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Formation and repair of oxidatively generated damage in cellular DNA

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Abstract

In this review article, emphasis is placed on the critical survey of available data concerning modified nucleobase and 2-deoxyribose products that have been identified in cellular DNA following exposure to a wide variety of oxidizing species and agents including, hydroxyl radical, one-electron oxidants, singlet oxygen, hypochlorous acid and ten-eleven translocation enzymes. In addition, information is provided about the generation of secondary oxidation products of 8-oxo-7,8-dihydroguanine and nucleobase addition products with reactive aldehydes arising from the decomposition of lipid peroxides. It is worth noting that the different classes of oxidatively generated DNA damage that consist of single lesions, intra- and interstrand cross-links were unambiguously assigned and quantitatively detected on the basis of accurate measurements involving in most cases high performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry. The reported data clearly show that the frequency of DNA lesions generated upon severe oxidizing conditions, including exposure to ionizing radiation is low, at best a few modifications per 10^6 normal bases. Application of accurate analytical measurement methods has also allowed the determination of repair kinetics of several well-defined lesions in cellular DNA that however concerns so far only a restricted number of cases.

Graphical Abstract

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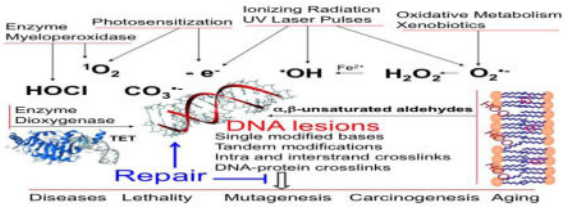

Keywords

Single base oxidation modifications; DNA oxidative degradation pathways; measurement of oxidatively generated DNA damage; repair of oxidized bases

Introduction

Oxidatively generated damage to cellular DNA is ubiquitous, being associated with oxidative, metabolism through the initial generation of the low reactive superoxide anion radical ($O_2^{\bullet -}$), the product of one-electron reduction of oxygen during aerobic respiration [1]. Subsequently, $O_2^{\bullet -}$ can be converted through enzymatic dismutation to hydrogen peroxide (H_2O_2) that, like its precursor, exhibits a very low reactivity toward biomolecules while it is involved in redox signaling. However, transition metals such as ferrous ions are able to reduce H_2O_2 according to the Fenton reaction giving rise to highly reactive hydroxyl radicals ($^{\bullet}OH$) that are implicated in most oxidative degradation pathways of cellular DNA under physiological may be exacerbated during inflammation and under various conditions. The formation of $O_2^{\bullet -}$ pathological situations including arteriosclerosis, type 2 diabetes, cancer and neurodegenerative diseases [2–5]. This is often accompanied by the activation of NO-synthases that triggers the release of nitric oxide ($^{\bullet}NO$) another poorly reactive radical although it can induce the generation of a powerful oxidant peroxynitrite ($ONOO^-$), a reactive nitrogen species (RNS), through the efficient combination of $^{\bullet}NO$ and $O_2^{\bullet -}$ [7]. The UVA component of solar radiation, a major environmental factor, is also able to damage DNA mostly through the formation of singlet oxygen ($^1O_2$), another reactive oxygen species (ROS) [8,9]. Ionizing radiation acting as both a generator of $^{\bullet}OH$ and a strong one-electron oxidant has been considered as the most biologically relevant oxidizing agent of DNA for more than 20 years since mid-50's [10–12]. Bystander effects that are mediated by the delayed generation of $^{\bullet}OH$ and $^{\bullet}NO$ have been shown to damage DNA in neighboring cells despite the fact that they are not directly hit with UV and ionizing radiations [13–16].

Earlier studies on the formation of oxidatively-induced damage to DNA have focused on pyrimidine bases mostly for analytical reasons. The discovery and tentative characterization of two isomers of 5(6)-hydroperoxy-6(5)-hydroxy-5,6-dihydrothymine in 1959 [17] provided a strong impetus toward mechanistic studies on oxidative degradation pathways of thymine, uracil and cytosine in the subsequent 20 years. Efforts were also made on the basis of gas chromatography-mass spectrometry measurements to gain information on the main $^{\bullet}OH$-mediated reactions of 2-deoxyribose moiety involved in the formation of DNA strand breaks [18,19]. The advent of high performance liquid chromatography (HPLC) a powerful separation tool largely replacing paper and thin-layer chromatography in the mid 70's allowed for the complete separation of complex mixtures of oxidized nucleosides

including thymidine hydroperoxides, polar degradation products of 2'-deoxyguanosine and modified short oligonucleotides. The development of other spectrometric methods included both 1-D and 2-D high field ^{1}H, ^{13}C and ^{15}N NMR and soft ionization mass spectrometry methods such as electrospray ionization. These advances permitted the structural assignment of complex oxidation products arising from polar purine components and more relevant DNA substrates such as short oligonucleotides. In addition, efforts were made to delineate the reactivity of defined base or sugar radicals upon their mild generation usually by UV activation of a photolabile precursor at a specific site within a defined sequence DNA fragment [20,21]. Theoretical calculation approaches gradually emerged through the development of highly performant *ab initio* DFT based computational and simulation methods, all of which have provided relevant information about the reactivity and energy requirements of key intermediates, which is not accessible experimentally [22–25]. Based on detailed structural and mechanistic studies, one may propose comprehensive oxidative degradation pathways of the main nucleic acid components upon exposure to $^{1}O_{2}$, $^{\bullet}OH$, one-electron oxidants, halogenating agents and cellular dioxygenases; this information has been recently critically reviewed in exhaustive surveys. In parallel, there have been numerous attempts to search for the presence of some of the oxidation products in cells and tissues that were previously characterized in model studies. As will be briefly reviewed in a subsequent section, most of the early assays that were designed and applied for the analysis of certain products for decades suffer from major drawbacks, often leading to an overestimation of the levels of base oxidatively induced modifications of up to two or three orders of magnitude. Several of these inaccurate measurements are still being used to support putative correlations involving possible biomarkers of oxidatively induced DNA lesions and biological end-points related to aging, neurological disease and cancer risk. The main aim of the present review article is to provide an updated overview of the current situation concerning the accurate characterization and quantitative formation of mostly oxidatively induced base modifications in nuclear DNA of cells and tissues. Emphasis is placed on HPLC coupled to electrospray ionization-tandem mass spectrometry (ESI-MS/MS) as the gold standard of analysis because of its accuracy, versatility and reproducibility, taking into consideration that DNA extraction and subsequent work-up remain critical sources of spurious oxidation of inherent and overwhelmingly abundant DNA components present in the analysis..

Main reactive endogenous and exogenous cellular oxidizing species and agents

Oxidatively generated damage to DNA may occur directly or indirectly through the action of various oxidizing species that result from metabolic processes and/or exposure to diverse chemical and physical agents. A brief description of the main biologically relevant oxidants that are able to trigger DNA degradation pathways is provided below together with information on their mode of action and preferred targets.

Reactive oxygen species

Hydroxyl radicals ($^{\bullet}OH$) are endogenously produced in cells by Fenton type-driven reactions and they react efficiently with most molecules at the site of generation. This explains why $^{\bullet}OH$ cannot be scavenged effectively, in contrast to numerous incorrect claims that

examine the addition of antioxidants to the cellular medium [26]. A convenient source of $^{\bullet}OH$ used to investigate radical-mediated oxidation reactions of cellular DNA involves the indirect effect of ionizing radiation through the radiolysis of bound water molecules. It is well documented from extensive model studies that $^{\bullet}OH$ reacts predominantly by adding to the 5,6-pyrimidine and 7,8-purine double bonds of nucleobases giving rise to radical intermediates to which O_2 may subsequently add or act as an one-electron oxidizing agent [27]. A second prominent oxidation reaction for thymine and 5-methylcytosine consists of $^{\bullet}OH$-mediated hydrogen abstraction from the methyl group leading to the formation of 5-(uracilyl) and 5-(cytosyl) methyl radicals, respectively [28,29]. Another efficient competitive $^{\bullet}OH$ reaction when considering the nucleotide monomeric units is the abstraction of a hydrogen atom from the three methine carbons and the 5-hydroxymethyl group of 2-deoxyribose, all being reactive sites due to the presence of a vicinal O atom [27]. The formation of carbon centered radicals at C3 and C5 followed in aerated aqueous solutions by the generation of peroxyl radical intermediates lead to the quantitative formation of strand breaks. This is not the case for the abstraction reaction initiated at C1 that gives rise to 2-deoxyribonolactone, also called an oxidized abasic site [19,30–32]. It may be noted that chemical reactions initiated by the formation of C4 radicals are more complex. One of the degradation products has been identified as 2-deoxypentos-4-ulose, another oxidized abasic site. In addition, a competitive oxidative pathway leads to the formation of DNA strand break bearing a 3'-phosphoglycolate residues with the concomitant release of malondialdehyde and non- modified nucleobase [31, 33,34]. However, large disparities have been observed in the quantitative formation of the various sugar oxidation products measured in isolated DNA using either an isotope-dilution gas chromatography-mass spectrometry assay [32] or a more recently designed HPLC-based method [34].

As already pointed out, H_2O_2 and $O_2^{\bullet-}$ together with its conjugate acid, the related minor hydroperoxide radical ($HO_2^{\bullet}$), do not exhibit any significant reactivity toward nucleobase and 2-deoxyribose DNA components in aqueous solutions DNA. However, it has been shown that $O_2^{\bullet-}$ but not O_2 [35,36] is able to react with the highly oxidizing radicals $G(-H)^{\bullet}$ that arise from deprotonation of the guanine radical cation by two competitive reactions [37]. Predominant addition of $O_2^{\bullet-}$ to $G(-H)^{\bullet}$ gives rise through a complex sequence of reactions leading to 2,2,4-triamino-5(2*H*)-oxazolone (Oz) [38] while relatively minor one-electron reduction leads to restoration of the guanine base in a chemical repair process. Similar $O_2^{\bullet-}$ addition reactions have also been observed for other highly oxidizing radicals that are derived from the one-electron oxidation of tyrosine and tryptophan, respectively [39,40].

Singlet oxygen ($^1O_2$) in the $^1\Delta$ state is another biologically relevant ROS that is mostly generated in cells by type II photosensitization mechanism through energy transfer from triplet excited molecules to molecular oxygen. One typical reaction involves endogenous photosensitizers, which upon excitation by the UVA component of solar radiation, lead to the predominant generation of $^1O_2$ as the main source of cellular DNA oxidation. Evidence has been provided from extensive model studies that the recombination of peroxyl radicals of various biomolecules can lead to the release of $^1O_2$ as the result of decomposition of transient tetroxides according to the concerted Russell mechanism [41]. However, no

evidence for the occurrence of such a mechanism has been found so far in cells. As a dienophile, $^1O_2$ reacts preferentially with unsaturated compounds that possess electron rich double bonds. This is the case with guanine as being the exclusive nucleic acid component susceptible to react with $^1O_2$ in aqueous solutions. Interestingly, synthetic naphthalene endoperoxides that are able to release pure $^1O_2$ upon gentle warming, and that can be labeled with stable isotopes (i.e., $^{18}O_2$), are available for mechanistic studies in both aqueous solution and cellular DNA [42,43].

Pyrimidine peroxyl radicals

In general, peroxyl radicals are weak oxidants and they tend to react very slowly with DNA. However, the situation is different for pyrimidine peroxyl radicals attached to oligonucleotides which, upon an initial radical reaction triggered by either $^{\bullet}OH$ or one-electron oxidants in aqueous solution, can induce additional damage as shown initially by the pioneering work of Box et al [43]. Evidence has been provided through detailed experimental and theoretical mechanistic studies that 5-(6)-hydroperoxy-5,6-dihydropyrimidyl radicals are able to add to vicinal guanine bases in a sequence dependent manner with a strong preference toward purines located on the 5'-end [45–47]. The use of [^{18}O]-labeled molecular oxygen showed that an ^{18}O atom is transferred from the peroxyl group of thymine to the C8 of vicinal guanine giving rise to a tandem nucleobase lesion consisting of formylamine and 8-oxo-7,8-dihydroguanine (8-oxoGua) [46]. It was estimated from an extensive mechanistic study that about 50% of 8-oxo-7,8-dihydropurine bases, including predominantly 8-oxoGua and to a lesser extent 8-oxo-7,8-dihydroadenine (8-oxoAde), are generated as part of tandem base lesions upon exposure of DNA to $^{\bullet}OH$ in aerated aqueous solutions [48]. As an initial study addressing the repair of oxidatively generated tandem base lesions, the two sequence isomers of vicinal 8-oxoGua and formylamine clustered damage were partly refractory to enzymatic removal by common base excision repair (BER) enzymes: endonuclease III and formamidopyrimidine DNA *N*-glycosylase [49]. Evidence was provided that 5-hydroperoxy-6-hydroxy-5,6-dihydrothyminyl, one of the two main peroxyl radicals induced by one-electron oxidation of a pyrimidine base in a thymine doublet, is able to abstract a hydrogen atom from the methyl group of the vicinal nucleobase. This was found to give rise to tandem base lesions consisting of 5,6-dihydroxy-5,6-dihydrothymine (ThyGly) on one side and either 5-formyluracil (5-FoUra) or 5-hydroxymethyluracil (5-HmUra) on the adjacent nucleobase [50]. Other examples of formation of oxidatively generated tandem lesions through the initial formation of defined peroxyl pyrimidine radicals in synthetic oligonucleotides using suitable photo-labile radical precursors are available. For example, evidence was provided that pyrimidine peroxyl radicals are also able to add to 3' or 5'-contiguous thymine nucleobases, thus generating tandem base modifications [51,52]. As a competitive minor reaction, peroxyl pyrimidine radicals were shown to abstract a hydrogen atom from the anomeric proton of the adjacent nucleoside leading to the formation of 2-deoxyribonolactone as part of a tandem sugar-nucleobase lesion. It was also demonstrated, in more recent model studies, that the peroxyl radical specifically generated in double stranded DNA fragments from appropriate photochemical precursor at C4 of a 2-deoxyribose moiety is able to induce the formation of DSBs [53,54]. However, no reports have so far been reported on the formation of the above described oxidatively-induced

tandem lesions in cellular DNA. This may be at least partly explained by the lack of sufficient sensitivity using standard HPLC-ESI-MS/MS instruments.

One-electron oxidants

Ionizing radiation, through its direct interaction with DNA components in the solid state, was the first physical agent used to abstract an electron from purine nucleobases and generate the corresponding base radical cations [55,56]. However, a different strategy was applied in aqueous solutions to gain mechanistic insights into one-electron oxidation degradation pathways of nucleobases whose susceptibility to undergo ionization decreases in the following order guanine > adenine > thymine > cytosine as an inverse correlation with their reduction potential [57]. Several type I photosensitizers including 2-methyl-1,4-naphthoquinone, riboflavin and benzophenone were selected for performing one-electron oxidation experiments [8,28]. This approach has allowed detailed mechanistic studies of the radical cations of purine and pyrimidine nucleobases in aqueous solution either as free components or when inserted into DNA. Another suitable photochemical technique to generate base radical cations consists of exposing DNA or its constituents to high intensity 266 nm nanosecond laser pulses. Increase in the pulse intensity favors bi-photonic ionization of the bases at the expense of [2+2] photocycloaddition of pyrimidine nucleobases. Increasing interest is devoted to the carbonate anion radical ($CO_3^{\bullet -}$) as a biologically relevant one-electron oxidant that is likely efficiently produced during inflammation. Favorable coupling of $O_2^{\bullet -}$ and $^{\bullet}NO$, emanating from the activation of NO-synthase gives rise to peroxynitrite that upon reaction with CO_2 generates nitrosoperoxycarbonate, the precursor of $CO_3^{\bullet -}$ and $^{\bullet}NO_2$ [58,59]. Purine and pyrimidine radical cations, thereby generated by these different agents, are able to undergo two main competitive reactions including deprotonation and hydration. Interestingly, the pathways leading to stable products from one-electron oxidants of nucleobases in aerated aqueous solutions converge with those of $^{\bullet}OH$-induced decomposition. As a consequence, many of the products of the two pathways are identical although there are marked differences in the yield of unstable intermediates and the final distribution of products. However, a peculiar property of the guanine radical cations, in addition to their ability to undergo hydration [60], is their tendency to react with other nucleophiles. A likely nucleophile in chromatin involves histone abundant lysine residues bearing a primary amino group, that can give rise to DNA-protein crosslinks [61,62]. Alternatively, the N3 atom of vicinal intrastrand thymine and opposite cytosine can yield intra and interstrand crosslinks [63–65]. Here, we will only discuss GT intra-strand cross-links that have been characterized so far in cellular DNA upon exposure to high intensity UVC laser irradiation [66].

One electron oxidation of adenine generates the adenine radical cation, which rapidly undergoes deprotonation to the neutral N6-adeninyl radical [67]. One- and two-photon photolysis of DNA-A tracts with energies inferior to the ionization potential were recently shown to generate *N*6-centered radicals nearly quantitatively in low temperate glasses [68]. The reaction of hypochlorite with adenine and cytosine in DNA, RNA and polynucleotides also lead to *N*-centered radicals through the decomposition of intermediate chloramines [69]. The chemistry of *N*-centered radicals of 2'-deoxyadenosine (dA) was studied by exposure of the corresponding photolabile phenylhydrazone derivatives to near-UV light

[70]. The major reactions of the nucleoside involved H-atom abstraction to regenerate dA, deamination to give 2'-deoxyinosine, and the formation of dA-dA dimers as determined by mass spectrometry analyses. Mason and co-workers [71] have developed an immune spin-trapping technique to detect DNA radicals as DMPO-DNA nitrone adducts by electron spin resonance (ESR). Using this method, they identified the same N^6-adduct of adenine as above by ESR and MS analyses upon exposure of purified DNA and cellular DNA to $CuCl_2$-H_2O_2 [72]. The characterization in cellular DNA of one-electron oxidation products of adenine with the exception however of 8-oxoAde and 4,6-diamino-5-formamidopyrimidine (FapyAde) awaits further experiments.

Peroxidases

Several mammalian heme peroxidases including myeloperoxidase (MPO), lactoperoxidase (LPO) and eosinophil peroxidase (EPO) are able to trigger the formation of hypohalous acid in inflammatory cells through the H_2O_2-mediated oxidation of related halides and pseudo halides including thiocyanate[73–76]. MPO-mediated oxidation reactions released from activated leukocytes catalyze the formation of hypochlorous acid (pKa = 7.46) in equilibrium with hypochlorite (OCl^-), andare very efficient to kill invading pathogens [77]. Evidence has been provided that both HOCl and hypobromous acid (HOBr) are efficient halogenating agents of cytosine, adenine and guanine in model compounds and isolated DNA and RNA [78–80]. *N*-Chloramines that may be generated by the reaction of HOCl with the amino groups of amino are also able to chlorinate nucleobases albeit with a lower efficiency than HOCl [81].

Measurement of oxidatively generated damage in cellular DNA

Much effort has been made during the last five decades to develop assays aimed at monitoring the formation of oxidatively in cellular DNA following. Early attempts at the beginning of the 70's focused on the measurement of radiation-induced 5,6-dihydroxy-5,6-dihydrothymine (ThyGly) also called "thymine glycol". For this purpose cellular DNA was pre-labeled with [^{3}H]- or [^{14}C]-thymidine before being subjected to gamma or X-irradiation. The formation of ThyGly was assessed indirectly as either 2-methylglycerol or acetol that arises from borohydride reduction [82] and alkali degradation treatment, respectively [83]. Thus, various attempts were made to monitor the formation and repair of ThyGly in bacterial and mammalian cells exposed to ionizing radiation [82,84,85]. These authors reported that ThyGly was generated in cellular DNA upon exposure to either UV radiation [83,85] or hematoporphyrin photosensitization [86]; however, these conditions do not trigger thymine oxidation. It was then confirmed that both assays suffered from major flaws. In particular, the occurrence of self-radiolysis most likely led to an overestimation of ThyGly by three to four orders of magnitude, thus explaining the previously mentioned inconsistencies [87]. Since then, numerous chromatographic based assays together with more global methods including immunological approaches and enzymatic assays were developed. More recently with the discovery of 5-hydroxymethylcytosine (5-HmCyt) as a relevant epigenetic mark, numerous efforts have been devoted to the design of next-generation sequencing and array-based hybridization methods capable of mapping this modified base and its oxidation products at the nucleoside level in the genome [88–90].

Detailed information on these different methods is available in several recently published comprehensive review articles [91–94]. In the present contribution, a brief summary of the respective strengths and limitations of the main methods that have been recently or are currently utilized is provided.

Immunoassays

Many attempts have been made over the years to develop immunological based methods including among others, the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA) for measuring oxidatively generated base damage in DNA. Initial efforts focused on the preparation of monoclonal and polyclonal antibodies directed against ThyGly [95–96]. Subsequently, interest was devoted to design immunoassays for detecting 8-oxoGua [97–99] after discovery of this guanine oxidation product [100]. As a common observation, the main limitation of the designed ELISA and radioimmunoassays (RIA) is the lack of specificity due to the weak antigenicity of single oxidized bases in DNA that show a notable cross-reactivity with the overwhelming natural base precursors within the 10^{-4} to 10^{-5} range. This is insufficient to detect low physiological levels of ThyGly, 8-oxoGua as well as most other oxidized bases in cellular DNA on the order of a few modifications per 10^{6} normal nucleosides. This has also been demonstrated for a monoclonal antibody directed against 5',8-cyclo-2'-deoxyadenosine (cdA), which is a bulkier tandem base-sugar modification [101]. In contrast, immunological detection of 5-HmCyt enzymatically generated in embryonic and neuronal cells is routinely achieved with 0.1 % of this modification with respect to cytosine [102–103]. Similarly, immunological detection of 5-formylcytosine (5-FoCyt) and 5-carboxycytosine (5-CaCyt) generated through iterative oxidation is not accurate because the modifications are present at least 100-fold less than 5-HmCyt. Antibodies against 8-oxoGua are still widely used for sensitive histochemical immunofluorescence detection in human and animal tissues with various clinical applications and basic research involving DNA repair [104–109]. However the specificity of these antibodies remains to be established under in vivo conditions. It may also be pointed out that the measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in human fluids including urine, plasma and saliva has been achieved by competitive ELISA using N45.1 monoclonal antibody [110,111]. It was recently confirmed that the immunological detection of 8-oxodG in urine is less quantitative than that achieved by HPLC-ESI-MS/MS due to the presence of interfering contaminants such as D-glucose and D-galactose [112,113].

Enzymatic assays

Enzymatic based methods have become available with the isolation, characterization and cloning of DNA repair enzymes with emphasis on DNA *N*-glycosylases that remove oxidatively generated nucleobase lesions. The aim of enzymatic approaches, which include the alkaline single cell gel electrophoresis assay, also called "comet assay" [114–117], the alkaline elution technique [118,119] and the alkaline unwinding assay [120] is to convert oxidized nucleobases in a more or less specific way into strand breaks through the transient formation of alkali-sensitive abasic sites. The quantitation of the strand breaks generated during the analytical step is achieved in a very sensitive manner under alkaline conditions using fluorescence emission measurement after appropriate dye staining. The assays in their

initial version allows for the quantitative detection of direct DNA nicks and so-called alkali-labile lesions including normal and oxidized abasic sites, ThyGly, 5-foUra, Oz), FapyAde and to a lesser extent 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) [121]. Additional strand breaks can be induced upon incubation of released oxidized DNA with the BER enzymes. Thus, bacterial formamidopyrimidine DNA *N*-glycosylase (Fpg) is used to recognize and indirectly measure 8-oxoGua, FapyGua and FapyAde [114,122]. The use of human 8-oxoguanine glycosylase 1 (hOGG1) made the detection of oxidized purine bases more specific since only 8-oxoGua and FapyGua are substrates for this enzyme [123]. In a complementary way, endonuclease III (endo III) is able to cleave the *N*-glycosidic bond of several oxidized pyrimidine nucleobases including ThyGly and 5-methyl-5-hydroxyhydantoin (HydThy) and related common oxidation products of cytosine such as 5,6-dihydroxy-5,6-dihydrouracil (UraGly) and related cytosine oxidation products. Usually, external calibration of the assays whose application requires only a small number of cells is carried out in parallel using ionizing radiation as a standard source of oxidatively generated damage to DNA assuming that the exposure of cellular DNA to 1 Gy gives rise to 3 single strand breaks and alkali-labile sites per 10^6 nucleosides [124]. One of the main advantages of these enzymatic assays with respect to HPLC-ESI-MS/MS assays is their high sensitivity, which benefits from the almost lack of adventitious oxidation of DNA samples during DNA release and subsequent analysis. This largely explains why the steady-state levels of 8-oxoGua in human cells obtained by DNA repair glycosylases are about 7 to 10 lower than those measured by either HPLC-ECD or HPLC-ESI-MS/MS [125–127]. It was also shown that Fpg-sensitive sites accumulate with age in the DNA of primary embryo fibroblast cultures [126] and hepatocytes from *ogg1*$^{-/-}$ nul mice [128]. Another relevant application dealt with the measurement of radiation-induced formation of Fpg- and endo III-sensitive sites in the DNA of human monocytes showing a linear increase within the dose range 0.1 – 0.5 Gy [129]. Enzymatic detection assays have several shortcomings that may slightly affect their general suitability. One problem concerns the lack of specificity of repair enzymes since quantitative information is provided on classes of damage and not on individual lesions. There are also uncertainties about the efficiency of the release of enzyme-sensitive sites since the ability to repair oxidized bases as part of tandem lesions is not as efficient as that of isolated oxidized bases [48,49]. In addition to applications already mentioned, the measurement of Fpg and endo III-sensitive sites has enlightened detailed mechanistic studies on oxidatively induced damage to cellular by UVA and visible light in which endogenous photosensitizers are implicated [8,9,115,130,131]. Increasing interest has also been devoted to human biomonitoring of genotoxic effects of environmental agents in particular nanomaterials [132] using the modified comet assay.

Gas chromatography-mass spectrometry (GC-MS)

Capillary gas chromatography coupled to mass spectrometry in the selective ion monitoring mode was introduced in the mid 80’s for measuring the formation of radiation-induced modifications of nucleobases including 12 oxidation products in DNA and model compounds. This method involves the strong acidic hydrolysis of DNA with concentrated formic acid (88%) at 150°C for 30 to 40 min in order to liberate DNA modifications as a free base [133].

Subsequently, the overwhelming normal nucleobases and related minor oxidation products are converted to trimethylsilylated derivatives by heating at 140°C for 30 min before GC-MS analysis. A different protocol at least in the initial step [134] was developed to monitor the formation of 5',8-cyclo-2'-deoxyguanosine (cdG) in isolated [135] and cellular DNA [136]. This involved the enzymatic release of the purine 5',8-cyclonucleosides using a cocktail of exonucleases and exonucleases. The acid based protocol was applied to the measurement of oxidatively generated base damage in cellular DNA a few years later [137]. Rapidly, major discrepancies up to 2 orders of magnitude became apparent between the yield of 8-oxoGua measured by GC-MS and HPLC coupled to electrochemical detection (ECD) [138], a method that is discussed in the next section. Several groups showed that the derivatization step appeared to be a major source of artefactual oxidation giving rise to the usual $^{\bullet}$OH-mediated oxidation products with a frequency of close to 10^{-4} (for a review article, see Cadet et al 1997 [139]. Another major drawback of acid hydrolysis of DNA is that it leads to the degradation of many oxidatively generated bases including FapyGua and FapyAde as it was unambiguously demonstrated [140]. Therefore, a mild acidic treatment involving hydrogen fluoride stabilized in pyridine has designed and when combined with a HPLC pre-purification step was shown to render quantitative GC-MS determination of both FapyAde and FapyGua in cellular DNA. An alternative version of the GC-MS assay allows one to overcome these two major shortcomings [141]. This version involves an alternative step to acid hydrolysis that is the enzymatic release of oxidized nucleobases by DNA repair *N*-glycosylases. Although this assay is very promising, there have only been a few recent applications [142,143]. Unfortunately, the analyses of DNA oxidatively modified lesions until mid-2000s' were dominated by the method using acid hydrolysis and GC-MS analyses giving way to a flood of unreliably high values and misleading correlations between the level of oxidized nucleobases and biological end-points, such cancer risk, aging and repair [144–147].

It is surprising that GC-MS analysis despite its well-documented shortcomings has received recent notice with questionable frequencies of 48 8-oxoG and 35 FapyG per 10^6 bases in the DNA of wild type strains of *Caenorhabditis elegans* [148].

High performance liquid chromatography-electrochemical detection (HPLC-ECD)

An assay for the sensitive detection of 8-oxodG, an oxidation product of 2'-deoxyguanosine [100] was developed using HPLC coupled to electrochemical detection (HPLC-ECD) operating in the oxidative mode [149]. This analytical method provided a strong impetus to the analysis of oxidatively induced lesions in cellular DNA. Very rapidly, this method became suitable for the detection of 8-oxodG in cellular DNA upon exposure to oxidizing agents [150,151] and in kidney DNA of rats treated with potassium bromate [152] a well-documented one-electron oxidant agent once metabolized. These studies together with numerous HPLC-ECD measurements during the following 15 years have fully established 8-oxodG as a relevant biomarker of DNA. This received further support from the comparative evaluation of the available methods for measuring 8-oxodG by the European Standard Committee on Oxidative DNA Damage (ESCODD), a consortium of 25 laboratories [127,153]. ESCODD concluded that both HPLC-ECD and HPLC-MS/MS unlike GC-MS in its basic version constitute robust methods. However, and this will be discussed in the next

section, DNA extraction and the subsequent work-up before HPLC measurements represent critical steps in which adventitious oxidative processes can occur with natural and relatively highly abundant nucleobases. In this respect, attempts were made to optimize the extraction step using the chaotropic method (i.e., with NaI as a salting out agent) and to minimize artefactual contribution [154]. It may be added that other electrochemically active modified nucleobases and nucleosides including 5-hydroxyuracil (5-OHUra), 5-hydroxycytosine (5-OHCyt), 8-oxoAde and FapyGua can also be detected by HPLC-ECD using higher oxidation potentials [155–157].

High performance liquid chromatography - mass spectrometry (HPLC-MS)

Early methods using one quadrupole (HPLC-MS) for the measurement of several oxidized 2'-deoxyribonucleosides usually show a limit of detection in the picomol range. This is clearly insufficient to detect oxidatively generated damage to cellular DNA under conditions where 30 to 50 μg of DNA are available and where the frequency of individual modifications reaches a few lesions per 10^6 nucleosides, a level that may only concern the most important modifications such as 8-oxoGua and ThyGly. Accordingly, it is rather surprising that attempts were made to monitor the formation of several radiation-induced oxidized nucleosides in cellular DNA through HPLC-MS measurements. The levels of reported 8-oxoGua and 8-oxoAde obtained by single quadrupole HPLC-MS, however, were between 50- and 100-fold greater [158,159] than those determined by HPLC-MS/MS [160,161]. Similarly, relatively high values for the 5*R* and 5*S* diastereomers of minor 5',8-cyclo-2'-deoxyribonucleosides were reported on the basis of HPLC-MS measurements[162]. Other questionable results still obtained by HPLC-MS analysis suggested that Sp, a secondary oxidation of 8-oxoGua, was generated preferentially at the expense of its precursor in *Escherichia* (*E.) coli* strains exposed to one-electron oxidants [163]. The apparent formation of elevated values of 8-oxodG, 8-oxodA and 5',8-cyclodA are explained by the erroneous detection provided by the use of only one quadrupole. Under these conditions, the possibility of detecting independent fragments arising from electrospray ionization of trace levels of impurities may give misleading and false identification of the targeted lesions in the selective ion monitoring (SIM) mode. So far, the only oxidized 2'-deoxyribonucleoside that has been accurately detected by HPLC-MS is 5-(hydroxymethyl)-2'-deoxycytidine (5-HmdC), the ten-eleven translocation (TET) mediated oxidation product of 5-methyl-2'-deoxycytidine (5-mdC) that accumulates in cellular DNA to the level of a few lesions per 10^3 nucleosides in embryonic stem cells (ESC) and brain tissues as detected by HPLC-MS [164,165]. However, attempts to detect 5-formyl-2'-deoxycytidine (5-FodC), the iterative enzymatic oxidation product that is present at the level of about one tenth to one hundred lower yields are problematic. This can be successfully achieved through by HPLC-ESI-MS/MS measurement [166] as is also the case for 5-carboxy-2'-deoxycytidine (5-CadC) [167] the more extensive oxidation product of 5-FodU.

High performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS)

HPLC-ESI-MS/MS and the sensitive HPLC-MS^3 version are recognized as gold standard methods for monitoring the formation of modified nucleosides in biological samples, including nuclear DNA and biological fluids such as urine, plasma and saliva [91–93,168–172].The success of this method lies in the versatility and sensitivity of electrospray

ionization that allows the efficient detection of a large number of oxidized DNA components with an optimum sensitivity that, on the average, extends into the femtomole range for modified 2'-deoxyribonucleosides using accurate multiple reaction monitoring mode [91,92]. Furthermore, the availability of synthetic [^{13}C]- and [^{15}N]-labeled internal standards allows for the use of isotope dilution as a means to correct for any losses during DNA digestion and changes in ionization efficiency during analysis [173–175]. Numerous successful HPLC-ESI-MS/MS measurements have been made to monitor the formation of several single and tandem oxidatively generated damage in cellular DNA following the initial detection of 8-oxodG in nuclear DNA and urine [176,177]. Although the method is highly accurate, there remains one major difficulty that restricts the use of HPLC-ESI-MS/MS. As already mentioned for other chromatographic methods, this is due to the unavoidable spurious oxidation of normal DNA bases (likely through the involvement of Fenton type reactions during DNA extraction and subsequent work-up) in particular during enzymatic digestion and release of individual DNA components. It should be noted that DNA is an excellent metal chelator, and thus, will bind potentially redox active metal ions during DNA extraction and release them during DNA digestion. Therefore, special attention was given during the ESCODD trial and subsequent studies [178–180] to develop methods to minimize the occurrence of artefactual oxidation. So far, this was partly achieved by the addition of transition metal chelators and anti-oxidants to the DNA sample extracts and digests prior to HPLC-MS/MS analysis. One major critical parameter that requires special attention is the amount of DNA used for the measurements in view of results indicating an inverse correlation between the level of artefactual oxidation and the quantity of extracted DNA [181]. Therefore, a minimum of 30 μg of DNA is required to minimize the contribution of spurious oxidation to levels that are lower than physiologically relevant values without at this stage being able to totally prevent artifactual autooxidation. This explains the difference of about 7 to 10 fold in the levels of 8-oxodGuo measured by chromatographic methods and the frequency of Fpg-sensitive site determined by either the comet assay or the alkaline elution technique, even if in the latter cases, there is a risk of underestimation. Such artefactual oxidation is the main reason why HPLC-MS/MS measurements are only useful for DNA samples in which the level of damage has been significantly increased by several fold, with respect to the steady-state levels, by exposure to either endogenous or exogenous oxidants as further illustrated in the following sections. It is also clear that HPLC-MS/MS is not an appropriate method to monitor the formation of oxidatively generated damage to DNA in mitochondrial DNA since there is a high risk of artifactual oxidation due to the relatively low amount of DNA available, usually in the low μg range with reasonable quantities of cell and tissue samples. The measurement of 8-oxoG and 8-oxodGuo, which is free of methodological artefactual oxidation, in human and animal fluids by HPLC-MS/MS will not be discussed in detail in this review (for recent reviews, see [111,171,172]. The origin of released 8-oxodG in urine still remains unclear particularly with purines since their metabolism involves enzymatic oxidation [182]. As a final remark, one should note the lack of accurate data on the formation of oxidatively induced damage to 2-deoxyribose in cellular DNA. So far only one attempt has been made to measure 2-deoxyribonolactone and 2-deoxyribonucleoside 5'-aldehyde in the DNA of TK6 human lymphoblastoid cells upon exposure to gamma rays using a GC-MS method with isotopic dilution [32]. However, the high yield of the latter oxidized 2'-deoxyribonucleoside, i. e.

0.22 lesions per 10^6 2'-deoxyribonucleosides and per Gy, or about 10-fold higher than that of 8-oxodG, suggests that the yield of this damage may be overestimated.

Single base modifications

Singlet oxygen

The search of $^1O_2$ oxidation products in cellular DNA has been greatly facilitated by extensive and detailed mechanistic studies [8,183,184] using a clean source of $^{18}[^1O_2]$ through the thermolysis of DHPN$^{18}O_2$ [42,185]. Abundant information is available on the $^1O_2$-mediated oxidation reactions of the guanine moiety of isolated 2'-deoxyribonucleoside and short oligonucleotides. This reaction is rationalized in terms of initial Diels-Alder [4+2] cycloaddition across the 4- and 8-carbons of the imidazole ring [186] (Fig 1). A relatively minor pathway involves rearrangement of guanine endoperoxides **1** that transform into 8-hydroperoxyguanine **2**, both which are relatively unstable [187,188]. Reduction of the 8-hydroperoxide group is likely to give rise to 8-hydroxyguanine [189–193] that is in dynamic equilibrium with the predominant 8-oxoGua tautomer in solution [121,186]. The reactivity of $^1O_2$ towards guanine bases in double stranded DNA has been shown to be much lower than that for either single stranded DNA or isolated 2'-deoxyguanosine [193,194]. In addition, a major change was observed in the product distribution of $^1O_2$ guanine oxidation products in isolated calf thymus DNA upon either exposure to type II photosensitizer [195–197] or incubation with DHPNO$_2$ [183,198]. The reaction with DNA appears to be far simpler than that observed for the isolated nucleoside. Thus, 8-oxoGua is predominantly product generated in DNA, whereas in sharp contrast, spiroiminodihydantoin (Sp) is predominantly formed at the expense of 8-oxoGua upon oxidation of the nucleoside [199]. This can be rationalized in terms of steric factors that prevent formation of the quinonoid intermediate and its subsequent hydration. It may be added that no evidence for the formation of FapyGua, a typical one-electron oxidation degradation product of guanine was observed [200]; thus, this excludes any significant contribution of charge transfer reaction as proposed earlier [201]. Incubation of human monocytes cells with [^{18}O]-labeled DHPNO$_2$ that has been shown to accumulate intracellularly led to the generation of [^{18}O]-labeled 8-oxodGuo in genomic DNA [200]. Levels as high as 2 lesions of 8-oxodG per 10^6 2'-deoxyribonucleosides, which represents about 12,000 lesions per nuclear DNA, were accurately quantified by HPLC-ESI-MS/MS using the multiple reaction monitoring (MRM) mode with [M+5] labeled 8-oxodG as the internal standard [91,173]. This clearly established the ability of $^1O_2$ to react with the guanine base in cellular DNA producing 8-oxodG according to the previously characterized mechanism involving initial [4+2] cycloaddition of $^1O_2$ across the 4 and 8 carbons of the purine ring. The possible formation of 8-oxoGua through radical reactions once DHPNO$_2$ penetrates into cells was ruled out. Further support for the specific guanine oxidation by $^1O_2$ in cells incubated with DHPNO$_2$ was provided by the detection of nicks induced in extracted DNA by bacterial Fpg [202], a DNA repair enzyme that is able to cleave the *N*-glycosidic bond of 8-oxoGua residues. On the other hand, there was no significant increase in the levels of strand breaks induced upon incubation of DNA with endonuclease III that essentially recognizes oxidized pyrimidine bases. It was also shown that the steady-state level of strand breaks and alkali-labile sites did not increase upon incubation of the cells with DHPNO$_2$. It

should be remembered that $^1O_2$ is mostly at the origin of nuclear 8-oxoGua measured in numerous types of cells [8,203] and human skin explants [204–206] exposed to UVA radiation as the result of a predominant type II photosensitization mechanism [205]. With respect to monocytes exposed to UVA, the relative contribution of $^1O_2$ compared to $^{\bullet}OH$ in the formation of 8-oxoGua was about 4:1 [115,207]. This ratio is likely lower in the DNA of UVA-irradiated melanocytes [208] due to the ability for melanin to photosensitize the generation of $O_2^{\bullet-}$, a potential precursor of $^{\bullet}OH$.

A relevant topic has recently emerged with assessment of the photosensitizing ability of 6-thioguanine (6-TGua) and related analogues including azathioprine and 6-mercaptopurine, which are well documented anticancer, immunosuppressant and anti-inflammatory agents that are efficiently incorporated into DNA [209–211]. It was reported that UVA irradiation of GM5399 primary human fibroblasts pre-incubated with azathioprine led to the induction of 8-oxodG in nuclear DNA as the result of photosensitized generation of $^1O_2$ [212]. It was recently estimated from a detailed photophysical study that the quantum yield of $^1O_2$ generation through energy transfer from triplet excited 6-TGua to $^3O_2$ was 24% [213], an approximate two-fold lower value than previously estimated [214]. UVA irradiation of 6-TGua in double-stranded DNA predominantly gives rise to guanine-6-sulfinate [215] as the result of efficient $^1O_2$ oxidation through the intermediacy of a peroxy precursor, as inferred from a theoretical study [216]². It would be of interest to search for the formation of both 8-oxoGua and guanine-6-sufinated in the DNA of cells pre-incubated with 6-TGua prior to UVA irradiation.

Hydroxyl radical

Relevant information has been gained concerning the formation of the major $^{\bullet}OH$-mediated oxidation products of the pyrimidine bases including thymine, cytosine and 5-methylcytosine in cellular DNA. This was achieved on the basis of HPLC-ESI-MS/MS measurements of modified 2'-deoxyribonucleosides and/or nucleobases that were enzymatically or chemically released from nuclear DNA from either human monocytes [160,161,173] or Fischer F98 glioma cells [217] subsequent to exposure to gamma rays. As depicted in Figure 2 the main $^{\bullet}OH$-mediated oxidation products of thymine (Thy) involved the *cis* and *trans* isomers of ThyGly, 5-hydroxymethylhydantoin (HydThy), 5-hydroxymethyluracil (5-HmUra) and 5-formyluracil (5-FoUra) as previously characterized in model studies including isolated DNA [28,65,218]. The formation of ThyGly is rationalized in terms of the initial addition of $^{\bullet}OH$ at C5 and to a lesser extent at C6 giving rise to transient **5** and **9** [27,219]. In a subsequent step, fast O_2 addition leads to 5,6-hydroxyhydroperoxides through related peroxyl intermediates. The formation of ThyGly is explained by reduction of the peroxide function whereas competitive rearrangement of the pyrimidine ring, through opening of the 5,6-bond and subsequent recyclization, leads to HydThy. The methyl group of Thy is also a major target for $^{\bullet}OH$ reaction such that it is subject to hydrogen abstraction thereby generating the 5-(uracilyl)methyl radical (**6**). Reduction and dehydration of transient 5-hydroperoxymethyluracil formed after O_2 addition to **6** gives rise to 5-HmUra and 5-FoUra, respectively. The oxidation reactions of cytosine (Cyt) triggered by $^{\bullet}OH$ present similarities and differences with respect to those identified for Thy (Fig 3) [29]. The 5,6-double bond of Cyt is the exclusive site of reaction with $^{\bullet}OH$

leading to the formation of about 90% of 5-hydroxy-5,6-dihydrocytosyl (**9**) and 10% of 6-hydroxy-5,6-dihydrocytos-6-yl radical (**11**). Relatively unstable 5,6-hydroxyhydroperoxides are expected to be generated after addition of O_2 to **9** and **11**. As observed for Thy, reduction of the Cyt hydroperoxides leads to the formation of 5,6-dihydroxy-5,6-dihydrocytosine products that in contrast to ThyGly are unstable [220,221]. Dehydration gives rise to 5-hydroxycytosine (5-OHCyt) whereas competitive deamination generates 5,6-dihydroxy-5,6-dihydrouracil (UraGly). In addition, opening of the 5,6-bond of Cyt 5,6-hydroxyhydroperoxides followed by hydrolysis and recyclization of the transient ureid likely comprise the rearrangement pathways involved in the formation of 5-hydroxyhydantoin (HydUra). Another peculiar feature of •OH-mediated oxidation reactions of Cyt deals with the intramolecular cyclization of 6-hydroperoxy-5-hydroxyl-5,6-dihydrocytosine [222] that, after opening of the endoperoxide and subsequent rearrangement, gives rise to 1-carbamoyl-4,5-dihydroxy-2-oxoimidazolidine (ImidCyt) (Fig 3) [29,223]. Related 5-mCyt oxidation products including 5,6-dihydroxy-5,6-dihydro-5-methylcytosine and 1-carbamoyl-4,5-dihydroxy-5-methyl-2-oxoimidazolidine have been identified in isolated DNA exposed to OH [217]. However, the latter modifications have not been accurately detected so far by HPLC-ESI-MS/MS in cellular DNA exposed to ionizing radiation likely due to the relatively low yields of formation. In contrast, 5-FoCyt and 5-HmCyt, which arise from the formation of 5-hydroperoxymethylcytosine (5-HypmCyt) following O_2 addition to initially generated 5-(cytosyl)methyl radicals (**12**)(Fig 4), were detected in a relative ratio of 10 to 1 respectively.

As already mentioned a significant proportion of •OH-mediated degradation products of guanine in cellular DNA are expected to result from reactions induced by pyrimidine peroxyl radicals either by addition to the vicinal bases or one-electron oxidation. However, •OH reacts efficiently with guanine as it does with other nucleobases in cellular DNA. A relatively minor fraction involves the addition of •OH to the C8 position of the purine moiety (close to 17% for free 2'-deoxyguanosine) giving rise to the reducing 8-hydroxy-7,8-dihydroguanyl radical **2** (Fig. 1) [219,224]. In contrast to what is observed with pyrimidine radicals, molecular oxygen is not able to add to intermediate 2 which rather is subject to one-electron oxidation by O_2 leading to the formation of 8-oxoGua. Interestingly, **2** tends to undergo competitive one-electron reduction in cellular DNA, likely by thiol compounds, thereby generating FapyGua [224] with a yield that is about two-fold higher than that of 8-oxoGua [160]. Similar degradation pathways are involved in the •OH-induced formation of 8-oxoAde and FapyAde (Fig 5) that are generated with about a 10-fold lower efficiency than that seen for related guanine degradation products. The lower efficiency of adenine degradation may largely be rationalized by the lack of a significant contribution of oxidation reactions triggered by pyrimidine peroxyl radicals that predominantly target guanine in double stranded DNA.

Oz, another •OH-mediated or one-electron oxidation productof guanine [38] that is formed by the addition of superoxide anion radical to highly oxidizing G(-H)• has been detected in liver DNA of diabetic rats [225]. The yield of Oz was found to be 10-fold lower than that of 8-oxoGua. It has been recently reported that 5-carboxamide-5-formamido-2-iminohydantoin

is a major •OH-mediated oxidation product of Gua in free nucleoside and oligonucleotides [226].

This should stimulate further studies aimed at searching for the formation of this rearrangement product of the purine ring of Gua in cellular DNA.

Attempts to search for the formation of 2-hydroxy-2'-deoxyadenosine, one of the main radiation-induced degradation products of the adenine moiety of cellular DNA [227] tentatively measured by GC-MS, however failed. Therefore, the relevance of 2-hydroxy-2'-deoxyadenosine as an adenine oxidation product is questionable since it was not detected using accurate HPLC-MS/MS in the DNA of THP1 cells exposed to γ –rays at doses up to 200 Gy [228].

One-electron oxidation

Overwhelming 8-oxoGua and several oxidized bases including ThyGly, 5-HmUra, 5-FoUra and 5-OHCyt have been detected by HPLC-ESI-MS/MS as the corresponding 2'-deoxyribonucleosides in cellular DNA upon ionization by UVC nanosecond high intensity laser pulses [66,161]. The formation of these base oxidation products may be rationalized in terms of the initial generation of purine and pyrimidine radical cations [28,186] followed by subsequent hydration and/or deprotonation reactions. Hydration reactions are highly specific giving rise to the 8-hydroxy-7,8-dihydroguanyl radical (**3**) from **4** (Fig 1) and the 6-hydroxy-5,6-dihydropyrimidin-5-yl radical (**8**) from **7** (Fig 2). In addition, competitive deprotonation of the thymine radical cation **7** leads to **6** [229,230] a radical which similar to **1**, **7** and **13** are generated by •OH. In addition, 8-oxoAde and FapyAde have been also measured in about 10-fold lower yield compared to related guanine degradation products. Their formation is rationalized in terms of initial formation of the radical cation **14** that is converted into the 8-hydroxy-7,8-dihydroadenyl radical **13** followed by either one-electron oxidation into 8-oxoAde or one-electron reduction into FapyAde (Fig 5). Therefore, it is not surprising that the five oxidized bases resulting from the fate of radicals **3**, **5**, **6**, **8** and **13** under aerobic conditions have also been identified as •OH-induced degradation products. However, there is a strong bias in the distribution of the oxidation products induced by ionization with respect to •OH. It was found that •OH-induced 8-oxoGua was formed with efficiency similar or even lower to that of 5-HmUra, 5-FoUra or 5-OHCyt. In contrast, 8-oxoGua generated by photoionization exhibits at least a 10-fold higher yield than any of the other four base oxidation products. The predominant formation of 8-oxoGua cannot be accounted for by the preferential ionization of guanine since photophysical studies showed that the four main base radical cations were induced with a similar efficiency upon laser irradiation. Therefore, it is reasonable to propose that subsequent to ionization of the bases, a redistribution of the radical cations thus produced takes place along the oligonucleotide chains, through long- and short-charge transfer mechanisms, as proposed for isolated DNA [231–233]. Guanine nucleobase, which exhibits the lowest ionization potential among DNA components, is the preferential sink for positive holes before they are converted into final degradation products.

Secondary oxidation reactions of 8-oxo-7,8-dihydroguanine

Several oxidized nucleobases including 8-oxoGua, 5-OHCyt and 5-hydroxyuracil, that show a much lower oxidation potential than normal bases, are susceptible to further degradation by one-electron oxidants including type I photosensitizers, $CO_3^{\bullet -}$ and organic radicals as demonstrated in model studies [234–243]. Major attention has been given to 8-oxoGua because it is about 100-fold more prone to one-electron oxidation and possesses a lower oxidation potential by about 0.5 eV compared to the parent molecule [234,235]. In neutral solution, spiroiminodihydantoin (Sp) was the main final degradation product of 8-oxoGua [236–238,241,242] which arises from an 1,2-acyl shift rearrangement of transient 5-hydroxy-7,8-dihydro-8-oxoguanine [244] initially proposed as a stable compound [245].The formation of guanidinohydantoin from the same intermediate was favored at acidic pH [246]. Interestingly, these studies have provided a strong stimulus to delineate the biochemical features of Sp, including the mutagenic potential [247,248] and associated repair by glycosylases [249–253]. The absolute configuration of 4*R* and 4*S* diastereomers of Sp 2'-deoxyribonucleosides was recently assigned [254,255]. However, only a few attempts have been made to search for the occurrence of secondary oxidation of 8-oxoGua in cellular DNA. In an initial study, the steady state level of Sp of 200 lesions per 10^6 Gua was reported to be at least one order of magnitude higher than 8-oxoGua in the DNA of wild type *E.coli* [163]. The same authors reported increases of Sp in Nei deficient *E. coli* compared to the wild type WT strain and unusually high increases when the cells were treated with 500 μM Cr(VI); the levels of Sp actually reached as much as 6,000 lesions per 10^6 Gua. It should be noted that the measurement of Sp was performed by HPLC-MS operating in the SIM mode, a method with shortcomings and well-documented lack of compound selectivity as already discussed (*vide supra*). The above extremely high value of Sp has to be compared with the relatively low level of secondary oxidation products of 8-oxoGua that were measured in the DNA of liver and colon tissues of *Rag2*$^{-/-}$ mice infected with *Helicobacter hepaticus* using the relevant and accurate HPLC-ESI-MS/MS method [256,257]. The frequency of Sp and Gh was found to be within the range of 1 to 7 lesions per 10^8 nucleosides, which is close to the limit of detection. These data show that the secondary one-electron oxidation of 8-Gua is modest even under conditions of infection in which inflammation triggers a massive production of $ONOO^-$, $CO_3^{\bullet -}$, and $^{\bullet}OH$. This is in agreement with chemical considerations that question the possibility that 8-oxoGua, present at a steady-state level of a only few residues per 10^6, can be a target for further oxidation among overwhelming normal bases when hole transfer process within DNA helix is restricted to 20 bp. This also explains why the proposed sacrificial role for 8-oxoGua [258,259] by acting as a preferential target of one-oxidation agents may protect canonical bases has been ruled out [260].

Halogenation of nucleobases

Hypochlorous acid (HOCl), a one-electron oxidizing and chlorinating agent is enzymatically generated by myeloperoxidase released from activated neutrophils during inflammation [261–264]. Exposure of SKM-1 cells to HOCl has been shown to induce the formation in DNA and RNA of the nucleosides of 5-chlorocytosine (5-ClCyt), 8-chloroguanine (5-ClGua) and 8-chloroadenine (5-ClAde) that were accurately measured by HPLC-ESI-MS/MS [265]. This may be rationalized by the transient formation of chloramines through the reaction of HOCl with the exocyclic amino group and subsequent rearrangement into the

corresponding chlorinated bases (Fig 6). 5-ClCyt is formed predominantly over 5-ClGua and 5-ClAde in the DNA and RNA in SKM-1 cells, with the latter biopolymer showing a higher susceptibility to HOCl reactions [265]. In a subsequent study, the halogenated pyrimidine base 5-ClCyt was higher in the DNA of diabetic patients than in healthy volunteers, suggesting that this product may be used as a relevant biomarker of inflammation [266]. This idea is supported by the [265] observation of a significant increase in the steady-state level of highly mutagenic 5-ClCyt [267] in the colon and liver of Rag2−/− mice that were subjected to chronic inflammation as the result of infection with *Helicobacter hepaticus* [256]. Similarly high values of this biomarker of inflammation, half of which were close or more than 10 5-ClCyt lesions per 10^8 normal bases, were assessed by HPLC-ESI-MS/MS in the human colon of patients suffering from inflammatory bowel disease [268].

Aldehyde adducts to amino-substituted bases

Lipid peroxidation is a crucial redox stress event, which has been associated with the development of a number of pathologies such as cancer, neurodegenerative and inflammatory diseases. The lipid peroxidation process generates a complex mixture of phospholipid products including hydroperoxides that can decompose leading to electrophilic derivatives such as aldehydes and epoxyaldehydes [269]. Lesions resulting from the reaction of DNA with breakdown products of lipid peroxides including malonyldialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 4-oxo-(2E)-nonenal (ONE), 2,4-decadienal (DDE), 4,5-epoxy-(2E)-decenal (EDE), hexenal, acrolein, and crotonaldehyde, have been detected at basal levels in human tissues and in clinical situations associated with redox stress disorders [270–272] (Fig 7).

MDA is one of the best studied lipid peroxidation products. This dialdehyde reacts with 2′-deoxyguanosine (dG), 2′-deoxyadenosine (dA), and 2′-deoxycytidine (dC), yielding the cyclic pyrimidopurinone 3-(2-deoxy-*β*-D-*erythro*-pentofuranosyl) pyrimido[1,2-a]purin-10(3H)-one (M_1dG), the acyclic N^6-(3-oxo-1-propenyl)-2′-deoxyadenosine (M_1dA) and N^4-(3-oxo-1-propenyl)-2′-deoxycytidine (M_1dC) adducts [273–275] (Fig 8). Basal levels of the promutagenic pyrimidopurinone adduct M_1dG have been detected in different human tissues [276–279]. Interestingly, it has been shown that the intake of dietary polyunsaturated fatty acids correlates with the formation M_1dG in female leukocytes [280]. Levels of M_1dGo from 0.004 to 9.15 adducts per 10^8 nucleotides were reported by Ma et al. [281] (2015) in human leukocyte DNA using a methodology based on LC/nano electrospray ionization high-resolution tandem mass spectrometry (HRMS/MS). Interestingly, M_1dG is oxidized to 6-oxo-M_1dG in genomic DNA of intact cells indicating a possible role of 6-oxo-M_1dG in the cellular consequences attributed to M_1dG [282].

The reaction of DNA bases with α,β-unsaturated aldehydes, yields cyclic-substituted propano adducts as the 1,N^2-propano-2′-deoxyguanosine (1,N^2–propanodG) formed by Michael addition at the exocyclic amino group followed by ring closure [272,283]. Background levels of 1,N^2–propanodG adducts resulting from acrolein, crotonaldehyde, and HNE reactions have been detected in DNA from different rodent and human tissues [284–286]. Using a method involving online HPLC/ESI/MS-MS, accurate determinations of 1,N^2–propanodG levels in DNA extracts of human cultured cells (3.43 ± 0.33 /10^8 dG) and

rat tissue (liver, 4.61 ± 0.69 /10^8 dG; brain, 5.66 ± 3.70 /10^8 dG; and lung, and 2.25 ± 1.72 /10^8 dG) have been performed [287]. Recently, 1,N^2–propanodG adducts with levels of 2.4–3.5 adducts per 10^8 nucleotides were detected in untreated human MRC5 cells. Cell treatment with crotonaldehyde increases the levels of propano adducts in a concentration-dependent manner [288]. Levels of 1,N^2–propanodG were shown to be higher in the liver DNA of glutathione-depleted rats [289] indicating that they are persistently formed by endogenous pathways.

High levels of 1,N^2-propanodG (20.8 fmol of 1,N^2-propanodG/mg creatinine) were present in urine samples from individuals exposed to urban air pollution [290]. Knowing that 1,N^2-propanodG promotes DNA miscoding in human cells, largely through G → T transversions, and can inhibit DNA replication [291], the monitoring of 1,N^2–propanodG levels may help protecting the health of urban populations.

Epoxidized α,β-unsaturated aldehydes, formed during the lipid peroxidation processes, can generate ethano or etheno derivatives upon reaction with DNA [270,271] (Fig 8). Several etheno adducts, 1,N^6-etheno-2′-deoxyadenosine (εdA), 3,N^4-etheno-2′-deoxycytidine (εdC),N^2,3-etheno-2′-deoxyguanosine (N^2,3-εdG), and 1,N^2-etheno-2′-deoxyguanosine (1,N^2-εdG), have been detected in cells as well as in rodent and human tissues [270,292–294]. Higher levels of εdA and εdC were found in chronic infections and inflammation [270,295]. Etheno adducts have been used as biomarkers for DNA damage resulting from the reactions of endogenous lipid peroxidation end products [296].

Etheno adducts are genotoxic and mutagenic as shown in vitro systems by primer extension assays and *in vivo* by site-specific mutagenesis in cells [297]. Heptanone-substituted 3,N^4-etheno-2′-deoxycytidine (heptanone-εdCyd) blocked DNA synthesis and increased miscoding in both bacteria and human cells [298]. Recently, Patra *et al* have analyzed incorporation events using steady-state kinetics and LC-MS analysis of primers extended opposite 1,N^6-ethenodA by recombinant human DNA polymerase [299]. The results showed a preference for purine pairing opposite 1,N^6-ethenodA and for -1 frameshifts.

Ten-eleven translocation (TET) oxidation

The methylation of Cyt to 5-mCyt plays a pivotal role in epigenetics as a chemical modification or a mark that regulates gene expression. For example, genes are silenced by the methylation of cytosine predominantly occurring in CpG dinucleotides and gene promoter regions [300]. The discovery of unusually high levels of 5-hydroxymethylcytosine (5-HmCyt) in Purkinje neurons [301], and the initial characterization of ten eleven translocation (TET) family enzymes that oxidize 5-mCyt to 5-HmCyt [302], led to a surge of research inaugurating a novel pathway that operates to remove 5mC marks in the genome during cell differentiation and tissue homeostasis [303]. TET enzymes (I-III) are α–ketoglutarate and Fe(II)-dependent dioxygenases that oxidize 5-mCyt by an iterative process to give 5-HmCyt, 5-FoCyt and 5-CaCyt in double stranded DNA [304] (Fig 9). Interestingly, vitamin C acts as a catalyst and enhances TET activity in cells and animals [305]. In the past 8 years, numerous approaches have been applied to quantify 5-HmCyt and related modifications in genomic DNA [306]. These approaches utilize various methods to separate 5-HmC from complex biological mixtures, including TLC, GC, CE, LC, with anion

exchange, reverse phase and HILIC solid phase chromatography, in combination with mainly mass spectrometry methods of detection [306,307]. Isotopic labels of 5-mCyt nucleoside and its oxidation products for MS analyses are either commercially available or fairly easy to synthesize starting with labeled dThd [217,308]. Thereby, the levels of 5-HmCyt vary appreciably from one tissue to another in mice, with the highest levels usually observed in the brain [165]. In humans, he levels of 5-hmCyt in blood DNA varies from 0.01 to 0.03% Cyt [309] while that in normal brain DNA varies from 0.54–1.34% Cyt [310,311]. It should be noted that the levels of 5-HmCyt in healthy cells and tissues are at least 100-fold higher than those of other oxidatively induced lesions modifications such as 8-oxoGua making quantification relatively easy without the problem of artifactual oxidation during sample preparation. Although it is possible to increase the formation of 5-HmCyt upon exposure of cells to ionizing radiation, the increase obtained at lethal doses of ionizing radiation is minor compared to the level of endogenous TET-mediated 5-HmC in cellular DNA [217]. The quantification of 5-HmCyt by LC-MS/MS may be achieved on a routine basis generally with about 1–2 μg of purified DNA; this corresponds to the extracted DNA from about 100,000 cells. The analyses of individual nucleobases or nucleosides of 5-HmCyt together with certain related modifications have been extended to the DNA of other organisms, mitochondrial DNA [311] mammalian RNA [312], and human urine [313]. Depending on the method and the instrument of analysis, the limit of detection of 5-HmCyt in cellular DNA is in the low- or sub-fmol range [314]. Indeed, a recent method with derivatization and MS detection reports the possibility of measuring 5-HmCyt from 20 cells [315]. The levels of 5-HmCyt in transformed cells in culture and cancerous tissue attain relatively low levels that may lead to difficulties in quantification using current methodologies. For example, the level of 5-HmCyt in brain tumors varies from 0.03 to 0.75 5-HmCyt/Cyt% [310,311]. Likewise the levels of related modifications, including 5-FoCyt, 5-HmUra, 5-HmUra, are usually 100-fold lower than those of 5-HmCyt under physiological conditions. As mentioned in previous sections, the analysis of 5-FoCyt and 5-CaCyt nucleosides by HPLC-ESI-MS/MS may be problematic because of the low levels present in certain cases. A recent study suggests that 5-HmUra may be produced by random enzymatic oxidation of thymine in DNA by TET [316]; however, the levels of 5-HmUra remain low compared to 5-HmCyt likely because of active and efficient DNA repair by single-strand selective monofunctional uracil DNA glycosylase [321].

Complex modifications

Intrastrand base lesions (G*-T*)

The formation of intrastrand cross-links (ICLs) involving one-electron oxidation of the guanine radical cation **3** was recently reported in cellular DNA [66]. This reaction constitutes a novel relevant example of nucleophilic addition reactions to **3** upon exposing HeLa cells to high intensity nanosecond UVC laser pulses followed by analyses of the damage by suitable enzymatic hydrolysis and subsequent HPLC-ESI-MS/MS measurement. The crosslink involved the formation of a covalent bond between C8 of guanine and N3 of thymine separated by an undamaged nucleotide residing between the modified bases (Fig 9). Importantly, the level of dG*-dT* crosslinks was found to increase with the intensity of laser pulses. The formation of dG*-dT* was a minor process with respect to the generation of

other base oxidation products that arise from either hydration or deprotonation of the corresponding radical cations. For example, the yield of dG*-dT* was about 800-fold lower than the predominant yield of 8-oxoGua. In addition, either 5-FoUra or 5-HmUra, the two methyl oxidation products of thymine were formed with a yield of 60-fold higher than dG*-dT*. Interestingly, the ability of **4** to form dG*-dT* and to a lesser extent dG*pdT*in which both modified bases are vicinal was previously observed in model studies involving single-stranded oligonucleotides [68] and DNA duplexes [318]. It was recently shown that DNA repair glycosylases were able to cleave the *N*-glycosidic bond of dG*-dT* and dG*dC-dT* [319] which were site-specifically inserted into oligodeoxynucleotides with a defined sequence [63] (Crean et al 2008).

Purine 5',8-cyclo-2'-deoxyribonucleosides

Support for •OH-mediated intramolecular cyclization of the C5'-yl sugar radical **15** of adenosine 5'-monophosphate and 2'-deoxyadenosine to the C8 position of the purine base moiety was gained from isolation and characterization of the corresponding cyclic ribonucleotide [320] and 2'-deoxyribonucleoside [321]. These modifications exist as either 5'*R* or 5'*S* diastereomers [322]. Similar reactions that give rise to (5'*R*) and (5'*S*)-5',8-cyclo-2'-deoxyguanosine (cdG) (Fig 10) were subsequently found to occur in free 2'-deoxyguanosine and isolated DNA [137]. A resurgence of interest for these intranucleotide crosslinks was noted after the 5'*R* and 5'*S* diastereomers of 5',8-cyclo-2'-deoxyadenosine (cdA) were found to be relevant substrates for the nucleotide excision repair pathway [323], an observation that was confirmed by another research group a few months later [324]. This discovery stimulated further mechanistic studies on the formation of both cdA and cdG [325,326] while major efforts were devoted toward better understanding the underlying repair mechanisms [327] and to delineate the effects of these tandem lesions on DNA replication and transcription [328,329]. In addition, attempts were made to search for the formation of purine 5',8-cyclo-2'-deoxyribonucleosides in cellular DNA. For this purpose, the two diastereomers of cdG and cdA were measured by GC-MS [330] and HPLC-MS [331,332] respectively: two methods that suffer from major drawbacks as already discussed (*vide supra*). Thus, (5'*S*)-cdA was detected in the DNA of primary keratinocytes of two normal human subjects upon exposure to a low dose of 5 Gy of X-rays with a frequency of 14 lesions/10^9 normal 2'-deoxyribonucleosides per Gy [162,333]. This yield appears to be highly overestimated because it is comparable to the yield of a much more common oxidation product, 8-oxodG, which was generated in gamma-irradiated human monocytes with a similar efficiency of 20 lesions/10^9 2'-deoxyribonucleosides per Gy [160]. It should be noted that the formation of (5'*S*)-cdA in isolated aqueous solutions of DNA under aerated conditions is a minor pathway and that the amount of (5'*S*)-cdA induced by •OH represents only 1% of 8-oxoG [325]. Furthermore, a dose of 2kGy of gamma rays was necessary, using an accurate HPLC-ESI-MS/MS method, to barely detect traces of (5'*S*)-cdA whose yield was estimated to be at most 0.2 lesion/10^9 2'-deoxyribonucleosides per Gy [325]. The very low efficiency of •OH induced formation of purine 5',8-cyclonucleosides in cellular DNA thus raises a question about their biological relevance; this idea actually received further confirmation from a recent study [334]. None of the four diastereomers of cdA and cdG was detected by HPLC-ESI-MS/MS measurements in the DNA of radiation-resistant hyperthermophilic *Thermococcus gammatolerans* archeon strains exposed to 5 kGy of

gamma radiation [334]! The questionable formation of purine 5',8-cyclo-2'-deoxyribonucleosides in cells is consistent with the low efficiency for •OH to generate C5'-yl radicals at either adenine or guanine nucleotides (less than 3% for each), which for the most part are efficiency scavenged by O_2 [325].

Other confusing aspects concern the steady-levels in nuclear DNA of both cdA and cdG that, in the past, have been measured by either capillary HPLC associated with MS^3 detection or, more recently, by nanoLC-NSI-MS/MS in the brain, kidney and liver tissues of several animals including healthy Long-Evans Agouti (LEA) rats and Long-Evans Cinnamon rats (LEC) bearing a genetic dysfunction as in human Wilson's disease [335,336]. Other studies have involved *Ercc*$^{-/\Delta}$ mice in which mutations led to a reduced expression of XPF-ERCC1 that affects NER efficiency and presumably leads to accumulation of both cdA and cdG with age [337,338]. Surprisingly, the steady-state levels of purine 5',8-cyclo-2'-deoxyribonucleosides were elevated reaching 1 cdA/10^6 2'-deoxyribonucleosides in the liver and brain tissues of LEA rats [336]. Again, the levels were comparable to the average levels of 8-oxodG, a more frequently oxidatively generated damage to DNA. It seems that the levels of cdA and CdG are overestimated by at least two orders of magnitude as also suggested by the elevated values of 5-FoUra and 5-HmUra that were measured in the DNA of LEA rats [335]. Thus, the level of 20 FoUra/10^6 2'-deoxyribonucleosides assessed in hepatic tissue corresponds to the amount observed in cellular DNA exposed to 1 kGy of gamma rays! Clearly, further studies are necessary to resolve these apparent discrepancies.

Interstrand cross-links

Insight into the formation of intrastrand crosslinks was gained from model studies that led to the isolation and characterization of four diastereomers of 6-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-2-hydroxy-3(3-hydroxy-2-oxopropyl)-2,6-dihydroimidazo[1,2-c]-pyrimidin-5(*3H*)-one in isolated DNA upon exposure to •OH in aerated aqueous solutions [339,340]. Interestingly, the four Cyt adducts were detected in the DNA of human THP1 monocytes exposed to gamma rays. Further information concerning the mechanism of generation of Cyt adducts was inferred from incubation of isolated and cellular DNA with bleomycin, a radiometric enediyne that is known to predominantly abstract the C4' hydrogen atom in double-stranded DNA [341]. The resulting carbon centred C4' radical similar to that generated by •OH is converted among other degradation pathways into the corresponding C4' oxidized abasic site (**C4-AP**) [33] after O_2 addition and elimination of the nucleobase (Fig 11). The abasic site or more likely its acyclic form [342] is prone to subsequent β-elimination, which is enhanced when either a cytosine or adenine base is located on the opposite strand [343]. This results in cleavage of the 3'-phosphodiester bond with the concomitant generation of **18**, a highly reactive conjugated keto-aldehyde, which can add to 4-amino group of 2'-deoxycytidine on the opposite strand giving rise to interstrand DNA cross-links (Fig 11). The proposed mechanism of ICL formation has received further support from subsequent model studies in which a photolabile precursor of the C4' radical was site-specifically inserted into DNA duplexes [343,344]. The formation of Cyt adducts that are present in untreated cells at a level of about 1–3 lesions per 10^8 nucleosides was found to increase upon exposure to gamma rays with a relative occurrence of 1% with respect to 8-oxoG. In contrast, incubation of cells with bleomycin leads to a

significant 4-fold increase in the frequency of the ICLs while the level of 8-oxoG is not affected. The search for the formation of similar cycloadducts to adenine in cellular DNA expected to be generated from other model studies [344] awaits further investigation.

Repair of oxidatively damaged cellular DNA

There is an abundance of literature concerning the enzymatic activity, substrate specificity and mechanistic aspects of diverse prokaryotic and eukaryotic DNA *N*-glycosylases that act on oxidized bases through the predominant base excision repair pathway as reviewed in several contributions to this issue. In contrast, there is a paucity of accurate information on the repair of individual oxidatively generated base damage in cellular DNA. This is mostly explained by difficulties that have been encountered in measuring well-defined DNA oxidatively induced modifications in isolated cells or animal tissues, particularly under mild oxidative stress conditions. Most relevant data concern the repair of 8-oxoG on the basis of HPLC-ECD measurements. In addition, an example of repair that does not implicate BER pathway is available [340].

Base excision repair of 8-oxoGua

In pioneering work, Kasai and co-workers were able to accurately monitor the formation of 8-oxoG by HPLC-CED in the hepatic DNA of mice after whole-animal exposure to γ-rays [151]. The average radiation-induced yield of 8-oxoG (20 lesions/10^9 bases per Gy) is similar to that measured by HPLC-MS/MS in γ-irradiated human monocytes, being three orders of magnitude lower than that observed in isolated DNA. Interestingly, evidence for the efficient repair of 8-oxoG was provided by a decrease of this oxidized base within a few hours during post-irradiation incubation. A similar observation was reported in mouse FM3A cells in which 8-oxoG was specifically induced in nuclear DNA by riboflavin sensitization to UVA radiation [345]. It was found that about 2/3 of the initial amount of 8-oxoG was repaired by about 2h post-irradiation. Other studies have confirmed that 8-oxoG was efficiently repaired in the DNA of animal tissues exposed to various oxidizing agents, including UVA radiation [346], ferric nitrilotriacetate [347,348], diesel exhaust particles [349] and asbestos [350]. The repair kinetics of $^1O_2$-specifically generated 8-oxoG in the DNA of liver and immortalized embryonic fibroblast cells lines from mice was determined from the analysis of Fpg-sensitive sites [128]. The removal of 50% of 8-oxoG lesions in both cell lines obtained from healthy mice required 4 h. This time-course compares with a 12 h period that was required in order to achieve a similar extent of repair in cells generated from *ogg1*$^{-/-}$ null mice that are partly deficient in BER excision ability.

Repair of Cyt adducts as part of interstrand DNA crosslink

The kinetics of removal of interstrand clustered DNA lesions generated by initial hydrogen atom abstraction at C4’ of the 2-deoxyribose moiety was assessed using HPLC-ESI-MS/MS by monitoring the gradual decrease of the levels of Cyt adducts generated in human monocytes upon treatment with bleomycin [340]. The repair rate of the Cyt adducts showed a typical biphasic phase and appears to be two-fold slower than that of 8-oxoG. Evidence has recently been gained, from model studies involving bacterial UvrABC complex, that nucleotide excision repair is also involved in the removal of the above interstrand lesions

giving rise to double strand breaks (DSBs) [351,352]. This constitutes a novel example of DSB generation arising from one radical hit as the result of DNA repair processing of a Cyt adduct containing interstrand crosslink. This involves incision of the damaged strand opposite to the nick that was initially generated by chemical reactions giving rise to the clustered lesion (Fig 11)

Conclusions and perspectives

Major progress has been made during the last two decades in the identification of oxidatively-induced damage to DNA and the mechanisms of formation of damage products, which include complex lesions such as tandem modifications and interstrand cross-links, DNA protein cross-links, in addition to predominant oxidized single bases. The development of HPLC coupled to tandem mass spectrometry that became available during the 90's has made it possible to accurately detect modifications in cellular DNA. In agreement with earlier measurements using either HPLC-ECD or enzymatic-sensitive sites, the steady-state level of 8-oxoG, an ubiquitous DNA oxidation modification, lies within the range of a few lesions per 10^7 2'-deoxyribonucleosides. This applies as well to other forms of oxidatively generated base modifications that have been measured in cellular DNA by HPLC-MS/MS methods. Another interesting conclusion is that the mechanism of formation of DNA products established from model studies in aerated aqueous solutions may be extrapolated in part to cellular DNA. The availability of capillary liquid chromatography associated with nanoelectrospray ionization tandem mass spectrometry (LC-NSI-MS/MS) displays a significant increase in the sensitivity of detection and represents a powerful tool for further investigations. A recent application of this method to mitochondrial DNA [353] has however shown the limitation of the approach when low amounts of nucleic acids are analyzed. The high values of 8-oxodG that varied within the range of 130–400 lesions/10^6 are likely due at least partly to the occurrence of artifactual oxidation which explains so far the lack of accurate data on the steady-state levels of 8-oxoGua in mitochondrial DNA. Other interesting potential applications of LC-NSI-MS/MS should focus on the search in cellular DNA of tandem base lesions that arise from the reactions of peroxyl pyrimidine radical with vicinal nucleobases. As discussed above, it remains to be established whether the occurrence of secondary oxidation products, most likely arising from one-electron oxidant-mediated reactions, is of biological significance. Another current conflicting issue that needs to be adequately addressed concerns the reported steady-state levels of purine 2'-deoxyribonucleosides that range widely between 0.2 and 10 *S*-cdA per 10^7 nucleosides, depending on the method employed [336,355]. It would be surprising that such large differences in the yield of *S*-cdA of up to a factor of 50 may only be explained by the diverse sources of DNA subjected to analysis.

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Highlights

- HPLC coupled to either ESI-tandem mass spectrometers or an electrochemical detector is appropriate for accurately monitoring oxidatively damage to cellular DNA.
- Seventeen single base lesions and several intra- and interstrand cross-links have been identified in cellular DNA as chemical or enzymatic oxidation products.
- Other base modifications arising from oxidative reactions included halogenated nucleobases and base adducts with reactive aldehydes from lipid peroxides.
- There is paucity of reliable data on the base excision repair kinetics of oxidized bases in cells at the exception of 8-oxo-7,8-dihydroguanine.

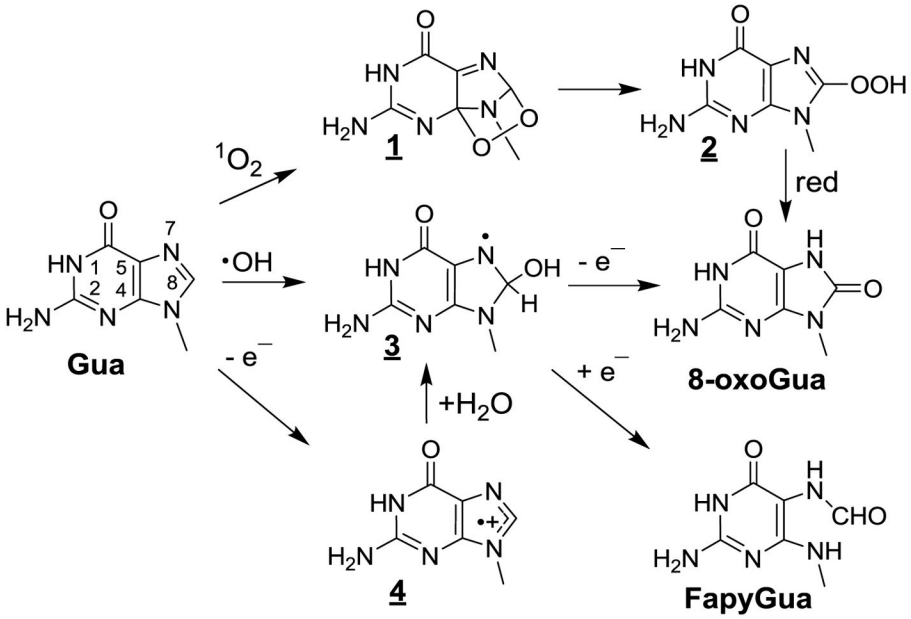

Figure 1.
Oxidation of guanine by OH, one-electron oxidants and singlet oxygen ($^1O_2$).

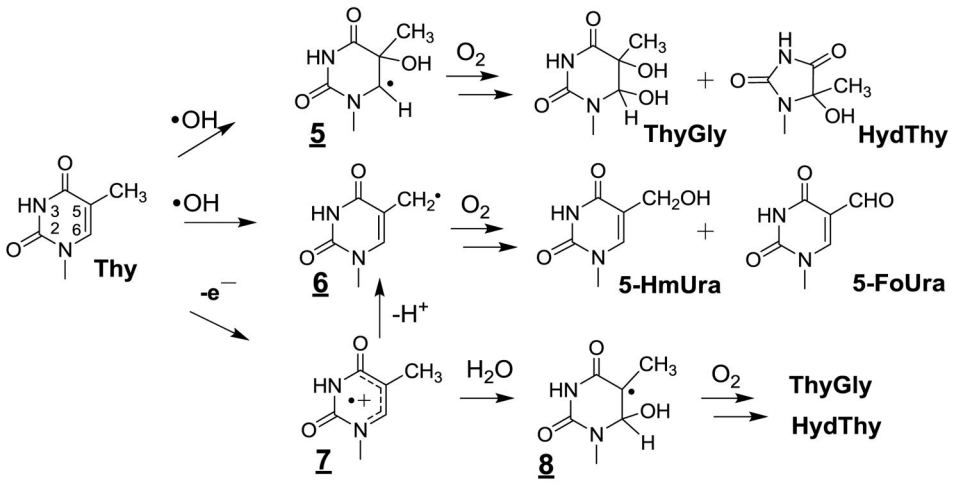

Figure 2.

Oxidation of thymine by OH and one-electron oxidation . Initial attack of OH occurs at both C5 and C6 positions to give the corresponding OH adducts (e.g. **5**). Analogous reactions occur for 5-methylcytosine (N3-C4=O → N3=C4-NH2).

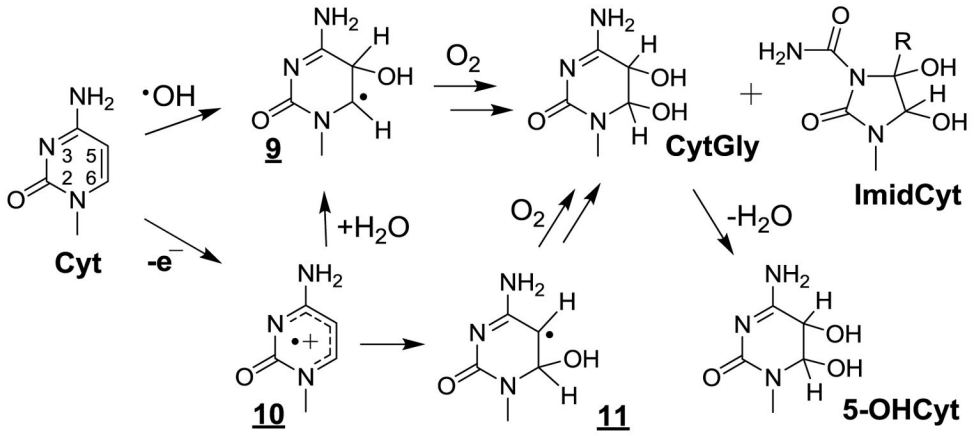

Figure 3.
Oxidation of cytosine by OH and one-electron oxidation.

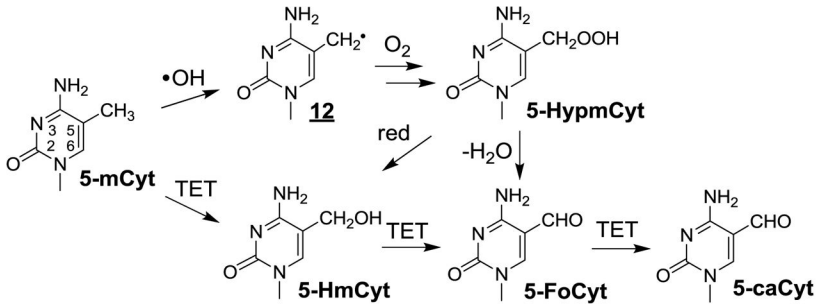

Figure 4.
Oxidation of 5-methycytosine by •OH and TET enzymes.

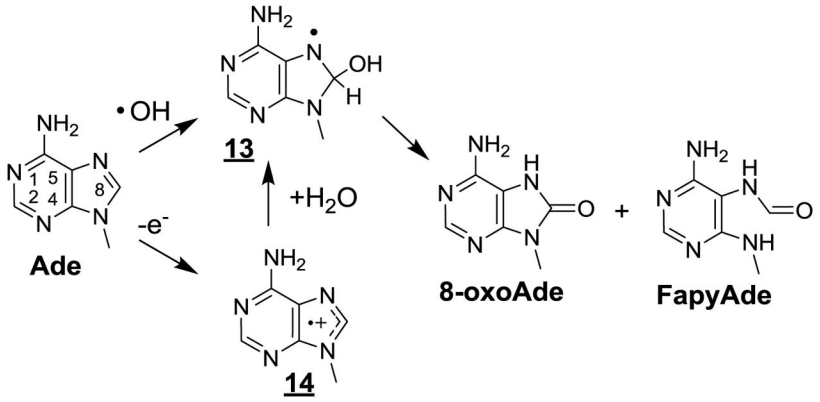

Figure 5.
Oxidation of adenine by OH and one-electron oxidants.

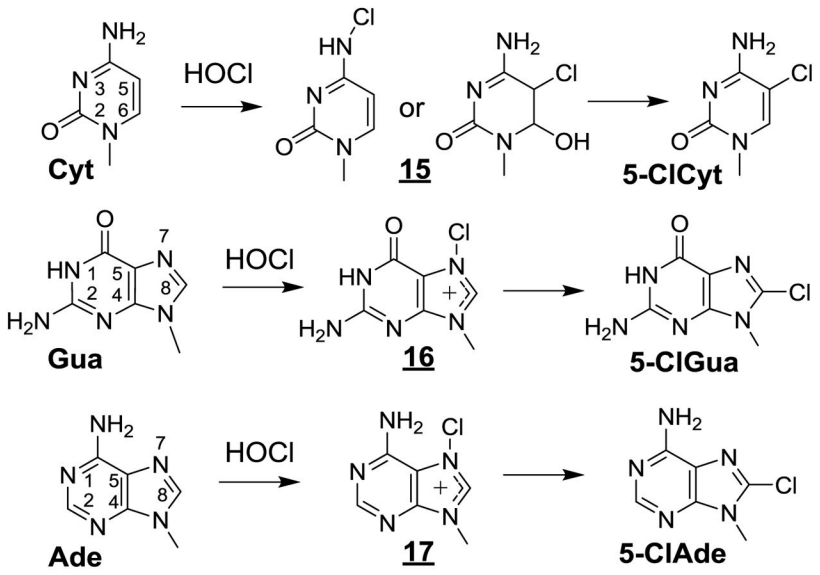

Figure 6.
Halogenation of DNA bases (Cyt, Gua and Ade)

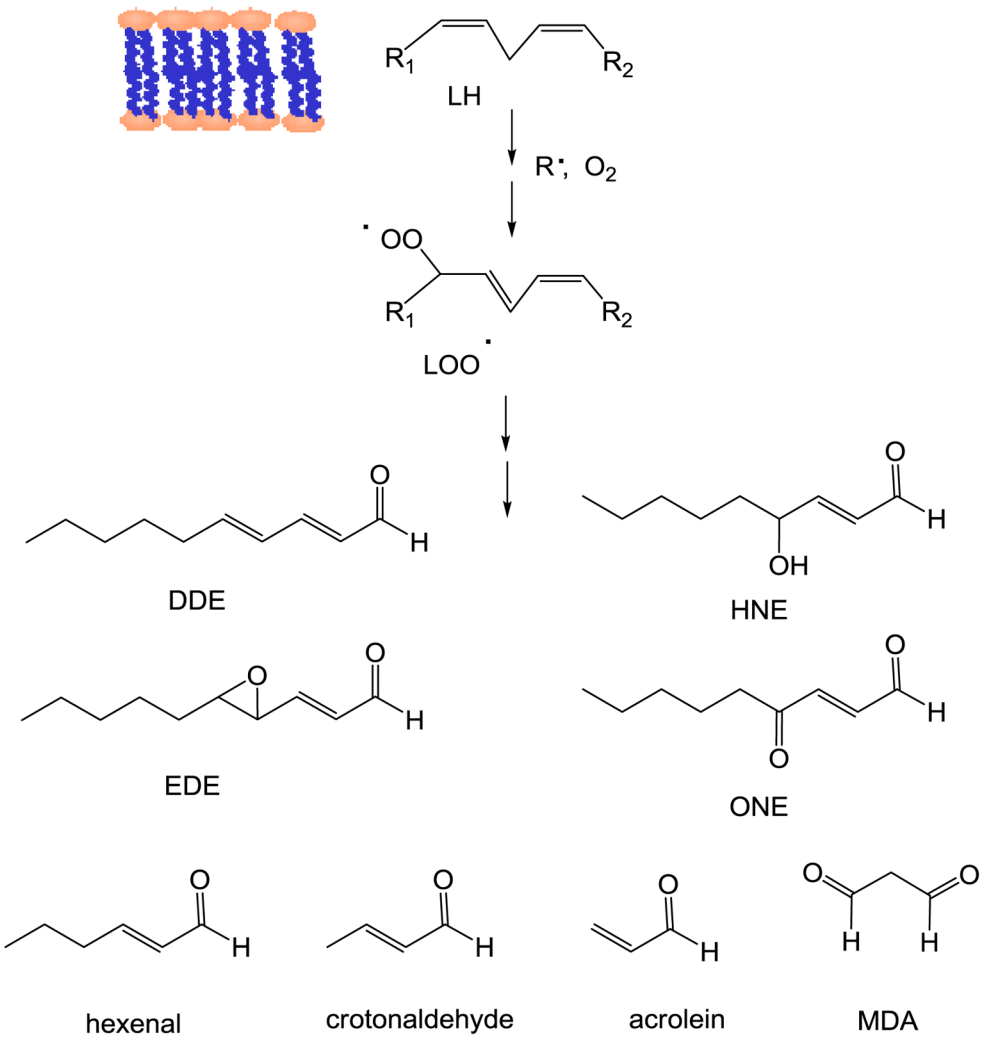

Figure 7.
Main α,β-unsaturated aldehydes arising from the decomposition of lipid peroxides.

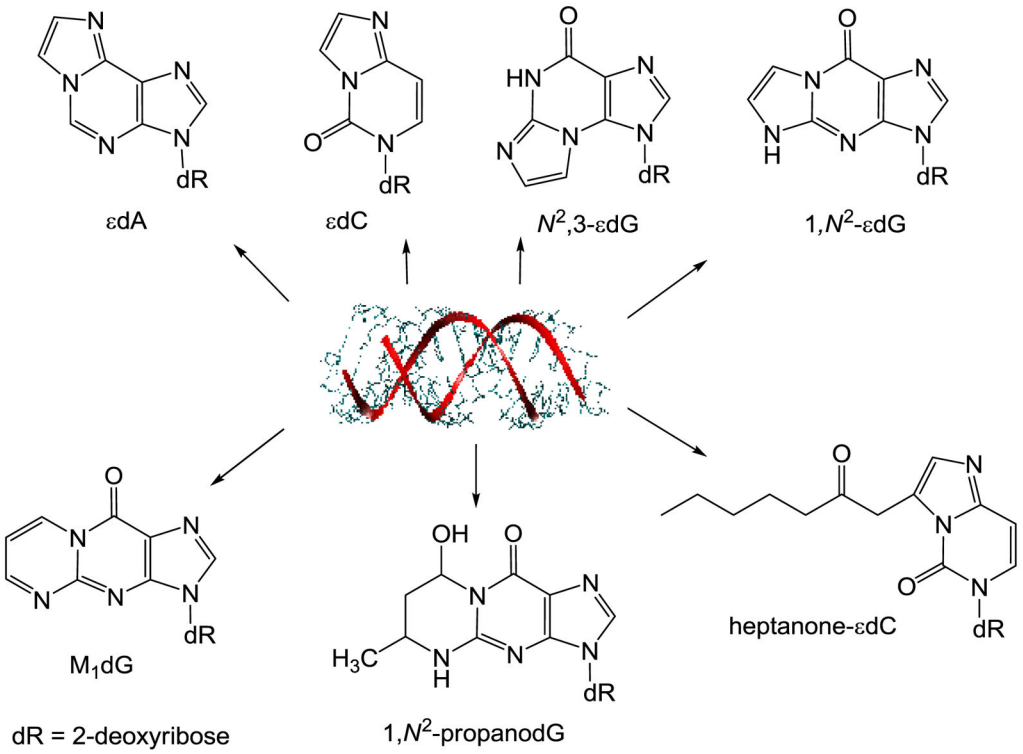

Figure 8.
Main adducts of α,β-unsaturated aldehydes to amino-substituted bases of 2'-deoxyribonucleosides

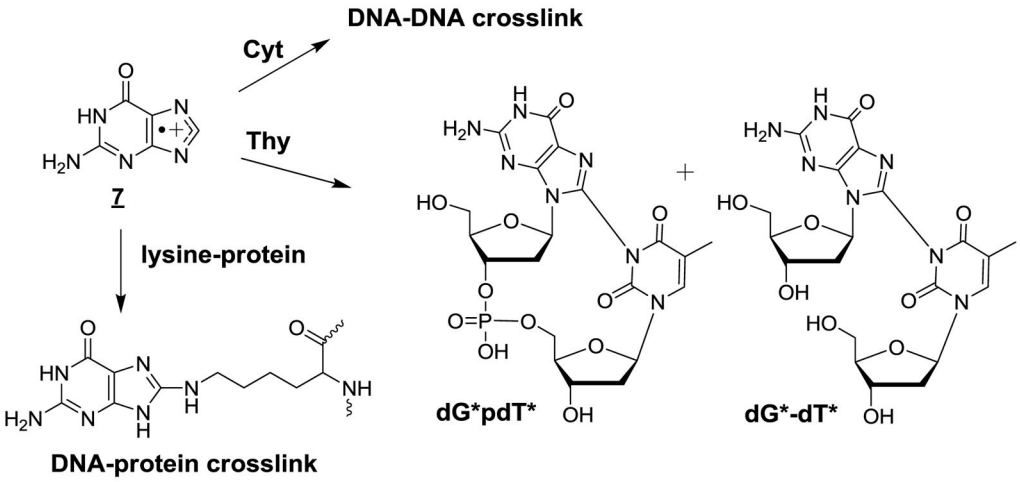

Figure 9.
Additional reactions of guanine radical cation **3**.

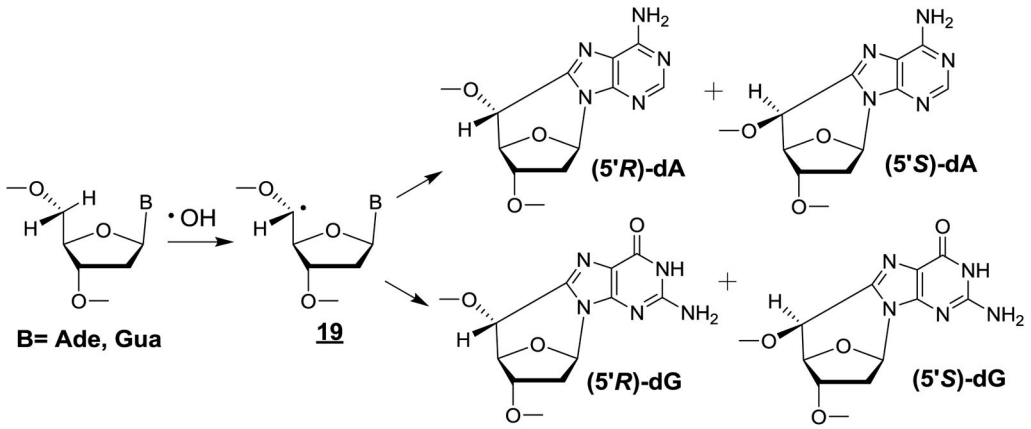

Figure 10.

Formation of purine 5',8-cyclo-2'-deoxyribonucleosides by ·OH-mediated hydrogen atom abstraction at C5'.

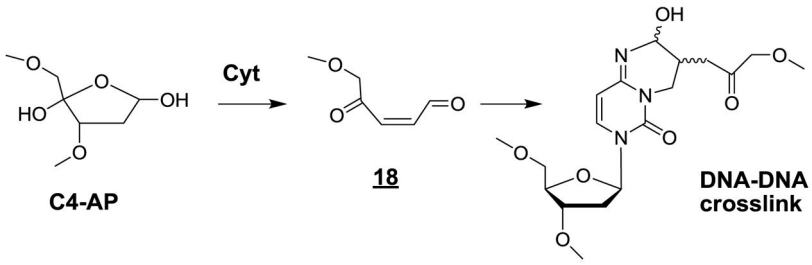

Figure 11.

Formation of a DNA interstrand crosslink by initial •OH-mediated hydrogen atom abstraction at C4'.

Table 1

Formation of oxidatively generated damage in cellular DNA.

DNA lesions	$^{\bullet}OH$	One-e oxidant	$^{1}O_2$	TET
5,6-Dihydroxy-5,6-dihydrothymine (ThyGly)	++	+		
5-Hydroxy-5-methylhydantoin (ThyHyd)	+			
5-Hydroxymethyluracil (5-HmUra)	++	+		
5-Formyluracil (5-FoUra)	++	+		
5-Hydroxycytosine (5-OHCyt)	+	+		
5,6-Dihydroxy-5,6-dihydrouracil (UraGly)	+			
1-Carbamoyl-4,5-dihydroxy-2-oxoimidazolidine (ImidCyt)	+			
5-Hydroxyhydantoin (UraHyd)	+			
5-Hydroxymethylcytosine (HmCyt)	+	+		+++
5-Formylcytosine (5-FoCyt)	+	+		++
5-Carboxylcytosine (5-CaCyt)				+
8-oxo-7,8-dihydroguanine (8-oxoGua)	++	++	++	
2,6-Diamino-4-hydroxy-5-formamidopyrimidine (FapyGua)	++			
8-Oxo-7,8-dihydroadenine (8-oxoAde)	+	+		
2,2,4-Triamino-5(*2H*)-oxazolone (Oz)		+		
Guanine-cytosine adduct Interstrand-crosslink (sugar-cytosine)	+	+		
(*5'S*)-5',8-cyclo-2'-deoxyadenosine (cdA)	?			

$^{+++}$Major damage: may reach a few lesions per 10^4 nucleobases.

$^{++}$Fair damage: between 10 and 100 lesions/10^8 nucleobases per Gy of ionizing radiation.

$^{+}$Minor damage: lower than 10/10^8 nucleobases per Gy of ionizing radiation.