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Gel electrophoretic detection of 7,8-dihydro-8-oxoguanine and 7,8-dihydro-8-oxoadenine via oxidation by Ir(IV)

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ABSTRACT

Two gel electrophoretic methods are described for detection of 7,8-dihydro-8-oxoguanine and 7,8-dihydro-8-oxoadenine based on their further oxidation with one-electron oxidants including $IrCl_6^{2-}$ and $IrBr_6^{2-}$. The products of nucleobase oxidation lead to enhanced piperidine-sensitive cleavage and to highly visible stop points in a primer extension assay. 8-oxoG and 8-oxoA lesions may be distinguished by the latter's inability to be oxidized by $IrBr_6^{2-}$ compared to $IrCl_6^{2-}$ Comparison is also made to oxidation by MnO_4^-.

Oxidative damage to DNA nucleobases commonly results in the formation of oxidized purines, particularly 7,8-dihydro-8-oxoguanine (8-oxoG) and, to a lesser extent, 7,8-dihydro-8-oxoadenine (8-oxoA) (1,2). The importance of these 8-oxopurine lesions necessitates good analytical techniques for their detection in DNA fragments. Currently, these lesions are quantified by total DNA digestion followed by HPLC analysis using electrochemical detection, since both 8-oxoG and 8-oxoA are more redox active than the four unmodified nucleobases (3,4). Thc alternative detection of 8-oxoG by enzymatic recognition and cleavage with Fpg suffers from the complication that several purine lesions are substrates for the enzyme (5). Furthermore, detection of 8-oxopurines by primer extension and DNA sequencing experiments is highly dependent upon the polymerase used. With the high temperature, high fidelity polymerases suitable for PCR, no stops or misincorporations are observed, although some mutations can be seen with other DNA polymerases (6,7). A PCR-compatible method would be particularly useful since this allows sequencing of samples as low as 5 fmol in quantity. We report here two methods based on (i) piperidine-induced DNA strand scission or (ii) primer extension using a thermally stable DNA polymerase that allow detection of 8-oxoG or 8-oxoA by gel electrophoresis.

In 1992, Chung *et al*.. reported that treatment of an 8-oxoG-containing synthetic oligodeoxynucleotidc with standard piperidine conditions (1 M, 90°C, 30 min) led to strand scission at the 8-oxoG site observable by gel electrophoresis (8). Subsequent studies by Cullis *et al*. demonstrated that 8-oxoG was not a piperidine-labile site (6% cleavage after 2 h, 1 M piperidine, 90°C); substantially less cleavage was observed in the presence of added β-mercaptoethanol (β-ME) that might quench the over-oxidation of 8-oxoG (9). 8-OxoA has never been reported as a piperidine-labile lesion (8). These observations suggest that 8-oxoG can be detected with piperidine treatment only after its further oxidation, either inefficiently with aerobic dioxygen or efficiently by judicious selection of a one-electron oxidant via photochemical (9,10) or chemical means (11,12). We have found that Ir(IV) is a highly selective oxidant that exclusively reacts with oxidized nucleobases, and that the oxidized products of 8-oxoG and 8-oxoA are highly piperidine labile, as evidenced by strand scission, under standard conditions. Furthermore, the Ir(IV)-oxidized lesions are stop points for Thermo Sequenase DNA polymerase using commonly employed conditions for primer extension and DNA sequencing.

Reagents and substrates. Reagents were purchased from the following sources: Na_2IrCl_6 and Na_2IrBr_6 from Alfa Aesar, $KMnO_4$ and piperidine (fresh!) from Acros, 8-oxoG and 8-oxoA phosphoramidites from Glen Research, dNTPs from Pharmacia, T4 polynucleotide kinase from New England Biolabs, Thermo Sequenase cycle sequencing kit and [γ-^{32}P]ATP from Amersham. Oligodeoxynucleotides were synthesized with an Applied Biosystems synthesizer (ABI 392B) using the manufacturer's protocols and incorporating 0.25 M β-ME into the final, manual deprotection step of oligos containing 8-oxopurines (13). Purification was carried out by PAGE using 20% polyacrylamide/7 M urea, and masses of oligos containing 8-oxoG were confirmed by negative ion electrospray MS. Oligos were 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP.

Oxidation and piperidine-induced cleavage. Oxidation reactions were carried out on three duplex substrates of related sequence: 5′-d(TCATGGGTC**X**TCGGT**Y**TA)-3′ where oligo **1** is native (**X** = G, **Y** = A) and oligos **2** (**X** = 8-oxoG, **Y** = A) and **3** (**X** = G, **Y** = 8-oxoA) each contain one 8-oxopurine. Each strand was annealed to the same complement **4**, the unlabeled oligodeoxynucleotide complement of **1**, by heating 1.1 eq. **4** with **1**, **2** or **3** at 90°C for 3 min and cooling to room temperature over a period of 3 h. Oxidations were carried out with 3 μM unlabeled duplex + 2 nCi of radiolabeled **1**, **2** or **3** and 100 μM $IrCl_6^{2-}$, $IrBr_6^{2-}$ or MnO_4^- in 10 mM NaP_i buffer (pH 7) with 100 mM NaCl. Ir(IV) reaction times were 60 min; MnO_4^- reaction time was 30 min. Reactions were quenched by addition of 2 mM HEPES/10 mM EDTA, pH

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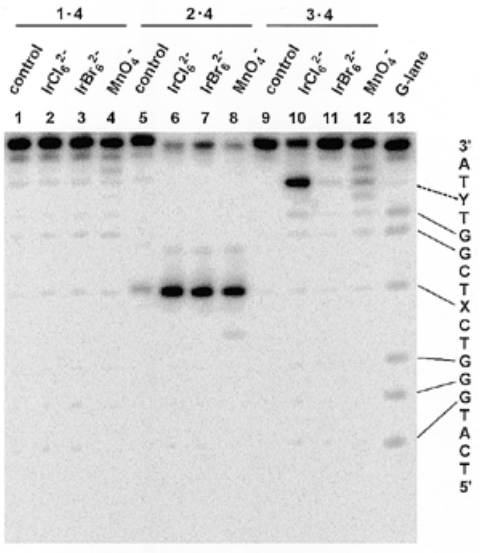

Figure 1. Analysis of piperidine-induced cleavage of duplex 18mers after oxidation of modified bases. All lanes were piperidine treated. Lanes 1–4: native duplex **1•4** (**X** = G, **Y** = A); lanes 5–8: **2•4** (**X** = 8-oxoG, **Y** = A); lanes 9–12: **3•4** (**X** = G, **Y** = 8-oxoA); lane 13: Maxam–Gilbert G lane. Reactions were conducted as described in the text. Control lanes were treated identically but without the addition of oxidant.

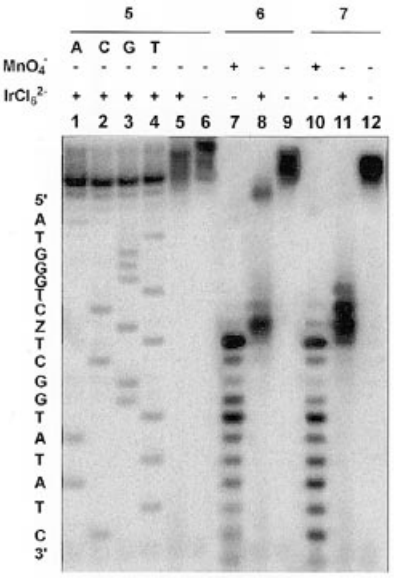

Figure 2. Analysis of extended primer complementary to 40mers after oxidation of modified bases. Lanes 1–6: native 40mer **5** (**Z** = G); lanes 7–9: **6** (**Z** = 8-oxoG); lanes 10–12: **7** (**Z** = 8-oxoA). Lanes 1–4 are Sanger sequencing lanes using ddTTP, ddGTP, ddCTP and ddATP, respectively. Reaction conditions are described in the text. Control lanes (6, 9 and 12) were not treated with oxidant.

7 for Ir(IV) and with 2 µl allyl alcohol for MnO_4^-. Reactions were individually dialyzed and lyophilized as previously described (14). Piperidine treatment was carried out with 1 M piperidine, 90°C, 30 min (14,15). Finally, fragments were separated by PAGE (20% polyacrylamide/7 M urea) and analyzed by phosphorimagery (Molecular Dynamics Storm 840) using ImageQuaNT software.

The results of oxidation and piperidine treatment of duplex 18mers are shown in Figure 1. Lanes 1–4 with a native duplex substrate show little background with Ir(IV) reagents and only slight reactivity of T1 (not shown) and T17 with MnO_4^-. Substrate **2** containing an 8-oxoG at position 10 was cleanly oxidized to a very piperidine-labile lesion by treatment with Ir(IV) (lanes 6 and 7). Without added oxidant, a faint band appeared after piperidine treatment (lane 5, 10% cleavage at 8-oxoG) that is presumably due to aerobic oxidation. MnO_4^- was also an effective oxidant (lane 8), although slight background reactions at T1 (not shown) and T8 were observed. Under the optimized conditions described above, both Ir(IV) reagents showed high conversion to one or more labile, oxidized products (lane 6, 80%, cleavage, lane 7, 70% cleavage at 8-oxoG) due to their high redox potentials, 0.90 V and 0.82 V versus NHE, respectively, compared to 8-oxoG (0.58 V versus NHE; ref. 16). MnO_4^- can react by either a one-electron mechanism ($E° = 0.76$ V versus NHE; ref. 17) or via dihydroxylation (or other mechanisms) with thymines, accounting for the background reactions of T in duplex DNA. Permanganate is particularly reactive with single-stranded thymine residues, and related studies of MnO_4^--mediated oxidation of 8-oxoG-containing single-stranded oligodeoxynucleotides additionally reported reactivity of guanines in the same strand (11,18). Thus, the utility of MnO_4^- for identifying 8-oxoG residues will be limited to duplex substrates.

Substrate **3** containing an 8-oxoA ($E° = 0.92$ versus NHE; ref. 16) at position 16 was oxidized by $IrCl_6^{2-}$ to a piperidine-labile lesion (Fig. 1, lane 10, 53% cleavage) but the weaker oxidizing agent $IrBr_6^{2-}$ was ineffective (lane 11). Oxidation with MnO_4^- was also largely ineffective at generating piperidine-sensitive cleavage at 8-oxoA (lane 12), but this may be due to conversion of 8-oxoA to a non-labile lesion (11). It is further noteworthy that the presence of a GGG duplex sequence of relatively low ionization potential (19) in the same strand did not compete with the 8-oxopurine sites during Ir(IV) oxidation. The results obtained for Ir(IV) now allow a convenient method for analysis of 8-oxoG versus 8-oxoA; both 8-oxopurines are visualized after oxidation with $IrCl_6^{2-}$ and piperidine treatment while only 8-oxoG leads to strand scission when oxidized with $IrBr_6^{2-}$. Importantly, the high conversion of 8-oxopurines to piperidine-labile lesions means that this method will be applicable to strands containing low percentage incorporation of 8-oxoG or 8-oxoA, and easily quantifiable cleavage will be observed.

Oxidation and primer extension analysis. $IrCl_6^{2-}$ and MnO_4^--mediated oxidation of three single-stranded substrates (1 µM, 15 µl) of sequence 5′-d(TCATGGGTC**Z**TCGGTATATCAGTGCTATCACATTAGTGTA)-3′ where **Z** = G (**5**). 8-oxoG (**6**) or 8-oxoA (**7**) were carried out as described above. For sequencing lanes, 3 µl aliquots of the over-oxidized DNA were combined with 2 µl (8 U) Thermostable Sequenase, 2 µl enzyme buffer (260 mM Tris–HCl pH 9.5, 65 mM $MgCl_2$), 2 µl 5′-end-labeled (2 µCi) primer [5′-d(TACACTAATGTGATAGCACT)-3′, 400 nM], and 8.5 µl H_2O. Then, 4 µl of this mixture was incubated with 4 µl of a ddNTP termination mix as described in the Thermo Sequenase cycle sequencing kit. For full extension, a 4 µl aliquot of the over-oxidized DNA was combined with 4 µl of a mixture containing 3 µl dNTP solution (1 mM each) plus enzyme, buffer, primer and water as above. After an initial incubation at 80°C for 10 min, samples were thermally cycled 15 times at 94°C (1 min), 55°C (1 min) and 72°C (1 min). At this point, 7 µl stop solution

(Thermo Sequenase kit) was added to each sample. Samples were heated at 95°C for 3 min, and then analyzed by PAGE (20% polyacrylamide/7 M urea) and imaged as above.

Table 1. DNA cleavage and polymerase stops observable (+) for 8-oxopurines by various methods

	8-oxoG	8-oxoA
Piperidine/β-ME	–	–
$IrCl_6^{2-}$ then piperidine	+	+
$IrBr_6^{2-}$ then piperidine	+	–
Sequenase	–	–
$IrCl_6^{2-}$ then Sequenase	+	+

Results of primer extension and DNA sequencing experiments are shown in Figure 2. As anticipated, substrates **5–7** led to complete extension when they were not subjected to further oxidation (lanes 6, 9 and 12). Treatment of the native strand **5** with $IrCl_6^{2-}$ also led to complete extension (lane 5), and sequencing lanes (1–4) confirm the fidelity of the strand, indicating that $IrCl_6^{2-}$ has no effect on unmodified nucleobases. In contrast, use of $IrCl_6^{2-}$ to further oxidize 8-oxoG (lane 8) or 8-oxoA (lane 11) leads to a clearly identifiable stop band at the site of the 8-oxopurine. Sequencing lanes for the $IrCl_6^{2-}$ reactions also confirmed the fidelity of the strand up until the stop point (data not shown). On the other hand, MnO_4^- oxidation occurred at many bases in addition to 8-oxoG (lane 7) and 8-oxoA (lane 10). Curiously, the stop points of polymerization after oxidation by $IrCl_6^{2-}$ versus MnO_4^- differ by one nucleotide with permanganate causing stops one base before that of iridate. This might suggest different oxidation products formed from these two reagents, and this possibility is under further investigation.

In summary, Ir(IV) appears to be the oxidant of choice for visualization of 8-oxopurines by gel electrophoretic methods employing either piperidine-sensitive cleavage or interruption of primer extension. Control of the oxidizing power of Ir(IV) allows differentiation of 8-oxoG and 8-oxoA, the latter being unreactive towards $IrBr_6^{2-}$. Since a number of other purine lesions (imidazolone, oxazolone, formamidinopyrimidines) (9,20) and pyrimidine lesions (5-hydroxypyrimidines, 5-formyluracil) (21) are known piperidine-labile sites (22) this method will be most informative when analysis is performed both with and without Ir(IV) treatment (Table 1).

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