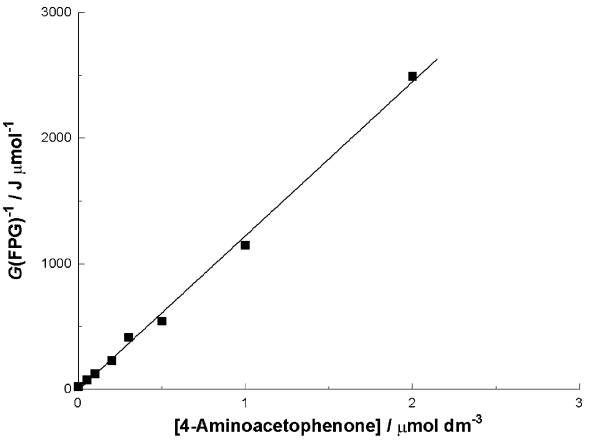

Fig. 4 Reciprocal plot of the attenuation of the yield of FPG sensitive sites, G(FPG), against the concentration of 4-aminoacetophenone according to competition kinetics (see text). The data set is fitted with a least mean square straight line of the form $y = mx + c$. The slope m of the fitted line is 1.23×10^9 MJ dm^3 mol^{-2}.

straight line fitted to the data set in Fig. 4. The value of m is 1.23×10^9 MJ dm^3 mol^{-2}. The value of c is obtained from Fig. 3 as $c = 1/(4.8 \times 10^{-2}\ \mu\text{mol J}^{-1}) = 21\ \text{J}\ \mu\text{mol}^{-1}$. The value of k_8 is imprecisely characterized in the literature, but a value of $k_8 = 0.2\ \text{s}^{-1}$ under the conditions we have used here (in neutral aqueous solution at room temperature) is consistent with the reports of two groups.[10–12] So the value of k_9 derived from Fig. 4 is $k_9 = 0.2 \times 1.23 \times 10^9/21 = 1.2 \times 10^7$ dm^3 mol^{-1} s^{-1}.

$$G(\text{FPG})^{-1} = G_0(\text{FPG})^{-1} \times \left(1 + \frac{[\text{AAP}]k_9}{k_8}\right) \qquad (1)$$

By constructing plots similar to Fig. 4 for the other aniline compounds, we arrive at estimates for the values of k_9 for each of them. These values are listed in Table 1.

Proton transfer in the reduction of guanyl radicals by anilines

The pK_a of the radical cation of guanosine is 3.9,[27] while that of guanine itself is 9.5.[27] At pH values between these limits, both species are uncharged. So at physiological pH values, the reduction of a DNA guanyl radical DNA–G(–H)$^\bullet$ to the original guanine DNA–G requires the transfer of a proton as well as of an electron. The only available reducing agent in solution is the aniline compound, and it is of course the source of the electron (Fig. 1). But the source of the proton is less clear. The pK_a of the radical cation of aniline (PhNH$_2^{\bullet+}$) is 7.1.[26] Radical cations derived from anilines bearing an electron withdrawing substituent are stronger acids than the radical cation of aniline itself.[26] They will deprotonate under the conditions we have used here (pH 7.0) and it is possible that they act as the proton source. But the radical cations of anilines bearing an electron donating substituent are weaker acids, and therefore poorer proton sources. It has been suggested that a likely alternative proton source is the complementary base pair partner (*i.e.*, cytosine) to which the guanyl radical is hydrogen bonded and which accepts a proton from it.[6,27,28] Cytosine protonated at N-3 has p$K_a = 4.3$.[6,27]

The invidual proton and electron transfer reactions are summarized by the schemes in Figs 5a and 5b. In Fig. 5a, the aniline compound acts as the proton source as well as the reducing agent. In Fig. 5b, the proton source is the conjugate acid of the complementary cytosine (symbolized as CH$^+$).

Table 1 Rate constants for the repair of DNA guanyl radicals by substituted aniline compounds (k_9, the rate constant for reaction 9 in Fig. 1)

Aniline derivative X–C$_6$H$_4$–NH$_2$	k_9/dm^3 mol^{-1} s^{-1}
4-CN	5.5×10^6
2-CF$_3$	4.4×10^6
4-CF$_3$	6.8×10^6
3-CF$_3$	5.5×10^6
4-COCH$_3$	1.2×10^7
4-H	2.8×10^7
4-I	2.5×10^7
2-CH$_3$	2.1×10^7
4-CH$_3$	4.1×10^7

Repair energetics

The individual steps in the reactions schemes in Fig. 5 are all simple single proton and/or electron transfers. It is possible to calculate the driving forces for these steps from literature data for values of acid dissociation constants and reduction potentials. The relevant data for anilines and for the DNA bases guanine and cytosine are summarized in Tables 2 and 3.

Although the aniline compounds we have used here remain unprotonated under the experimental conditions (all their cations have pK_a < 7), the pK_a values of their radical cations spans the physiological range. Therefore the reduction potentials of the more acidic radical species are lowered at pH 7. For example, the reduction potential of the radical derived from 4-aminoacetophenone at pH 7 is $E_7 = +1.14 - (\log_e 10 \times RT/F) \times (7.0 - 6.1) = +1.09$ V. Values of the reduction potentials under the experimental conditions (pH 7) for the radicals of the other aniline derivatives are listed in Table 4. Similarly, the reduction potential of the DNA guanyl radical at pH 7 is $E_7 = +1.47 - (\log_e 10 \times RT/F) \times (7.0 - 3.9) = +1.29$ V.

The driving forces for the electron transfer steps in Figs 5a and 5b are derived from differences in reduction potentials. For example in the case of reaction 9a with 4-aminoacetophenone as the aniline compound, the transfer of an electron from the aniline ($E = +1.14$ V) to the DNA guanyl radical ($E = +1.14$ V)

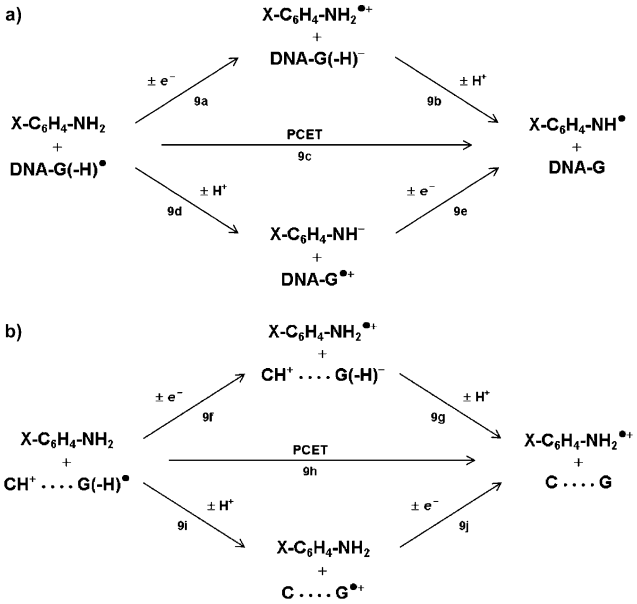

Fig. 5 **a)** Reaction scheme showing the individual proton and electron transfers involved in the reductive repair of a DNA guanyl radical DNA–G(–H)$^\bullet$ by an aniline derivative X–C$_6$H$_4$–NH$_2$ (reaction 9 in Fig. 1) such that the aniline compound is the source of both the proton and the electron. The three pathways shown here are: (1) electron before proton (reactions 9a and 9b); (2) both coupled together (PCET, reaction 9c); and (3) proton before electron (reactions 9d and 9e). **b)** Reaction scheme depicting the individual proton and electron transfer steps in the reduction of a DNA guanyl radical by an aniline compound (reaction 9 in Fig. 1), where the aniline is the source of the electron but the proton is supplied by the conjugate acid of the complementary cytosine base (CH$^+$) to which the guanyl radical is hydrogen bonded. The three pathways shown here are: (1) electron before proton (reactions 9f and 9g); (2) both coupled together (PCET, reaction 9h); and (3) proton before electron (reactions 9i and 9j).

Table 2 Literature values of acid dissociation constants for substituted anilines (X–C_6H_4–NH_2), their conjugate acids (X–C_6H_4–NH_3^+), and their radical cations (X–C_6H_4–$NH_2^{\bullet+}$); and values of reduction potentials for the radical cations of these derivatives

Aniline derivative X–C_6H_4–NH_2	pK_a X–C_6H_4–NH_2 ↔ X–C_6H_4–NH^- + H^+	pK_a X–C_6H_4–NH_3^+ ↔ X–C_6H_4–NH_2 + H^+	pK_a X–C_6H_4–$NH_2^{\bullet+}$ ↔ X–C_6H_4–$NH^\bullet$ + H^+	E°/V (X–C_6H_4–$NH_2^{\bullet+}$/ X–C_6H_4–NH_2)[f]
4-CN	22.7[a]	1.74[c]	4[d]	+1.32[d]
	25.3[b]			
2-CF_3		2.10[c]	4.5[e]	+1.30[e]
4-CF_3	27.0[b]	2.57[c]	4.8[d]	+1.28[d]
3-CF_3	25.4[a]	3.49[c]	5.5[e]	+1.27[e]
	28.2[b]			
4-$COCH_3$	25.4[b]	2.19[c]	6.1[d]	+1.14[d]
4-H	30.6[b]	4.58[c]	7.1[d]	+1.02[d]
4-I		3.78[c]	7.1[d]	+1.02[d]
2-CH_3		4.39[c]	7.9[e]	+1.01[e]
4-CH_3	31.7[b]	5.07[c]	8.5[d]	+0.92[d]

[a] In EtOH–DMSO.[56] [b] In DMSO.[57] [c] See reference 58. [d] See reference 26. [e] See reference 59. [f] On the NHE scale.

Table 3 Acid dissociation constants and reduction potentials for cytosine and guanine bases

Reaction	pK_a	Couple	E/V[a]
CH^+ ↔ C + H^+	4.3	G(–H)$^\bullet$/G (at pH 7)	+1.29
G ↔ G(–H)$^-$ + H^+	9.5	G(–H)$^\bullet$/G(–H)$^-$	+1.14
$G^{\bullet+}$ ↔ G(–H)$^\bullet$ + H^+	3.9	$G^{\bullet+}$/G	+1.47

[a] On the NHE scale.

Table 4 Reduction potentials of substituted aniline compounds at pH 7

Aniline derivative X–C_6H_4–NH_2	E_7/V
4-CN	+1.14
2-CF_3	+1.15
4-CF_3	+1.15
3-CF_3	+1.18
4-$COCH_3$	+1.09
4-H	+1.00
4-I	+1.00
2-CH_3	+1.01
4-CH_3	+0.92

has a driving force $\Delta G = -F\Delta E = -96.5 \times 10^3 \times (+1.14 - 1.14) = 0$ kJ mol^{-1}.

The driving forces for the proton transfer steps are derived from pK_a differences. For example in the case of 4-aminoacetophenone the driving force for reaction 9b, the transfer of a proton from the substituted aniline radical cation (pK_a = 6.1) to the DNA guanine anion (pK_a of conjugate acid = 9.5), is $\Delta G = -RT\log_e K = 2.3RT\Delta pK_a = 2.3 \times 8.31 \times 298 \times (6.1 - 9.5) = -19$ kJ mol^{-1}. If the proton is transferred before the electron (reaction 9d), from the neutral aniline species (pK_a = 25.4) to the DNA guanyl radical (pK_a of conjugate acid = 3.9), the driving force is highly unfavorable because the aniline species is such a poor acid: $\Delta G = 2.3RT\Delta pK_a = 2.3 \times 8.31 \times 298 \times (25.4 - 3.9) = +122$ kJ mol^{-1}.

The driving forces for the proton coupled electron transfer steps are derived from differences in reduction potentials at pH 7 (differences in E_7 values). In the case of 4-aminoacetophenone, the driving force for reaction 9c is $\Delta G = -F\Delta E_7 = -96.5 \times 10^3 \times (1.29 - 1.09) = -19$ kJ mol^{-1}.

Because the individual proton and electron transfers in Figs 5a and 5b are part of a thermodynamic cycle, the driving forces are independent of the order of the individual proton and electron transfers: $\Delta G_{9a} + \Delta G_{9b} = \Delta G_{9c} = \Delta G_{9d} + \Delta G_{9e}$. The driving force for reaction 9e was obtained using this identity. The calculations described above for 4-aminoacetophenone were repeated for all of the other aniline derivatives we used. The results are summarized in Table 5. In some cases the pK_a of the aniline derivative was not available in the literature (see Table 2), and it was not possible to estimate the driving forces for the steps in the proton first route (reactions 9d and 9e). These calculations were also repeated for reactions 9f to 9j, where the complementary base (the *N*-3 conjugate acid of cytosine, symbolized as DNA–CH^+) is assumed to be the proton source. These results are also summarized in Table 5.

The estimated driving forces listed in Table 5 are subject to several sources of uncertainty. We have assumed that the data in Table 3 are applicable to all of the guanine residues in plasmid DNA. This is not strictly true, because base sequence effects on both acid dissociation constants of up to 1.6 units[45] and reduction potentials of up to 0.08 V[46] have been reported with oligonucleotides. These correspond to 9 and 8 kJ mol^{-1} respectively. Differing values for the acid dissociation constants of aniline derivatives of up to 3 units (Table 2) are equivalent to

Table 5 Driving forces for the reduction of a DNA guanyl radical by a substituted aniline compound, where the source of the proton is either the aniline compound (reactions 9a to 9e in Fig. 5a) or the complementary cytosine base (reactions 9f to 9j in Fig. 5b)

	ΔG/kJ mol^{-1}								
	e^- first	H^+ second		PCET		H^+ first		e^- second	
Aniline derivative X–C_6H_4–NH_2	9a/9f	9b	9g	9c	9h	9d	9i	9e	9j
4-CN	+17	−31	−30	−14	−12	+122	+2	−136	−14
2-CF_3	+15	−28	−30	−13	−14		+2		−16
4-CF_3	+14	−27	−30	−13	−16	+132	+2	−145	−18
3-CF_3	+13	−23	−30	−10	−17		+2		−19
4-$COCH_3$	0	−19	−30	−19	−30	+122	+2	−142	−32
4-H	−12	−14	−30	−26	−41	+152	+2	−178	−43
4-I	−12	−14	−30	−25	−41		+2		−43
2-CH_3	−13	−9	−30	−22	−42		+2		−44
4-CH_3	−21	−6	−30	−27	−51	+158	+2	−185	−53

17 kJ mol^{-1}. Electrostatic work effects have also been ignored. These are estimated to be of the order of 2 kJ mol^{-1}.[23]

Repair mechanism

Despite the large uncertainty (see above), the driving forces of the proton first pathway (where the aniline derivative acts as the proton source, reaction 9d) are clearly extremely endoergonic (over +100 kJ mol^{-1}, Table 5). Such an unfavorable step would be far too slow to make any significant contribution to the observed reaction.[23] Similar effects have been reported in other systems.[47] The driving force dependence of the rate constant for an electron transfer reaction may be used to eliminate from consideration some of the other reaction pathways shown in Figs 5a and 5b. The Marcus treatment[48] asserts that this relationship can be described in a differential form by eqn. (2), where λ represents the reorganization energy. Although we cannot assign a value to λ, eqn. (2) still predicts that a plot of $RT\log_e k_9$ against the driving force ΔG should have a slope less steep than $m = -\frac{1}{2}$ if the reaction is exoergonic but steeper than $m = -\frac{1}{2}$ if it is endoergonic.[49,50] In Figs 5a and 5b, there are six steps that involve an electron transfer. These are reactions 9a, 9c, 9e, 9f, 9h, and 9j. Since reactions 9a and 9f are equivalent, and reaction 9e can be eliminated because its necessary precursor reaction 9d is kinetically irrelevant (see above), this leaves four possibilities. Two of these (reactions 9 and 9h) are plotted according to eqn. (2) in Fig. 6. The slopes of the fitted straight lines are $m = -0.31$ (reaction 9c) and $m = -0.14$ (reaction 9h). A slope of $m = -0.14$ (not shown in Fig. 6) also applies to reaction 9j, which has a very similar driving force to that of reaction 9h. All of these values are consistent with eqn. (2). The relationship between the kinetics and energetics of reaction 9a are inconsistent with eqn. (2), since a slope of $m = -0.14$ (also not shown in Fig. 6) is too shallow for a reaction whose driving force spans the range $\Delta G = ca.$ ± 20 kJ mol^{-1}.

$$\frac{\partial}{\partial \Delta G}(RT\log_e k) = \frac{1}{2} + \frac{\Delta G}{2\lambda} \qquad (2)$$

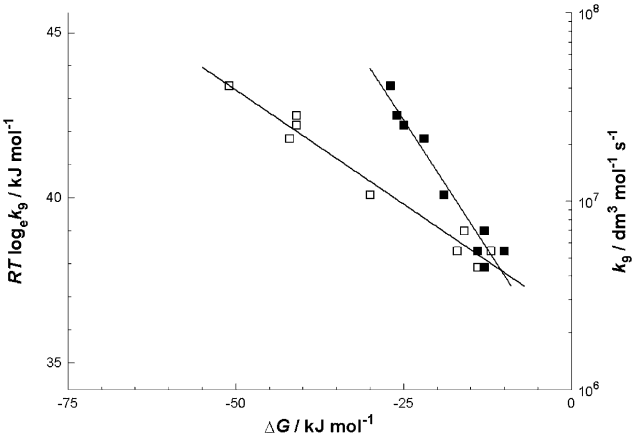

Fig. 6 Dependence of the rate constant k_9 on the driving force. The value of $RT\log_e k_9$ (taken from Table 1) is plotted against the driving force ΔG for reactions 9c (■) and 9h (□), taken from Tables 5a and 5b respectively. Each data set is fitted with a least mean square straight line of the form $y = mx + c$. The values of the slopes m are −0.31 and −0.14.

Therefore the possible pathways appear to be a proton coupled electron transfer with either aniline (reaction 9c) or the conjugate base (reaction 9h) supplying the proton, or the proton first electron second route with the conjugate base acting as the proton source (reactions 9i, 9j). In all of these three cases, the proton transfer either precedes the electron transfer or takes place on the same time scale.

Conclusions

The DNA guanyl radical is the major intermediate produced by the direct effect of ionizing radiation. It is also formed by other electron removal processes such as photoionization and photosensitization. Characterizing its reactivity is therefore of central importance to understanding the mechanisms of oxidative DNA damage and its repair. Mild reducing agents are able to reverse the oxidative damage. An important class of mild reducing agents widely distributed in nature and well known in pharmacology are the aromatic amines, here exemplified by aniline derivatives. We find that anilines are able to repair oxidative DNA damage by returning the missing electron. This electron transfer reaction is associated with a proton transfer reaction because of the acidities of the species involved. The driving force dependence of the rate constant for the reaction implies that the electron transfer is mechanistically coupled with or even subordinate to a preceding proton transfer. This observation is consistent with the importance attached by other workers to the transfer of protons in the formation or repair of oxidative DNA damage.[11,27,28,51–55]

Experimental

Biochemicals

A sample of plasmid pHAZE (10,327 base pairs in length) was kindly supplied by Dr W. F. Morgan (Department of Radiation Oncology, University of Maryland). It was grown to a large scale, isolated, and purified as described previously.[33] The *E. coli* base excision repair endonuclease formamidopyrimidine-DNA *N*-glycosylase (FPG) was obtained commercially (Trevigen).

Irradiation

Plasmid pHAZE was γ-irradiated in aqueous solution with a AECL GammaCell-1000 isotopic instrument (caesium-137, 662 keV γ-ray photon). The dose rate of 335 rad min^{-1} (5.6×10^{-2} Gy s^{-1}) was determined with the Fricke dosimeter.[34] The aqueous solutions (each aliquot was 27 μl in volume) contained plasmid pHAZE (25 μg ml^{-1}, equivalent to 7.7×10^{-5} mol dm^{-3} nucleotides or 3.7×10^{-9} mol dm^{-3} plasmids); sodium phosphate (5×10^{-3} mol dm^{-3}, pH 7.0); sodium thiocyanate (1×10^{-3} mol dm^{-3}), sodium perchlorate (1.1×10^{-1} mol dm^{-3}); and aniline or one of eight derivatives of it (1×10^{-8} to 1×10^{-4} mol dm^{-3}). The aniline compound (X–C_6H_4–NH_2) was one of the following: 4-aminobenzonitrile (X = 4-CN); 2, 3-, or 4-(trifluoromethyl)aniline (X = 2-CF_3, 3-CF_3, or 4-CF_3); 4-aminoacetophenone (X = 4-$COCH_3$); aniline itself (X = 4-H); 4-iodoaniline (X = 4-I); *o*- or *p*-toluidine (X = 2-CH_3 or 4-CH_3).

Enzyme incubation

After irradiation, each 27 μl aliquot was mixed with 3 μl of a solution containing the enzyme FPG so that the final FPG concentration was either zero or 3×10^{-6} g ml^{-1}. This is equivalent to a final activity of 30 units ml^{-1} (a unit is defined as the formation of 10^{-12} mol of single strand breaks (SSB) from abasic sites after incubation at 37 °C for 60 min). The resulting solutions were incubated at 37 °C for 30 min and then assayed for SSB formation by gel electrophoresis.

Measurement of strand break yields

The formation of single strand breaks (SSB) in plasmid pHAZE was detected and quantified using agarose gel electrophoresis. The procedures for digital video imaging of intercalated ethidium fluorescence and for calculating the radiation chemical yield (referred to as the *G* value, having units of mol J^{-1}) of SSBs have been described previously.[33] Introduction of SSBs into the plasmid converts the supercoiled form into the open circle form.

The supercoiled form contains no SSBs, and the open circle form contains one or more SSBs.

Assuming that the SSBs are distributed according to Poisson statistics, the total number of SSBs can be estimated from the fraction of the plasmid that is free of SSBs. This SSB free fraction is just the mole fraction of the supercoiled form of the plasmid. The D_0 dose is defined as the radiation dose required to reduce the fraction of the supercoiled form to $1/e$ (approximately 37%) of its value in the absence of irradiation. Numerically D_0 is equal to the reciprocal of the slope m of a straight line fitted to a semi logarithmic dose yield plot. Assuming a Poisson distribution, at the D_0 dose there is a mean of one SSB per plasmid target, so that the concentration of the SSB product is equal to the concentration of the plasmid substrate (3.9×10^{-9} mol dm^{-3}, see above). The radiation chemical yield (referred to as the G value) for SSB formation (symbolized G(SSB)) is calculated by dividing this concentration by the value of D_0.

Note that the strand break assay is able to detect stable guanine oxidation products at the level of about one per plasmid. This represents a yield of guanine modification of about 1 in 5000 (assuming one quarter of the 10,327 base pairs are guanines) or 0.02%. Second order effects due to inter radical reactions or product accumulation are therefore unlikely to be significant.

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