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Review

Imidazole Ring-Opened DNA Purines and Their Biological Significance

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Fragmentation of purine imidazole ring and production of formamidopyrimidines in deoxynucleosides (Fapy lesions) occurs upon DNA oxidation as well as upon spontaneous or alkali-triggered rearrangement of certain alkylated bases. Many chemotherapeutic agents such as cyclophosphamide or thiotepa produce such lesions in DNA. Unsubstituted FapyA and FapyG, formed upon DNA oxidation cause moderate inhibition of DNA synthesis, which is DNA polymerase and sequence dependent. Fapy-7MeG, a methylated counterpart of FapyG-, a efficiently inhibits DNA replication *in vitro* and in *E.coli*, however its mutagenic potency is low. This is probably due to preferential incorporation of cytosine opposite Fapy-7MeG and preferential extension of Fapy-7MeG:C pair. In contrast, FapyA and Fapy-7MeA possess miscoding potential. Both lesions in SOS induced *E.coli* preferentially mispair with cytosine giving rise to A→G transitions. Fapy lesions substituted with longer chain alkyl groups also show simult aneous lethal and mutagenic properties. Fapy lesions are actively eliminated from DNA by repair glycosylases specific for oxidized purines and pyrimidines both in bacteria and eukaryotic cells. Bacterial enzymes include *E.coli* formamidopyrimidine-DNA-glycosylase (Fpg protein), endonuclease III (Nth protein) and endonuclease VIII (Nei protein).

Keywords: Alkylated Fapy lesions, DNA glycosylases, DNA synthesis, FapyA, FapyG, Mutations

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Abbreviations: FapyA, 4,6-diamino-5-formamidopyrimidine; FapyG, 2,6-diamino-4-hydroxy-5-formaindopyrimidine; Fapy-7MeG-2,6, diamino-4-hydroxy-5N-methyl-formamidopirymidine; Fpg protein, formamidopyrimidine-DNA glycosylase from *E. coli*; GC/IDMS, -SIM, gas chromatography/isotope dilution mass spectrometry with selected ion monitoring.

Introduction

Oxidizing and certain alkylating agents are able to trigger imidazole ring opening of adenine and guanine and formation of formamidopyrimidines (Fapy lesions) in DNA. The family of imidazole ring-opened purines contains oxidatively formed unsubstituted FapyA and FapyG as well as N7 (or C8)-substituted Fapy lesions with adducted groups ranging from one to few carbon atoms (Fig. 1). Oxidatively formed FapyA and FapyG are ubiquitous in DNA and they are also formed efficiently during cancer radiotherapy. Certain chemotherapeutic agents also increase lability of imidazole ring. Although many bacterial and eukaryotic DNA glycosylases excising from DNA Fapy lesions have been described, only few studies on the consequences of their presence in DNA are available. The present review will highlight the actual knowledge on the effect of these lesions on the rate and fidelity of DNA synthesis, mutations and cancer induction.

Physicochemical Properties of Fapy Lesions in Deoxynucleotides and in DNA

Although the mechanism of formation of unsubstituted and substituted Fapy lesions is different (redox reactions *versus*

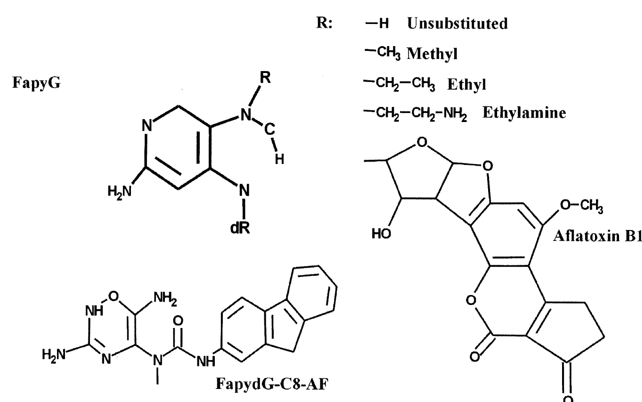

Fig. 1. Fapy-guanine lesions with possible substituents at N(7) or C(8).

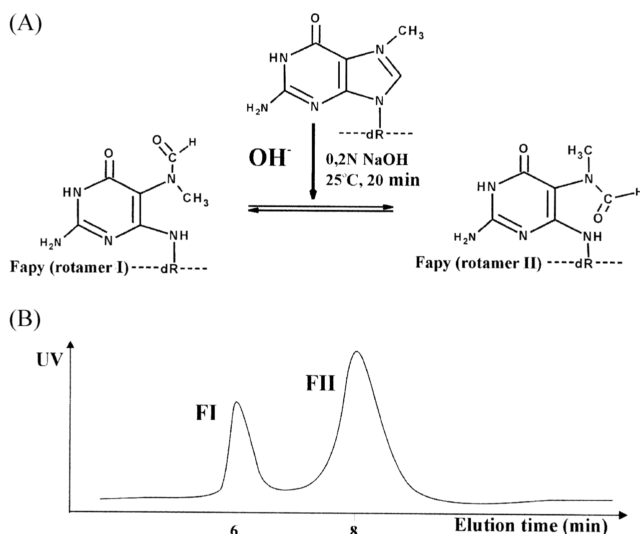

Fig. 2. Imidazole ring opening of 7-methylguanine in alkali and two rotameric forms separated by HPLC.

solvolysis), both share common structural and chemical characteristics. Imidazole ring rupture increases the number of degrees of freedom and enables rotation about N7 and C8 atoms in guanine residue. Theoretically four rotameric forms are possible. Experimentally, two rotamers of Fapy-7MeG, Fapy-7MeA and Fapy-7EtG, with rotation about N7 were separated by HPLC (Fig. 2) (Boiteux *et al.*, 1984; Tudek *et al.*, 1998; Tudek *et al.*, 1999). Rotamers of Fapy lesions bearing bulky adducts have also been found (Smela *et al.*, 2002). Rotation about N7 probably occurs also in unsubstituted, oxidatively formed Fapy lesions. However, HPLC analysis does not allow to separate rotameric forms of unsubstituted FapyA and FapyG (unpublished). In native DNA over 80% of Fapy-7MeG occurs in the form of rotamer II. In free solutions both rotamers are found in equilibrium in comparable amounts (Boiteux *et al.*, 1984). Imidazole ring opening of Fapy-7MeG stabilizes N-glycosidic bond. Only recently monomers and polymers containing unsubstituted Fapy-dA and Fapy-dG have been synthesized (Greenberg *et al.*, 2001). Using these oligonucleotides half time for N-glycosidic bond breakdown in pH 7.0 was estimated to be 103 h for Fapy-dA. Fapy-dG appeared to be 25-times more resistant to hydrolysis than Fapy-dA in the same conditions (Greenberg *et al.*, 2001).

Imidazole ring breakdown of 7MeG increases flexibility of the base and changes stacking between adjacent bases. However destabilization of the double helix seems to be rather moderate. Decrease of thermal stability of Fapy-7MeG paired with C equals -3.9ºC, which is comparable to that caused by 8-oxoG:C pair (-3.5ºC) (Asagoshi *et al.*, 2002). Presence of unsubstituted FapyA and FapyG also decreases melting points of duplexes by 1-7ºC, depending on the opposite base in the complementary strain, with the exception of FapyG:A mismatch, which melts higher than G:A duplex (Haraguchi *et al.*, 2002). FapyG with a bulky adduct of aflatoxin B_1 changes markedly the structure of DNA duplex, but differently than its precursor G-AFB_1 adducts (Smela *et al.*, 2002).

Formation of Fapy Lesions from Alkylated Bases

Alkylating agents are chemical carcinogens created not only as products or by-products of many branches of industry, but also during cellular metabolism. For example, the cellular methyl group donor, S-adenosylmethionine reacts non-enzymatically with DNA to produce 7-methylguanine and 3-methyladenine at the calculated rate of 10^3 modified bases/human genome/day (Rydberg and Lindahl, 1982). During inflammatory processes, activated macrophages produce reactive nitrogen species, of which N_2O_3 may react with cellular secondary amines forming very reactive nitrosoamines (Oshima *et al.*, 1991). The main product of DNA methylation, N7-methylguanine (7MeG) is detected in native human DNA, and its level reaches few adducts per 10^7 of normal bases (Szyfter *et al.*, 1996). It has been predicted by Lawley and Brookes that withdrowal of electrons from the purine ring by an alkyl group will destabilize imidazole ring and glycosidic bond. Accordingly, 7MeG undergoes further processing yielding either apurinic/ apyrimidinic (AP) sites or imidazole ring-opened derivative, 2,6-diamino-4-hydroxy-5N-methyl-formamidopyrimidine (Fapy-7MeG) (Singer and Grunberger, 1983; Boiteux *et al.*, 1984). Imidazole ring-opening of purines in DNA substituted in N7 position with methyl or ethyl groups occurs very slowly in physiological conditions, but is accelerated in alkali (Haines *et al.*, 1962). Fapy-7MeG was, however found in the liver of rats treated with N,N-dimethylnitrosamine, or 1,2-dimethylhydrazine, and in rat bladder epithelial DNA after treatment with N-methylnitrosourea (Beranek *et al.*, 1983; Kadlubar *et al.*, 1984).

The rate of imidazole ring-opening of purines is mediated by the structure of substituent in N7 position of guanine and occurs easily with phosphoramide mustard or ethyleneimine adducts (Hemminki, 1984). Half time of imidazole ring opening for guanine N7-ethyleneamine derivative equals 11 min in pH 7.0 and exceeds the rate of depurination of adducted base, which is 42 min in pH 6.00. Compounds commonly used in cancer chemotherapy. eg. cyclophosphamide or thiotepa produce such kinds of DNA damages (Hemminki and Kallama, 1986). Chemical carcinogen, aflatoxin B1 adds to N-7 position of guanine and the adducts can further break down to two secondary lesions, the apurinic (AP) site and AFB_1-formamidopyrimidine (Fapy) (Busby and Wogan, 1984). Fapy-adducts are persistent several days and weeks in DNA (Smela *et al.*, 2002).

One of the minor lesions formed upon DNA non-enzymatic methylation is 7-methyladenine (Lawley and Brookes, 1963). 7MedA is extremely labile in DNA, and is spontaneously depurinated about three orders of magnitude faster than from ribonucleotides. $T_{1/2}$ for 7MeA depurination from DNA equals

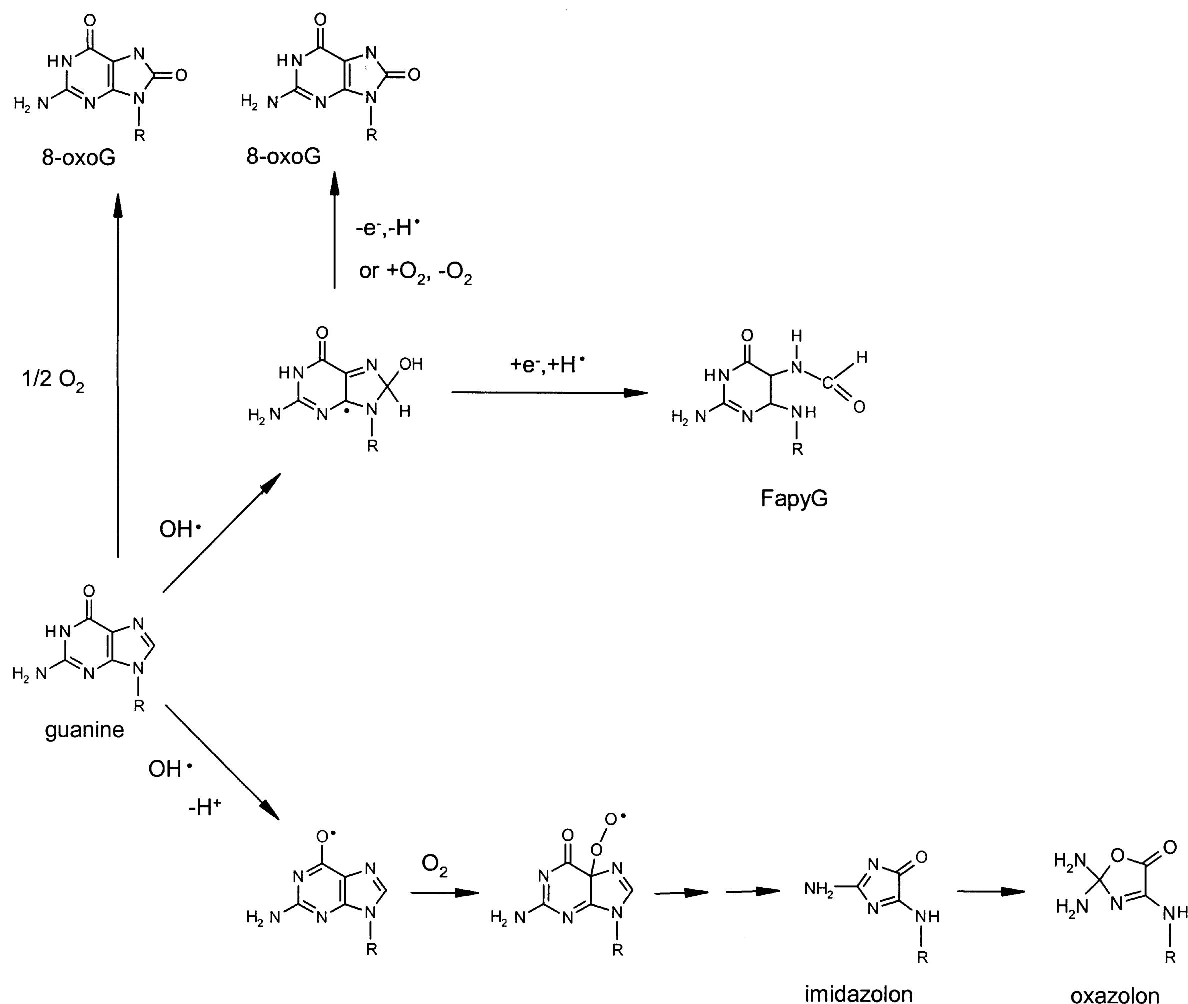

Fig. 3. Major products of guanine oxidation by reactive oxygen species.

only 3 hrs in 37°C, pH 7.00, while for 3MeA-26 hrs and for 7MeG-155 hrs (Singer and Grunberger, 1983).

In alkali 7MedA converts into Fapy-7MedA, which migrate in HPLC as 2 peaks of rotamers similarly to Fapy-7MeG, however with different time of retention (Tudek *et al.*, 1999).

Spontaneous formation of Fapy-7MeA is very likely to occur in ribonucleic acids. Studies on alkylation of free adenosine revealed that 7-methyladenosine converts spontaneously to Fapy-7-methyladenosine in neutral conditions (Singer *et al.*, 1974). In polyA, 7MeA also spontaneously converts into product(s) migrating in HPLC similarly to those observed after action of NaOH on alkylated polyA. More than 50% of 7MeA is converted into Fapy-7MeA in 24 hrs (Tudek *et al.*, 1999). The biological significance, if any, of Fapy-7MeA formation in RNA has not been studied.

Imidazole Ring-opening of Purines by Oxidation

Reactive oxygen species are generated in organisms by γ and UV radiation, biotransformation of chemicals and during cellular metabolism. The most reactive species, hydroxyl radicals are produced from H_2O_2 and O_2 in Haber-Weiss and Fenton reactions in the presence of transient metals ions (von Sonntag, 1987).

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

$$Me^{3+} + O_2^{\bullet -} \rightarrow Me^{2+} + O_2 \quad (2)$$

$$Me^{2+} + H_2O_2 \rightarrow Me^{3+} + OH^{\bullet} + OH^- \quad \text{(3, Fenton reaction)}$$

The attack of hydroxyl radicals on DNA results in single or double strand breaks, generation of abasic sites, base and sugar lesions. Hydroxyl radicals cause ionization of purines at the C8 position to form unstable 8OHG or 8OHA radicals. Such radicals can subsequently undergo further oxidation to form 8-hydroxypurines, or reduction (Fig. 3) leading to imidazole ring-opening and formation of Fapy (Fujita and Steenken, 1981). Hydroxyl radicals may also add to the C4 carbon of the guanine ring leading to the formation of oxidising neutral radical through subsequent dehydration. In the presence of oxygen, the latter compound is converted into

an imidazolone, which is further hydrolysed into 2,2-diamino-4-[(2-deoxy-β-D-erythro-pento-furanosyl)amino]-5-(2H)-oxazolone (Cadet *et al.*, 1994). 8OHG and the oxazolone are the major oxidation products of guanine upon exposure of DNA to ionising radiation in aerated aqueous solution (Douki and Cadet, 1996). However, formamidopyrimidines FapyG and FapyA are also efficiently generated by hydroxyl radicals (Dizdaroglu *et al.*, 1991). These two lesions are known to increase in H_2O_2 treated mammalian cells (Dizdaroglu *et al.*, 1991a), in cells irradiated with X or γ rays (Zastawny *et al.*, 1997) and in cancer cells (Olinski *et al.*, 1992). Additionally, illumination of DNA with UVA, aside to pyrimidine dimers, causes formation of FapyA (Doetsch *et al.*, 1995). Hydroxyl radical generating system-hypoxanthine / xanthine oxidase / Fe^{3+} / EDTA, has been shown to induce formation of unsubstituted Fapy lesions as major damages in double stranded DNA (900-fold increase of FapyGua and 40-fold increase of FapyAde) (Aruoma *et al.*, 1989), as well as in the single stranded DNA (Graziewicz *et al.*, 1999; Graziewicz *et al.*, 2000).

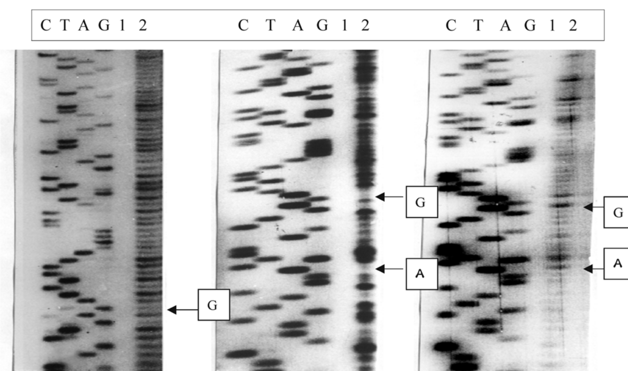

Fig. 4. DNA synthesis *in vitro* performed in the presence of all four dNTPs by: T7 DNA polymerase (left panel), Klenow fragment of polymerase I from *E.coli* (middle panel) and DNA polymerase β from calf thymus (right panel). Lanes: C, T, A, G correspond to the reference sequence. Lanes 1 show the products of DNA synthesis performed on unmodified template. Lanes 2 show the products of DNA synthesis on the template oxidised by Fenton reagent hypoxanthine/xanthine oxidase/Fe^{+3}/EDTA. Arrows point to adenine and guanine sites in the template where inhibition of DNA synthesis is different for 3 DNA polymerases.

Biological Properties of Fapy Residues

Effect on DNA synthesis First direct demonstration of imidazole ring-opened guanine behaviour during replication was obtained for methylated template, where Fapy-7MeG was introduced by alkali treatment of dimethylsulfate modified poly[d(G-C)]. These studies showed that Fapy-7MeG blocks chain elongation by *E.coli* DNA polymerase I, but does not induce mispairing with either dAMP or dTMP (Boiteux and Laval, 1983). It was subsequently shown that *E.coli* DNA polymerase I exo$^-$ and T4 DNA polymerases were inhibited one base before Fapy-7MeG (OConnor *et al.*, 1988). Fapy-7MeG inhibited also DNA synthesis in *E.coli* cells, revealing very efficient killing potential; one Fapy-7MeG residue per lethal hit was calculated (Tudek *et al.*, 1992). Recent *in vitro* studies utilizing oligodeoxynucleotide with a single Fapy-7MeG residue demonstrated that this modified base constitutes a strong, but not absolute block to DNA synthesis by *E.coli* DNA polymerase I Klenow fragment proficient and deficient in 3' → 5' exonuclease, permitting translesion synthesis with a limited efficiency. Inhibition of DNA synthesis by Fapy-7MeG is stronger than that of 8-oxoG, but weaker than that of apurinic site. Analysis of nucleotide insertion and extension efficiencies (V_{max}/K_m) shows that the extension step constitutes a major kinetic barrier to DNA synthesis, and thus DNA polymerase incorporates nucleotide opposite Fapy-7MeG and stops.

An attempt to clarify the possible effect on DNA replication of unsubstituted FapyA and FapyG was undertaken by studying DNA synthesis *in vitro* by three DNA polymerases: T7 DNA polymerase, Klenow fragment of DNA polymerase I from *E. coli* and β polymerase from calf thymus on M13mp18 phage DNA template, in which FapyG and FapyA were introduced by the Fenton reagent-hypoxanthine / xanthine oxidase / Fe^{+3} / EDTA (Graziewicz *et al.*, 2000). This modification predominantly introduced FapyA and FapyG to DNA, and its level 4-fold exceeded the level of oxidised pyrimidines, as measured by gas chromatography/mass spectroscopy with selected ion monitoring (GC/MS-SIM). DNA synthesis by both prokaryotic DNA polymerases was inhibited less frequently by oxidised purines in the template than by oxidised pyrimidines. In contrast, adenine and guanine sites in modified template that were bypassed by prokaryotic DNA polymerases, arrested calf thymus repair polymerase β (Fig. 4). An efficient DNA synthesis was also observed on poly(dA) template in which FapyA content increased 16-fold upon modification with Fenton reagent. This suggested that FapyA is rather a weak inhibitor of DNA synthesis, and this suggestion was further supported by the observation that digestion of M13 DNA with formamidopyrimidine_DNA-glycosylase (Fpg protein), which excised predominantly FapyA, created additional chain terminations opposite almost all adenine residues (Graziewicz *et al.*, 2000). The ability of FapyA to inhibit DNA synthesis depended on the sequence context: almost no inhibition of DNA replication was observed in poly (dA) run; a little more when adenine was surrounded by cytosine residues; more when adjacent to thymine and the strongest inhibition was observed in some adenine and guanine rich sequences. Klenow DNA polymerase was similarly, but less efficiently inhibited by FapyA.

FapyG residues were suggested to possess analogous moderate toxic properties, dependent both on the nucleotide sequence and DNA polymerase, since the degree of inhibition

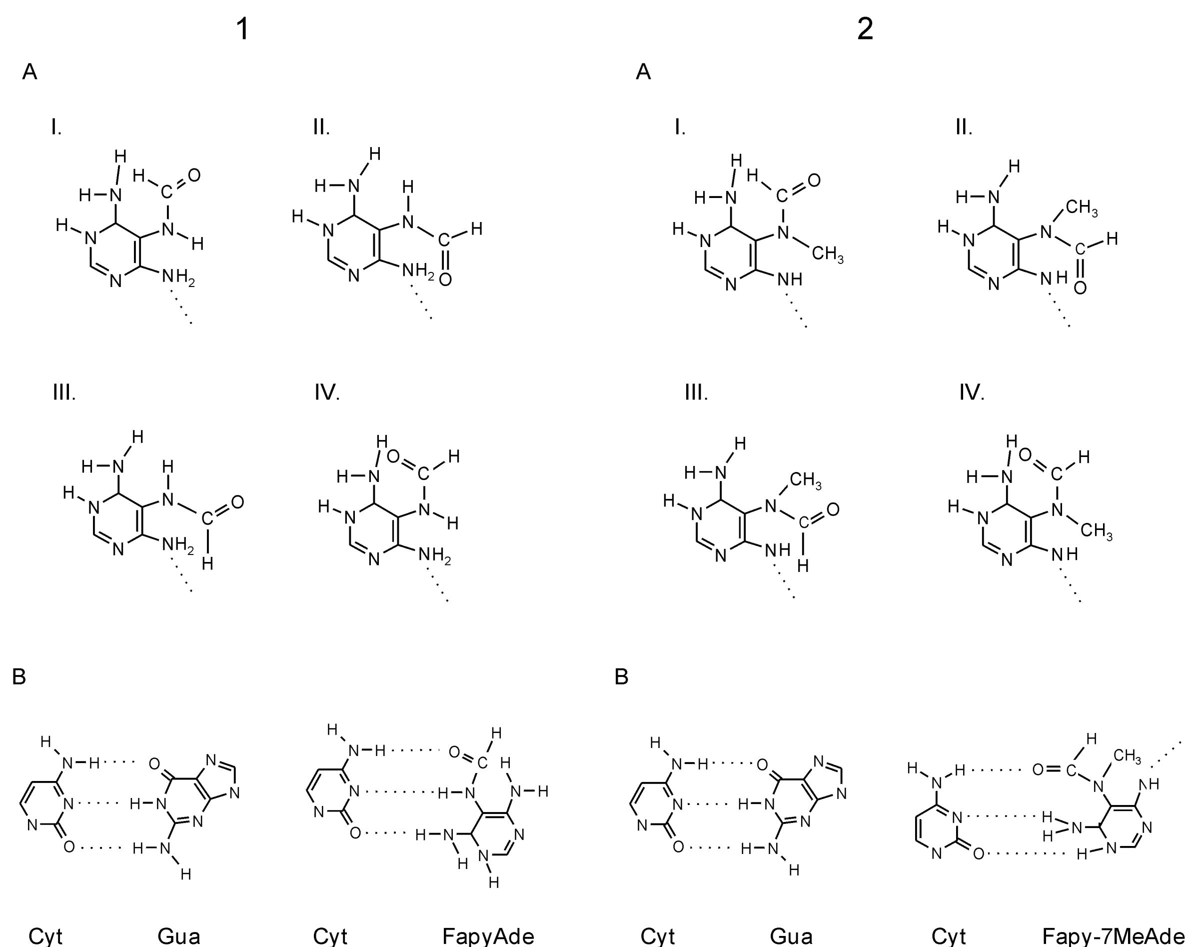

Fig. 5. Fapyadenine and Fapy-7methyladenine. (A) Four possible rotameric forms. (B) Hydrogen bonding face of guanine and appropriate rotamers of FapyA and Fapy-7MeA that resemble hydrogen bonding face of guanine and are able to pair with cytosine.

of DNA synthesis at the same guanine sites in the template was different for three DNA polymerases studied. It was proposed that oxidative DNA damage might induce conformational changes in the DNA template. These changes would, in turn, influence in different way processivity and fidelity of DNA polymerases. Rotation of the formyl group around N7 is expected in unsubstituted Fapy. It is likely that nucleotide sequence close to the Fapy can influence this conformational change and favour one of rotameric forms of the base. Conformation of the active site of DNA polymerase can, in turn, determine the efficiency of bypassing such lesion. Therefore it was suggested that DNA synthesis arrest by FapyA and FapyG is moderate for prokaryotic DNA polymerases and depends both on the neighbouring nucleotide sequence and interaction with the active site of DNA polymerase.

Miscoding properties Studies on mutagenic properties of N7-substituted FapyA and FapyG have shown that treatment with NaOH of methylated ssDNA of M13 phage created mutagenic base derivatives giving rise to A → G transitions (60-fold increase) and G → T and G → C transversions (2-3-fold increase) in *E.coli* cells under SOS conditions (Tudek *et al.*, 1992). Partial elimination of Fapy-7MeG by digestion of M13 DNA with *E. coli* formamidopyrimidine-DNA glycosylase (Fpg protein, which excises Fapy-7MeG and cleaves phosphodiester bond at the AP site left after base liberation) diminished the frequency of G → T and G → C transversions (Tudek *et al.*, 1999), suggesting that these mutations derived from Fapy-7MeG. However, mutagenic potency of Fapy-7MeG is very low and DNA polymerase III or V (SOS conditions) incorporate mainly C opposite Fapy-7MeG, but infrequently also A or G. These initial findings were confirmed by Asagoshi *et al.* (2002) who studied incorporation of nucleotides opposite Fapy-7MeG in deoxynucleotide by Klenow fragment of DNA polymerase I. When Fapy-7MeG was bypassed, dCMP was preferentially inserted opposite the lesion, while relative efficiencies of incorporation of dTMP and dAMP were 10^4-fold lower, and dGMP even 10^7-fold lower in comparison to dCMP. The primer terminus containing a Fapy-7MeG:C pair was also most efficiently extended (Fapy-7MeG:C (relative f(ext) = 1)

> Fapy-7MeG:T ($4.6 \times^{10-3}$)) Fapy-7MeG:A and Fapy-7MeG:G (extension not observed)). Thus, Fapy-7MeG is a potentially lethal but very weakly, if at all, premutagenic lesion.

In contrast Fapy-7MeA seems to be a potent miscoding lesion. Confirmation of this contention comes from observation that depurination of 7MeA from M13 DNA before imidazole ring-opening in alkali resulted in dramatic 9-fold decrease of the frequency of $A \rightarrow G$ transitions, suggesting that mutagenic adenine modification specifically triggering $A \rightarrow G$ transitions originated from 7MeA and most probably was Fapy-7MeA. Fapy-7MeA would be approximately two orders of magnitude more mutagenic than Fapy-7MeG, since as a minor lesion (40-fold less abundant in DNA than Fapy-7MeG) was giving rise to 7-fold more mutations than Fapy-7MeG (Tudek *et al.*, 1999).

Mutations deriving from oxidatively formed FapyA and FapyG, induced by Fenton reagent, hypoxanthine / xanthine oxidase / Fe^{3+} / EDTA, were analysed in M13mp18 phage DNA transfected to SOS-non-induced and induced *E. coli* (Graziewicz *et al.*, 1999). Such DNA modification resulted in the increase of $A \rightarrow G$ transitions, which were strictly dependent on the induction of the SOS system. Since in the modified DNA, FapyA constituted one of the major base damages, it is possible that these mutations derived from FapyA. Their induction involves DNA polymerase V, which is able to bypass this moderately toxic lesion, however with decreased fidelity. One of possible FapyA rotamers resembles hydrogen bonding face of guanine and might pair with cytosine during replication (Fig. 5). In the same experiment practically no increase in SOS-dependent guanine mutations were observed (Graziewicz *et al.*, 1999). It is then possible that unsubstituted FapyG, similarly to Fapy-7MeG is lethal. but not mutagenic, or with limited miscoding potency.

Other indirect evidence show that Fapy lesions adducted in N7 position with longer chains also reveal simultaneous lethal and mutagenic properties. Lethal and mutagenic properties of imidazole ring-opened purines have been demonstrated in mammalian cells, in which overproduction of repair protein for divergent Fapy residues, *E.coli* formamidopyrimidine-DNA glycosylase (Fpg protein) diminished lethal and mutagenic effect of thiotepa and aziridine (Cussac and Laval, 1996; Gill *et al.*, 1996).

Carcinogenic potency of aflatoxin B_1 was correlated with the presence of its imidazole ring-opened guanine adduct in DNA (Hsieh and Atkinson, 1991, Smela *et al.*, 2002). In reconstituted studies it was observed that the frequency of AFB_1-FAPY derived $G \rightarrow T$ mutations in *Escherichia coli* approximately 6-fold exceed the frequency of mutations caused by AFB_1-N7G adduct (imidazole ring-closed). It was also observed that one of AFB_1-FAPY rotameric forms is a very potent block of DNA synthesis, even when DNA polymerase of lowered replication fidelity was used (*Salmonella typhimurium* MucAB DNA polymerase; Smela *et al.*, 2002). Thus mutations must have arrived from the other rotamer, more easily bypassed by DNA polymerase. These observations supply a direct proof of importance of rotation about N7 in Fapy lesions in formation of the appropriate configuration that can be tolerated by the active centre of DNA polymerase in translesion synthesis through these toxic lesions.

Enzymatic Excision of Fapy Residues from DNA

DNA glycosylase/AP-lyase specific to unsubstituted and substituted formamidopyrimidines as well as 8-hydroxyguanine-Fpg protein was cloned in *E. coli* (Boiteux *et al.*, 1987). The affinity of the enzyme toward a spectrum of known substrates differs markedly depending on the structure of excised base. Fapy-7MeG and 8OHG are repaired very efficiently, with K_m being as low as 0.6-8 nM (Boiteux *et al.*, 1990; Castaign *et al.*, 1992). Size of the substituent in the N7 or C8 position of imidazole ring-opened guanine seems to be an important determinant of the enzyme activity, since the K_m value for excision of Fapy-7-ethylguanine is 7-fold higher than for excision of Fapy-7MeG (Tudek *et al.*, 1998) and for guanine imidazole ring-opened adduct of aminofluorene- over 10-fold higher (Boiteux *et al.*, 1989). In eukaryotic cells, repair of 8OHG, FapyG and of FapyA is realised by separate enzymes. In *Saccharomyces cerevisiae* two glycosylases specific for 8OHG-OGG1 and OGG2 were described (Hazra *et al.*, 1998; Karahalil *et al.*, 1998) and separate proteins, NTG1 and NTG2 excising Fapy as well as oxidized pyrimidines (Senturker *et al.*, 1998).

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