Biochimica et Biophysica Acta 1790 (2009) 16–24

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Excision of the oxidatively formed 5-hydroxyhydantoin and 5-hydroxy-5-methylhydantoin pyrimidine lesions by *Escherichia coli* and *Saccharomyces cerevisiae* DNA *N*-glycosylases

Didier Gasparutto [a,*], Evelyne Muller [a], Serge Boiteux [b], Jean Cadet [a]

[a] *Laboratoire des Lésions des Acides Nucléiques, Service de Chimie Inorganique et Biologique UMR-E3 CEA-UJF, INAC, DSM, CEA-Grenoble, F-38054 Grenoble Cedex 9, France*
[b] *Laboratoire de Radiobiologie de l'ADN, UMR 217 CNRS-CEA, iRCM, DSV, CEA, F-92265 Fontenay-aux-Roses, France*

ARTICLE INFO

Article history:
Received 24 July 2008
Received in revised form 25 September 2008
Accepted 1 October 2008
Available online 17 October 2008

Keywords:
Oxidized DNA base lesion
Hydantoin nucleobase
DNA *N*-glycosylase
Base excision repair
Modified oligodeoxyribonucleotide

ABSTRACT

Background: (5*R**) and (5*S**) diastereomers of 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin (5-OH-dHyd) and 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxy-5-methylhydantoin (5-OH-5-Me-dHyd) are major oxidation products of 2′-deoxycytidine and thymidine respectively. If not repaired, when present in cellular DNA, these base lesions may be processed by DNA polymerases that induce mutagenic and cell lethality processes.

Methods: Synthetic oligonucleotides that contained a unique 5-hydroxyhydantoin (5-OH-Hyd) or 5-hydroxy-5-methylhydantoin (5-OH-5-Me-Hyd) nucleobase were used as probes for repair studies involving several *E. coli*, yeast and human purified DNA *N*-glycosylases. Enzymatic reaction mixtures were analyzed by denaturing polyacrylamide gel electrophoresis after radiolabeling of DNA oligomers or by MALDI-TOF mass spectrometry measurements.

Results: In vitro DNA excision experiments carried out with endo III, endo VIII, Fpg, Ntg1 and Ntg2, show that both base lesions are substrates for these DNA *N*-glycosylases. The yeast and human Ogg1 proteins (yOgg1 and hOgg1 respectively) and *E. coli* AlkA were unable to cleave the *N*-glycosidic bond of the 5-OH-Hyd and 5-OH-5-Me-Hyd lesions. Comparison of the k_{cat}/K_m ratio reveals that 8-oxo-7,8-dihydroguanine is only a slightly better substrate than 5-OH-Hyd and 5-OH-5-Me-Hyd. The kinetic results obtained with endo III indicate that 5-OH-Hyd and 5-OH-5-Me-Hyd are much better substrates than 5-hydroxycytosine, a well known oxidized pyrimidine substrate for this DNA *N*-glycosylase.

Conclusions: The present study supports a biological relevance of the base excision repair processes toward the hydantoin lesions, while the removal by the Fpg and endo III proteins are effected at better or comparable rates to that of the removal of 8-oxoGua and 5-OH-Cyt, two established cellular substrates.

General significance: The study provides new insights into the substrate specificity of DNA *N*-glycosylases involved in the base excision repair of oxidized bases, together with complementary information on the biological role of hydantoin type lesions.

1. Introduction

Cellular genomes are stable but can be damaged by both exogenous environmental agents and endogenously generated reactive species, and also as the result of errors during replication [1]. In that respect, reactive oxygen species are probably the most important source of spontaneous damage to DNA and more than 50 base modifications have been identified so far [2–4]. A majority of the resulting chemical alterations that induce structural modifications of DNA are known to be mutagenic and are possibly involved in acceleration of telomere shortening, aging process and human diseases, including cancer [5–9]. The mutagenicity of the oxidatively generated DNA lesions, which is expressed during their replication by DNA polymerases, has been extensively studied and is now well documented [10–12]. Fortunately, the genome alterations may be

Abbreviations: 5-OH-Hyd, 5-hydroxyhydantoin; 5-OH-dHyd, 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin; 5-OH-5-Me-Hyd, 5-hydroxy-5-methylhydantoin; 5-OH-5-Me-dHyd, 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxy-5-methyl-hydantoin; 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 5-OH-Cyt, 5-hydroxycytosine; 5-OH-dCyd, 5-hydroxy-2′-deoxycytidine; Oz, 2,2,4-triamino-5-(2*H*)-oxazolone; Ox, oxaluric acid; Tg, 5,6-dihydroxy-5,6-dihydrothymine; Ug, 5,6-dihydroxy-5,6-dihydrouracil; DHT, 5,6-dihydrothymine; 5-OH-DHT, 5-hydroxy-5,6-dihydrothymine; ESI-MS, electrospray ionization-mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; AlkA, *E. coli* 3-methyladenine DNA *N*-glycosylase II; endo III, *E. coli* endonuclease III; endo VIII, *E. coli* endonuclease VIII; Fpg, formamidopyrimidine DNA *N*-glycosylase; yNtg1, endonuclease III-like glycosylase 1 of *Saccharomyces cerevisiae*; yNtg2, endonuclease III-like glycosylase 2 of *Saccharomyces cerevisiae*; yOgg1, 8-oxo-guanine DNA *N*-glycosylase 1 of *Saccharomyces cerevisiae*; hOgg1, human oxo-guanine DNA *N*-glycosylase

* Corresponding author. Tel.: +33 04 38 78 45 58; fax: +33 04 38 78 50 90.
E-mail address: didier.gasparutto@cea.fr (D. Gasparutto).

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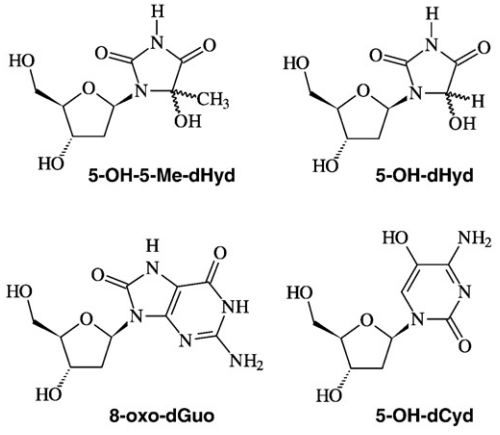
doi:10.1016/j.bbagen.2008.10.001

removed from cells by a variety of enzymatic repair processes, with increasing evidence for overlapping between the repair systems, leading in most cases to the restoration of the integrity of the biopolymer [13–16]. Thus, most of the oxidized bases are eliminated by the base excision repair pathway [11,17–21]. Homologues of several DNA *N*-glycosylases, which act in the initial step of the repair process by cleaving the *N*-glycosidic bond between the modified nucleobase and the sugar-phosphate backbone, have been identified in bacterial, yeast and mammalian cells [17,22–24]. This leads to the excision of the damaged moiety as a free base together with the concomitant generation of an abasic site within DNA. The resulting abasic site is converted to a single strand break by the action of an AP-endonuclease with subsequent removal of the sugar residue. Then, gap-filling is achieved by a DNA polymerase and finally a DNA ligase completes the repair process. Some DNA *N*-glycosylases are quite specific such as uracil DNA *N*-glycosylase, whereas others are able to recognize several different types of damaged nucleobases [11,17].

Among the four DNA 2′-deoxyribonucleosides, 2′-deoxycytidine and thymidine have been the subject of comprehensive studies aimed at characterizing related oxidative degradation pathways. Information on the structure of the latter nucleoside alterations and their mechanisms of formation is now available [25–32]. Among these, the (5*R**) and (5*S**) diastereomers of 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxy-hydantoin (5-OH-dHyd) and the (5*R**) and (5*S**) diastereomers of 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxy-5-methylhydantoin (5-OH-5-Me-dHyd), have been shown to be major oxidation decomposition products of 2′-deoxycytidine and thymidine respectively (Scheme 1). Both hydantoin residues have been shown to be generated upon exposure of the pyrimidine moieties of nucleosides to several oxidizing agents or processes including radiation-induced ·OH radical [26,29,30], excited type I photosensitizers operating by one-electron abstraction [26,31] and ozone [27,32]. In particular 5-OH-dHyd was found to be the major decomposition product of O_3-mediated oxidation of 2′-deoxycytidine [27]. The formation of both hydantoin derivatives may be rationalized in terms of initial oxidation of the 5,6-double bond followed by opening of the pyrimidine ring and a decarboxylation reaction leading to the loss of the carbon at position 6 prior a final cyclization step resulting in a ring contraction [25]. Recently, one-electron oxidation of 5-hydroxypyrimidine nucleobases, namely 5-hydroxyuracil (5-OH-Ura) and 5-hydroxycytosine (5-OH-Cyt) lesions, has been shown to give rise to 5-hydroxyhydantoin modifications *via* the formation of isodialuric and dialuric acid intermediates [33–35].

Examining the mutagenicity and repair of DNA oxidation products and providing a structural basis for these effects are of fundamental importance for understanding their genotoxic properties. Thus,



Scheme 1. Chemical structure of 5-OH-dHyd and 5-OH-5-Me-dHyd, resulting from oxidatively generated modifications of 2′-deoxycytidine and thymidine respectively. The structures of 8-oxo-dGuo and 5-OH-dCyd lesions, used as reference substrates in repair experiments, are also provided.

Table 1
Oligonucleotide sequences used in the present work

Oligonucleotides	Sequences (5′ to 3′)
1	CAC TTC GGA (**5-OH-Hyd**)CG TGA CTG ATC T
2	CAC TTC GGA (**5-OH-5-Me-Hyd**)CG TGA CTG ATC T
3	CAC TTC GGA (**8-oxoGua**)CG TGA CTG ATC T
4	CAC TTC GGA (**5-OH-C**)CG TGA CTG ATC T
5A	AGA TCA GTC ACG ATC CGA AGT G
5C	AGA TCA GTC ACG C̄TC CGA AGT G
5G	AGA TCA GTC ACG G̲TC CGA AGT G
5T	AGA TCA GTC ACG T̲TC CGA AGT G

Sequences 5A, 5C, 5G and 5T represent the complementary strands with the four possible nucleobases opposite the lesion.

determination of the biological role of 5-hydroxyhydantoin and 5-methyl-5-hydroxyhydantoin bases has been made possible by the synthesis of site-specific modified oligonucleotides [36,37]. Availability of such probes facilitates the assessment of the mutagenic potential of the damage during DNA synthesis by DNA polymerases. Thus, primer extension studies suggest that 5-OH-5-Me-dHyd and 5-OH-dHyd act as potential blocking lesions for replicative DNA polymerases, inducing cell lethality *in vivo* [36]. Moreover recent data, obtained by Woodgate et al. [38,39], clearly indicate that hydantoin lesions may be efficiently by-passed by low fidelity polymerases involved in the translesional synthesis (TLS) pathway. The replication data thus obtained, which necessitate to be extended by complementary *in cellulo* assays, reveal that both hydantoin lesions may be involved in mutagenesis and carcinogenesis processes if not efficiently eliminated by the cellular repair machineries.

The lesion-containing oligonucleotides constitute suitable probes for the delineation of the substrate specificity of DNA repair enzymes [40–42]. Thus, 5-OH-dHyd and 5-OH-5-Me-dHyd-containing oligomers (Table 1) were used in the present repair study to assess the substrate specificity of several DNA *N*-glycosylases. DNA base damage excision experiments were performed in order to check whether the latter oxidatively generated base lesions were substrates for endonuclease III (endo III), endonuclease VIII (endo VIII), formamidopyrimidine DNA *N*-glycosylase (Fpg or MutM), yeast Ntg1 protein (yNtg1), yeast Ntg2 protein (yNtg2), yeast and human Ogg1 proteins (yOgg1 and hOgg1) and 3-methyladenine DNA glycosylase II (AlkA). K_m and V_{max} values were determined to assess the excision efficiency of 5-OH-Hyd and 5-OH-5-Me-Hyd by Fpg and endo III, by comparison with 8-oxoGua and 5-OH-Cyt. The relatively high values of the V_{max}/K_m ratio for the removal of both hydantoin nucleobases, that may efficiently undergo epimerization between the 5*R** and 5*S** stereoisomers by chain-cycle tautomerism, suggest that repair of these oxidized bases by Fpg and endo III proteins could be of biological importance.

2. Experimental

2.1. Materials

T4 polynucleotide kinase, Klenow fragment (exo⁻) of *E. coli* DNA polymerase I, [γ-^{32}P]-ATP, dNTPs, NAP-25 Sephadex and MicroSpin G-25 columns were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). *Taq* DNA polymerase from *Thermus aquaticus* was purchased from Boehringer Mannheim (Mannheim, Germany). Endonuclease VIII was from Trevingen-Interchim (Montluçon, France). Endonuclease III, Fpg, Ntg1, Ntg2, yOgg1, hOgg1 and AlkA were prepared as described previously [43–47]. ZipTip$_{c18}$ columns were from Millipore (Milford, MA).

2.2. Oligonucleotide synthesis

All the oligonucleotides used in the present study (Table 1) were synthesized by standard phosphoramidite chemistry using an Applied Biosystems Inc. 392 DNA synthesizer. The 5-hydroxyhydantoin- and

the 5-hydroxy-5-methylhydantoin-containing oligonucleotides 1 and 2 were prepared as previously described [36,37]. The 8-oxodGuo-containing oligonucleotide 3 was prepared using a commercially available phosphoramidite monomer of 8-oxo-7,8-dihydro-2′-deoxyguanosine (GlenResearch, Sterling, VA). The 5-OH-dCyd-containing oligonucleotide 4 was synthesized using the 5-hydroxy-2′-deoxycytidine phosphoramidite as previously reported [48]. Unmodified oligonucleotides 5A, 5C and 5G were deprotected in concentrated aqueous ammonia solutions (32%) for 15 h at 55 °C. Oligonucleotides 1, 2 and 4 were deprotected in concentrated ammonia solutions (32%) for 4 h at 25 °C. Oligonucleotide 3 was deprotected in a concentrated ammonia solution of 0.25 M β-mercaptoethanol for 15 h at 55 °C in order to prevent further oxidation of 8-oxoGua during the deprotection step. After evaporation of the solvent under vacuum, the crude 5′-DMTr oligonucleotides were purified and deprotected on-line by reversed-phase HPLC using a polymeric phase column [48]. The purity and homogeneity of the collected fractions were controlled by HPLC and denaturing PAGE analyses. Oligonucleotides 1–4 were further purified by PAGE using a 20% polyacrylamide/7 M urea gel and then desalted using NAP-25 Sephadex columns. The integrity of the oligonucleotides was assessed by both MALDI-TOF mass spectrometry (positive mode) and ESI mass spectrometry analyses (negative mode).

2.3. Radiolabeling of oligonucleotides

Oligonucleotides (50 pmol) were labeled at the 5′-end with 5 μCi of [γ-^{32}P]-ATP (2 pmol, 10 mCi/mL) upon incubation with T_4 polynucleotide kinase (5 units) in 10 μL of supplied buffer at 37 °C for 30 min. Then, the reaction was stopped by addition of 1 μL of a 0. 5 M EDTA solution (pH=8). Un-incorporated [γ-^{32}P]-ATP was removed by purification of the oligonucleotides on MicroSpin column G-25 columns.

2.4. Excision of the hydantoin lesions by DNA N-glycosylase proteins

2.4.1. Determination of substrate specificity

DNA repair experiments were carried out with Fpg, endo III, endo VIII, Ntg1, Ntg2, yOgg1, hOgg1 and AlkA proteins using site-specifically modified double-stranded DNA fragments that contained either a unique 5-hydroxy-5-methylhydantoin or a single 5-hydroxyhydantoin residue as the base substrate. Typically, 1 pmol of the 5′-[^{32}P]-end-labeled modified 22-mer oligonucleotide 1 or 2 was annealed to 2 pmol of the non labeled complementary strand 5A, 5C, 5G or 5T, by heating the resulting solutions at 80 °C for 5 min and subsequent slow cooling to 4 °C, at least, for 3 h. The enzymatic reactions were performed in a 10 μL solution of 20 mM Tris–HCl, 1 mM EDTA, 100 mM KCl (pH=7.5) for Fpg and endo III, 25 mM Tris–HCl, 50 mM NaCl, 2 mM EDTA (pH=7.6) for Ntg1, Ntg2, and Ogg1 proteins, 10 mM Hepes, 1 mM EDTA, 50 mM NaCl (pH=7.5) for endo VIII protein and 70 mM Hepes–KOH, 1 mM EDTA, 5 mM 2-mercaptoethanol (pH=7.8) for AlkA, at 37 °C for 30 min with increasing concentrations of the enzymes. It should be noted that an additional heating step, at 90 °C for 5 min in a 1 M piperidine solution, has been performed for AlkA protein. The reactions were stopped by addition of 5 μL formamide dye. Samples were then loaded onto a 20% polyacrylamide/7 M urea gel in TBE buffer. The electrophoresis was carried out at 1600 V for 2 h. The products of the reaction were then analyzed by phosphorimaging.

2.4.2. Kinetic studies of excision by Fpg and endo III proteins including determination of V_{max} and K_m

The concentration range of modified oligonucleotides 1, 2, 3 or 4, in the presence of 2 equiv. of the complementary strand 5G (for 1 and 4), 5A (for 2) or 5C (for 3) was 0.1 to 3 μM. Substrate concentrations were chosen so that the Michaelis–Menten curves reach a plateau. For each reaction that was performed in a volume of 10 μL, the amount of ^{32}P-labeled oligonucleotide was 1 pmol. The concentrations of Fpg (30. 2 kDa) were 2.6, 1.3 and 7.8 ng/μL for the 8-oxodGuo, 5-OH-dHyd and 5-Me-5-OH-dHyd-modified duplexes, respectively. The concentrations of endo III (23 kDa) were 6.0, 1.5 and 1.5 ng/μL for the 5-OH-dCyd, 5-OH-dHyd and 5-OH-5-Me-dHyd-modified duplexes, respectively. The enzymatic reactions were allowed to proceed at 37 °C for either 8, 10 or12 min in the presence of Fpg or 6, 4 or 8 min in the presence of endo III, for the reference lesions (8-oxodGuo or 5-OH-dCyd) and the studied DNA modifications (5-OH-dHyd or 5-OH-5-Me-dHyd). The reactions were stopped by adding the formamide dye. The samples were subjected to a 20% denaturing PAGE, and the resulting gel was analyzed by phosphorimaging. Bands corresponding to cleavage products and unreacted oligonucleotides were quantified by non-linear least-squares fitting of the data points using Microcal Origin, on the basis of at least three separate experiments. Reaction velocity (V_{max}) was expressed in picomoles of substrate per min, while the

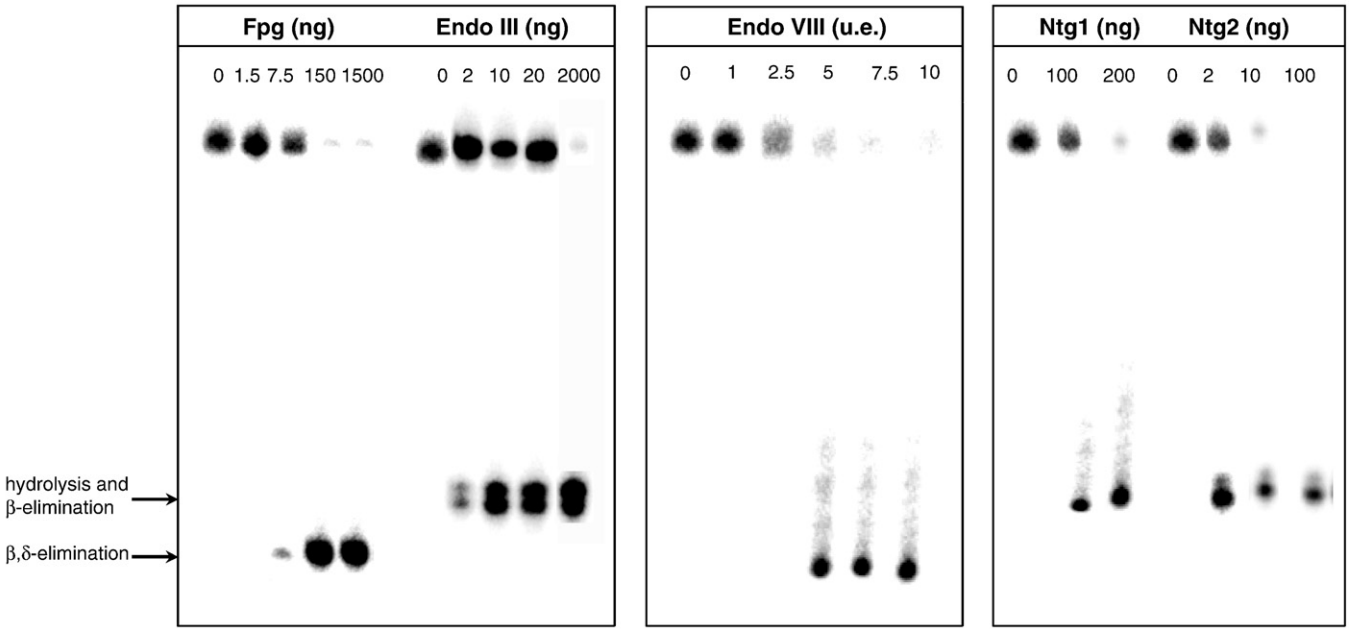


Fig. 1. Denaturing PAGE analysis of the cleavage products upon incubation of 22-mer DNA duplexes 1/5G with Fpg, endo III, endo VIII, Ntg1, and Ntg2.

substrate concentration was expressed in either nanomolarity or micromolarity.

3. Results

5-OH-Hyd and 5-OH-5-Me-Hyd are abundant lesions generated by exogenous or endogenous oxidative processes within isolated DNA [25] and have been tentatively detected in cells [49,50]. They are formed in high yields at pyrimidine sites by ·OH radical-mediated oxidation [25,26,28,29], UV-A sensitized one-electron oxidation reactions [31] and ozone perepoxidation [27,32] in aerated aqueous solutions. Only a few experimental results on the biological consequences of the latter hydantoin derivatives have become available in the recent years. Thus, primer extension assays by either *Taq* polymerase or the Klenow exo$^-$ fragment show that dAMP is mainly inserted opposite 5-OH-dHyd with no full extension of the primer (Gasparutto et al., unpublished results). In addition, previous primer extension studies show that 5-OH-5-Me-dHyd acts as a blocking lesion for the *Taq* polymerase and the Klenow exo$^-$ fragment [36]. Indeed, the DNA chain extension by *Taq* polymerase was stopped in front of the lesion since only small amounts of nucleotides, likely to be dAMP and dGMP, were inserted opposite 5-OH-5-Me-dHyd with no fully extended primer. Klenow exo$^-$ fragment-mediated polymerization leads to incorporation of mainly dAMP opposite the lesion. However the enzyme was not able to extend the 5-OH-5-Me-dHyd/dA pair beyond the damage in the presence of the four dNTPs. More recently, McDonald et al. have clearly shown that hydantoin lesions may be efficiently processed by Y-family DNA polymerases, such as *Sulfolobus solfataricus* polymerase IV. The latter low fidelity polymerase involved in the TLS pathway can overpass hydantoin lesions inducing mutagenesis [38]. The latter primer extension results suggest that 5-OH-Hyd and 5-OH-5-Me-Hyd may be mutagenic *in vivo*. Due to the potentially mutagenic properties of the 5-OH-5-Me-dHyd and 5-OH-dHyd lesions, it is relevant to study how they are removed by the base excision repair process, which involves several types of DNA *N*-glycosylases for the initial excision step of the modified nucleobases [17].

3.1. Action of various E. coli and eukaryotic DNA N-glycosylases on 5-hydroxyhydantoin containing oligonucleotides

The main substrates for Fpg protein are 8-oxo-7,8-dihydroguanine (8-oxoGua) and formamidopyrimidine modifications of purine bases [43,51]. More recently other oxidized bases including oxaluric acid [52], 2,2,4-triamino-5-(2*H*)-oxazolone [53], spiroiminodihydantoin and guanidinohydantoin derivatives [54] have been shown to be efficiently recognized and excised from DNA duplexes by Fpg. It was also found that several modified pyrimidine bases, such as 5-hydroxycytosine, 5-hydroxyuracil, thymine glycol and 5,6-dihydrothymine are recognized and excised by Fpg [55,56]. Endonuclease III is the product of the *nth* gene. The enzyme exhibits both *N*-glycosylase and 3′-apurinic/apyrimidinic (AP) lyase activities [17]. Previous studies have shown that endo III recognizes and excises several oxidized and fragmented thymine and cytosine residues [36,55–57]. More recently, it was reported that modified purine nucleobases namely 2,2,4-triamino-5-(2*H*)-oxazolone [53], oxaluric acid [52], spiroiminodihydantoin and guanidinohydantoin [54] are recognized and efficiently excised by endo III. It may be added that *E. coli* endo VIII protein exhibits substrate specificity similar to that of endo III [58,59]. Thus, endo VIII glycosylase is likely to act as a back-up repair system for excision of oxidized pyrimidine residues and may operate in complement to endo III enzyme when the level of damage to DNA increases significantly within the cell [60]. More limited information is available on the specificity and action mode of yeast Ntg1 and Ntg2 enzymes, which were more recently isolated and characterized. Both Ntg1 and Ntg2 genes from *Saccharomyces cerevisiae* encode proteins whose sequences are significantly homologous to those of *E. coli* endo III, with overlapping substrate specificities that include several oxidized pyrimidine bases [61–65]. Interestingly, Ntg1 and Ntg2 are able to excise several modified purine bases [45]. The yOgg1 and hOgg1 proteins are DNA *N*-glycosylase/AP lyase enzymes which are functionally homologous to the *E. coli* Fpg enzyme; they are able to remove mostly guanine lesions such as 8-oxoGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 2,6-diamino-4-hydroxy-5-N-methyl-formamidopyrimidine (Me-FapyGua) [46,61,66–69]. It was clearly shown that yOgg1 efficiently excised 8-oxo-7,8-dihydroadenine when the latter damaged was present opposite cytosine [70]. Up to now, only 5,6-dihydrothymine and two oxidized pyrimidine bases including 5,6-dihydroxy-5,6-dihydrothymine and 5-hydroxyuracil have been identified as weak Ogg1 substrates [71]; the latter glycosylase showing a high specificity towards alterated purines. 3-Methyladenine DNA glycosylase II (AlkA) is an *E. coli* monofunctional enzyme, that exhibits a *N*-glycosylase without AP-lyase activity with a broad substrate specificity for alkylated DNA [17,47]. Moreover, 5-formyluracil and 5-(hydroxymethyl)uracil, two oxidized pyrimidines, are efficiently removed by AlkA [72].

To obtain further information on the substrate specificity of the eight studied DNA *N*-glycosylases (*vide supra*), toward the 5-OH-dHyd moiety,

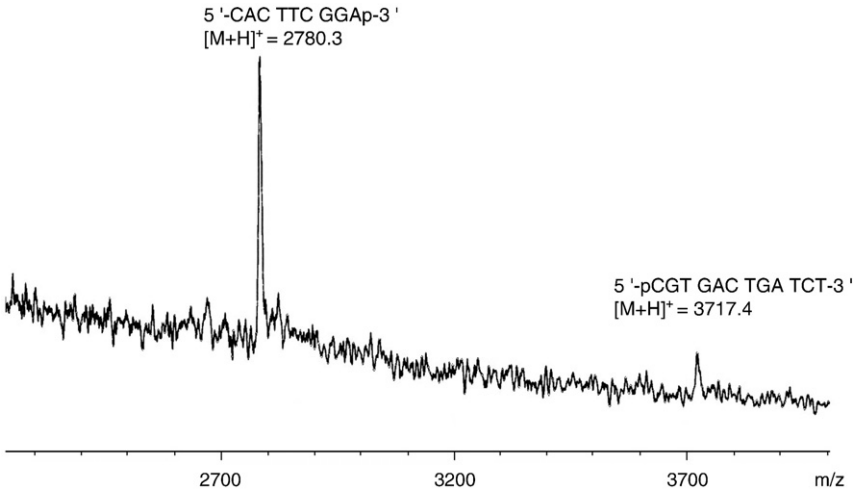
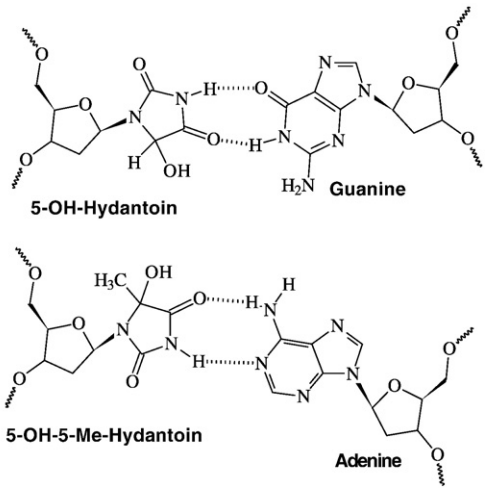


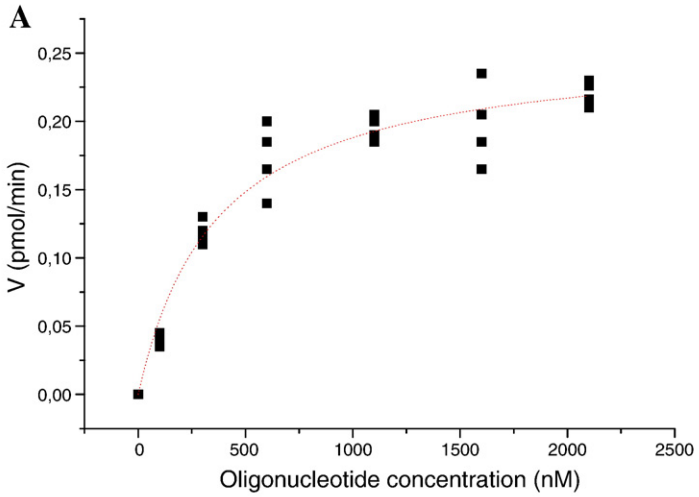
Fig. 2. MALDI-TOF-MS analyses of the mixture of oligonucleotides arising from the incubation of the 22-mer DNA duplex 1/5G with Fpg protein. Typically, 10 pmol of lesion-containing oligonucleotide duplex were incubated at 37 °C for 30 min with Fpg protein (50 ng/μL) in 10 μL standard enzymatic reaction buffer. The products were precipitated twice in ammonium citrate buffer and cold ethanol, prior to be subjected to MALDI-TOF mass spectrometric measurements.



Scheme 2. Postulated structures of 5-OH-Hyd/G and 5-OH-5-Me-Hyd/A base pairs (that may be formed within oxidized DNA) inserted into DNA duplexes and used to determine kinetic parameters of Fpg- and endo III-mediated excision reactions of both hydantoin lesions.

the modified 22-mer DNA duplexes (1/5A, 1/5C, 1/5G and 1/5T) (Table 1) that contain the damage opposite any of the four main normal DNA bases were prepared. Then, 5-OH-dHyd excision by the repair enzymes was assessed by monitoring the oligodeoxyribonucleotide strand breakage on the basis of the results of polyacrylamide gel electrophoresis analyses. Thus, it was shown that endo III, yNtg1 and yNtg2 proteins, which act primarily on modified pyrimidine bases, are able to cleave the modified DNA duplex at the site of 5-OH-dHyd (Fig. 1), regardless of its opposite base. Moreover, it appears clearly that endo VIII is able to remove the damage from the double-stranded DNA, confirming the high degree of functional similarity between this enzyme and endo III (Fig. 1). More surprisingly, it was found that 5-OH-Hyd was also a substrate for the Fpg protein since the oligonucleotide was quantitatively cleaved at the modified site using 100 ng (10 ng/μL) of the latter repair enzyme (Fig. 1). Interestingly, 5-OH-Hyd lesion is efficiently excised by the Fpg protein regardless of the base located on the opposite strand (data not shown). This provides further support to some recent observations, which indicate that Fpg exhibits a much broader substrate specificity that was initially expected, being able to remove several fragmented and rearranged purine and pyrimidine lesions.

To obtain additional information on the processing of the 5-OH-dHyd lesion by the latter repair enzyme, MALDI-TOF MS analysis of the cleavage reaction mixture was carried out. As it was already shown, MALDI-TOF mass spectrometry is a powerful tool to investigate lesion processing within DNA fragments by repair enzymes, thus allowing us to gain insights into mechanistic aspects of oligonucleotide cleavage [36,56,73]. It should be remembered that conclusions of earlier studies were suggestive of a β-δ-elimination mechanism concerning the mode of action of Fpg protein [74]. Under the experimental conditions of cleavage and analysis used in the present work, Fpg when acting on 5-OH-Hyd containing oligonucleotides, gave rise to fragments with molecular weighs corresponding to those of the expected products of a β-δ-elimination mechanism. Thus, two peaks, one at $[M+H]^+$ = 2780.3 Da corresponding to the 9-mer oligonucleotide released 5′ to the lesion 5′-CAC TTC GGAp-3′ ($[M+H]^+$ calculated = 2779.8 Da), the other at $[M+H]^+$ = 3718.2 Da corresponding

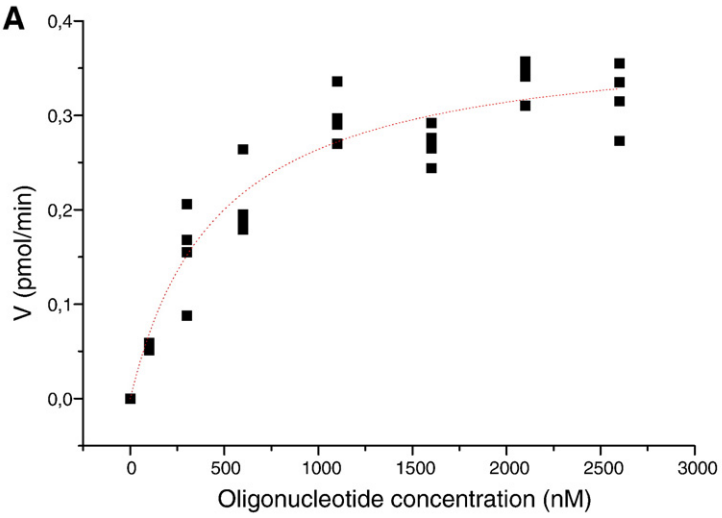


B

Enzyme	Substrate[(a)]	V_m (pmol/min)	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} nM^{-1}) x 10^{-3}
Fpg	5-OH-dHyd/G	0.149 (0.007)	1030 (123)	0.35 (0.01)	0.337
Fpg	5-Me-5-OH-dHyd/A	0.257 (0.012)	365 (60)	0.10 (0.01)	0.273
Fpg	8-oxoGua/C	0.216 (0.026)	600 (20)	0.25 (0.03)	0.418

[(a)]Designated by the base pair at the site of the lesion

Fig. 3. (A) Michaelis–Menten kinetics. Cleavage of 5-OH-5-Me-Hyd-containing oligonucleotide by Fpg (7.8 ng/μL). (B) K_m and V_{max} values for the removal of 5-OH-Hyd, 5-OH-5-Me-Hyd and 8-oxoGua by Fpg (1.3, 7.8, 2. 6 ng/μL respectively). Numbers in parentheses indicate standard errors. The values are the mean of at least three separate experiments.



B

Enzyme	Substrate[(a)]	V_m (pmol/min)	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} nM^{-1}) x 10^{-3}
endo III	5-OH-dHyd/G	0.387 (0.021)	467 (89)	0.31 (0.02)	0.663
endo III	5-Me-5-OH-dHyd/A	0.194 (0.024)	926 (295)	0.16 (0.02)	0.167
endo III	5-OHC/G	0.192 (0.009)	897 (122)	0.08 (0.009)	0.086

[(a)]Designated by the base pair at the site of the lesion

Fig. 4. (A) Michaelis–Menten kinetics. Cleavage of 5-OH-Hyd-containing oligonucleotide by endo III (1.5 ng/μL). (B) K_m and V_{max} values for the removal of 5-OH-Hyd, 5-OH-5-Me-Hyd and 5-OH-Cyt by endo III (1.5, 1.5, 6 ng/μL respectively). Numbers in parenthesis indicate standard errors. The values are the mean of at least three separate experiments.

to the 12-mer oligonucleotide released 3′ to the damage 5′-pCGT GAT TGA TCT-3′ ($[M+H]^+$ calculated = 3717.4 Da were observed for the 22-mer substrate oligonucleotide 1 (Fig. 2).

The yeast and human functional homologues of Fpg, namely yOgg1 and hOgg1 proteins, were unable to excise efficiently 5-OH-Hyd when paired with the four normal nucleobases, as already observed for the 5-OH-5-Me-Hyd lesion [36]. This result confirmed the high specificity of Ogg1 for 8-oxo-7,8-dihydroguanine and formamidopyrimidine bases, with no significant capability to excise modified, contracted or fragmented pyrimidine residues. Finally, 5-OH-dHyd and 5-Me-5-OH-dHyd containing 22-mer oligonucleotides 1 and 2, respectively, were incubated with AlkA (*N*-glycosylase activity) followed by treatment in 1 M solution of piperidine at 90 °C during 5 min in order to induce the cleavage of putative AP-sites. It was found that the 2 latter lesions were not excised by the AlkA enzyme (data not shown).

3.2. Comparative kinetic parameters of excision of hydantoin lesions from duplex DNA by Fpg and endo III proteins

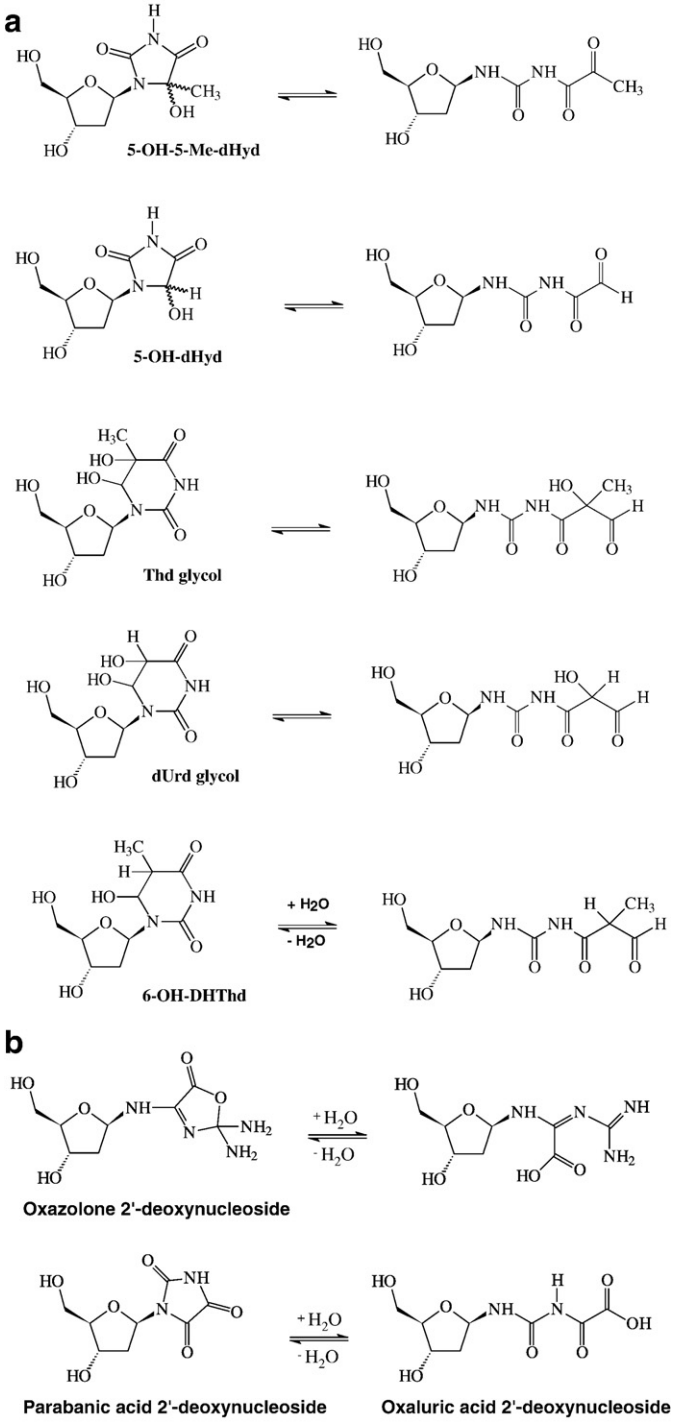
In order to determine kinetic parameters of Fpg- and endo III-mediated excision reactions of 5-OH-dHyd and 5-OH-5-Me-dHyd lesions and then to assess the relative efficiency in comparison with reference substrates, four modified DNA duplexes were used. This was achieved by annealing the modified oligonucleotides 1-4 with their complementary strand 5G, 5A, 5C and 5G respectively (Scheme 2). Experiments in which the concentration of the probes was varied were carried out, and values of V_{max} and K_m were determined for each of the lesions. Kinetic values obtained either with Fpg for 8-oxoGua, 5-OH-Hyd and 5-OH-5-Me-Hyd or with endo III for 5-OH-Cyt, 5-OH-Hyd and 5-OH-5-Me-Hyd are also reported for comparison purpose (Figs. 3B and 4B). Consideration of the k_{cat}/K_m values, listed in Fig. 3B, indicates that removal of 8-oxoGua, a preferential substrate for Fpg, is only 1.2-fold more efficient in comparison to 5-OH-Hyd and 1.5-fold in comparison to 5-OH-5-Me-Hyd. The kinetic constants of the Fpg protein for excision of both hydantoin lesions and 8-oxoGua are very similar; this is suggestive of a significant protecting role for Fpg against deleterious consequences of these damaged pyrimidine bases in prokaryotic cells.

The relative efficiency of removal of 5-OH-Hyd-, 5-OH-5-Me-Hyd-, and 5-OH-Cyt-containing oligonucleotides by endo III was also determined. Site-specifically modified duplexes 1/5G, 2/5A and 4/5G were subjected to endo III cleavage. Experiments in which the concentration of the substrate was varied were performed and values of V_{max} and K_m were determined for each modified duplex (Fig. 4A, 5-OH-Hyd excision by endo III). As shown in Fig. 4B, 5-OH-Hyd and 5-OH-5-Me-Hyd are much better substrates for endo III than 5-OH-Cyt as inferred from the comparison of the k_{cat}/K_m constants. Moreover, the processing of 5-OH-dHyd by the latter DNA repair enzyme is now favored by approximately 4-fold with respect of that of 5-OH-5-Me-Hyd. Interestingly the removal of 5-OH-Hyd is favored by 7.6-fold in comparison to 5-OH-Cyt. The latter value is closed to that previously reported for 5,6-dihydroxy-5,6-dihydrothymine (Tg) (7.0-fold higher for Tg than for 5-OHC) [54] and 5,6-dihydroxy-5,6-dihydrouracil (Ug) [75].

4. Discussion

The available comparative studies together with the present findings, clearly show that the pyrimidine lesions which exhibit a linear structure or can undergo ring-chain tautomerism at C6-N1 or

C5-N1 bond, namely Tg, Ug, ring-fragmented thymine C5-hydrate, 6-hydroxy-5,6-dihydrothymine (6-OH-DHT) [76,77], 5-OH-Hyd and 5-OH-5-Me-Hyd, are the best substrates for endo III (Scheme 3). These observations are confirmed by recent results, which have shown that oxidized purine bases with an acyclic structure, such as 2,2,4-triamino-5-(*2H*)-oxazolone (Oz) and oxaluric acid (Ox) (Scheme 3), are excellent substrates for endo III in comparison with the 5-OH-Cyt residue [52,53].



Scheme 3. Structures of ring-opened base products and modified bases which exhibit a ring-chain tautomerism that result from pyrimidine (a) and purine (b) base oxidation, efficiently excised by Fpg and endo III proteins.

Thus, on the basis of the latter studies in which the kinetic parameters have been determined from lesion-containing duplex oligonucleotides, a tentative substrate classification can be done for Fpg and endo III proteins. Regarding the Fpg protein, it is now well documented that the best substrates are 8-oxoGua and 2,6-diamino-4-hydroxy-5-formamidopyrimidine. However, the present study together with other recent investigations [52,53] have shown that Fpg may recognize and remove several ring-opened base products and modified bases which exhibit a ring-chain tautomerism leading to a dynamic equilibrium between a linear form and cyclic structures, with the same order of magnitude or a slightly less efficiency, namely Ox, Oz, 5-OH-Hyd, 5-OH-5-Me-Hyd, 6-OH-DHT and Tg lesions; the latter lesions being processed with a higher preference than 5-OH-Cyt or DHT residues, oxidized or saturated pyrimidine bases that have been identified as poor substrates. Moreover, several data reported in the literature show that the endo III protein preferentially excises a large variety of oxidatively generated lesions in the following order: Ug ~ Tg ~ Oz ~ Ox > 5-OH-Hyd ~ 5-OH-5-Me-Hyd > 5-OH-Cyt > DHT. It may be noted that such comparisons of various substrates for endo III and Fpg proteins, presented here, include lesions inserted into different sequence contexts and the use of different batches of enzymes that could lead to potential value variations between the reported studies. However, it clearly appears that the ring-opened base products are excellent substrates for both DNA *N*-glycosylases that however exhibit different initial specificities.

The excision activities of DNA *N*-glycosylases, such as endo III and Fpg repair enzymes, toward a large panel of ring opened base lesions may be explained by the high lability of the *N*-glycosidic linkage. Furthermore the *N*-glycosidic bond of ureids is usually labile, thus favoring its hydrolysis. Moreover the loss of planarity of the nucleobases with a fragmentated ring that also leads to the lack of stacking with neighboring bases induces a structural distortion and the adoption of an extrahelical orientation for the base residues. This should favor efficient enzymatic processing, regardless of the opposite base. Another parameter to be considered for the excision of linearized residues is the possible accomodation by the active sites of both endo III and Fpg of such small acyclic nucleobases that generate a weak steric hindrance. As a result, the linear fragmented nucleobases may be easily inserted into the active site of the repair enzymes and then processed. Moreover the presence of several carbonyl and amino functions in the fragmented bases structure, able to form bonds with the amino acids localized in the enzyme catalytic pocket, may be involved in the recognition and the stabilization of the damaged DNA-protein repair complex. Finally, our present proposal (efficient removal of damaged nucleobases linked to their abilities to adopt an opened-ring structure) is based on several experimental results obtained recently in our laboratory and others by using a set of linear lesion-containing DNA probes (*vide supra*). We agree on the fact that a more definitive answer is awaiting forthcoming by performing structural and molecular modelling studies in order to better ascertain the current working hypothesis.

5. Conclusion

In conclusion, evidence was provided that 5-OH-Hyd is a good substrate for the Fpg, endo III, endo VIII, yNtg1 and yNtg2 repair enzymes, which exhibit both a *N*-glycosylase and a AP-lyase activity. In contrast, 5-OH-Hyd and 5-OH-5-Me-Hyd are not substrates for the yeast and human Ogg1 proteins and the AlkA enzyme. Values of k_{cat}/K_m for the Fpg-mediated removal of the latter oxidatively generated pyrimidine lesions revealed that 8-oxoGua is a slightly better substrate than 5-OH-Hyd and 5-OH-5-Me-Hyd. The results obtained with endo III indicate that 5-OH-Hyd and 5-OH-5-Me-Hyd are much efficiently removed than 5-OH-Cyt, another oxidized pyrimidine base. Thus, the present study supports a biological relevance of these excision processes, while the removal of hydantoin lesions by the Fpg

and endo III proteins are effected at better or comparable rates to that of the removal of 8-oxoGua and 5-OH-Cyt, two established cellular substrates. Regarding the excision repair of these hydantoin lesions in humans, hNth, hNeil1 and hNeil2 proteins, which are homologs of endo III and endo VIII enzymes respectively, should be the major actors that act at removing the latter base damage from cellular DNA (work is in progress in our laboratory to clarify that point). Finally the present study brings new lights on the capabilities for prokaryotic and eukaryotic organisms to deal with biologically deleterious hydantoin lesions that cannot be tolerated by cells due to their lethal and mutagenic potential.

Acknowledgments

We thank Christine Saint-Pierre (INAC/SCIB/LAN-CEA Grenoble) for the MALDI-TOF mass spectrometry analyses. Authors are grateful to the Comité de Radioprotection (Electricité de France) for financial support.

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