Benzene-Derived N^2-(4-Hydroxyphenyl)-Deoxyguanosine Adduct: UvrABC Incision and Its Conformation in DNA

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Running title:

Conformation and UvrABC Incision of N^2-4-HOPh-dG

Abbreviations:

HQ, hydroquinone; *p*-BQ, *p*-benzoquinone; N^2-4-HOPh-dG, N^2-(4-hydroxyphenyl)-2'-deoxyguanosine; AP, apurinic/apyrimidinic; UvrABC, *E. coli* UvrABC complex; NER, nucleotide excision repair; BER, base excision repair; Exo III, Exonuclease III; Endo IV, endonuclease IV; APE1, human AP endonuclease 1; MD, molecular dynamics.

Abstract

Benzene, a ubiquitous human carcinogen, forms DNA adducts through its metabolites such as *p*-benzoquinone (*p*-BQ) and hydroquinone (HQ). N^2-(4-Hydroxyphenyl)-2'-deoxyguanosine (N^2-4-HOPh-dG) is the principal adduct identified *in vivo* by ^{32}P-postlabeling in cells or animals treated with *p*-BQ or HQ. To study its effect on repair specificity and replication fidelity, we recently synthesized defined oligonucleotides containing a site-specific adduct using phosphoramidite chemistry. We here report the repair of this adduct by *Escherichia coli* UvrABC complex, which performs the initial damage recognition and incision steps in the nucleotide excision repair (NER) pathway. We first showed that the *p*-BQ-treated plasmid was efficiently cleaved by the complex, indicating the formation of DNA lesions that are substrates for NER. Using a 40-mer substrate, we found that UvrABC incises the DNA strand containing N^2-4-HOPh-dG in a dose- and time-dependent manner. The specificity of such repair was also compared with that of DNA glycosylases and damage-specific endonucleases of *E. coli*, both of which were found to have no detectable activity toward N^2-4-HOPh-dG. To understand why this adduct is specifically recognized and processed by UvrABC, molecular modeling studies were performed. Analysis of molecular dynamics trajectories showed that stable G:C-like hydrogen-bonding patterns of all three Watson-Crick hydrogen bonds are present within the N^2-4-HOPh-G:C base pair, with the hydroxyphenyl ring at an almost planar position. In addition, N^2-4-HOPh-dG has a tendency to form more stable stacking interactions than a normal G in B-type DNA. These conformational properties may be critical in differential recognition of this adduct by specific repair enzymes.

Keywords: Benzene; hydroquinone; *p*-benzoquinone; DNA adduct; UvrABC, Nucleotide excision repair; adduct conformation, molecular modeling

1. Introduction

Benzene is a common environmental pollutant with wide usage in industry (Wallace, 1996). Human exposure to benzene comes from gasoline and automobile combustion products, and from certain industries such as oil refineries and petrochemical/rubber manufacturing (IARC, 1982; NTP, 2005). Cigarette smoking is another major source as it is estimated to account for about half of the entire nationwide exposure to benzene (Wallace, 1996). Benzene is classified as a human carcinogen by the International Agency for Research on Cancer (IARC) and National Toxicology Program (NTP) (IARC, 1982; NTP, 2005). In rodents, benzene exposure has been demonstrated to cause tumors at multiple organ sites (Huff et al., 1989). Several human epidemiological studies (*e.g.*, Sorahan et al., 2005; Yin et al., 1996) have revealed a relationship between occupational benzene exposure and an increased incidence of leukemia, primarily acute myeloid leukemia. An increased risk of breast and lung cancer among benzene exposed populations has also been suggested from limited epidemiological or population-based studies (Costantini et al., 2009; Hansen, 2000; Petralia et al., 1999; Yin et al., 1996).

Benzene must be metabolized to more reactive compounds to exert its effects. Despite extensive investigation of the biological effects of benzene and its major metabolites for many years, the precise mechanisms underlying benzene mutagenicity and carcinogenicity remain unclear. Nevertheless, several possible mechanisms have been proposed with focuses on interactions of benzene with different cellular components as well as resulting structural and biological impacts. One of the mechanisms concerns the formation of mutagenic DNA damage by benzene

metabolites in certain types of target cells/tissues, particularly the hematopoietic progenitor cells in the bone marrow (McDonald et al., 2001; Whysner et al., 2004). DNA adducts have been detected in these cells or tissues following exposure to benzene or its metabolites (Whysner et al., 2004). The role of DNA adduct formation is also supported by the evidence from induced mutagenesis following transfection of *p*-benzoquinone (*p*-BQ)- or hydroquinone (HQ)-damaged shuttle vectors into cultured mammalian cells (Gaskell et al., 2004, 2005; Nakayama et al., 2004).

A major class of DNA adducts found to be formed *in vitro* by *p*-BQ or HQ are the exocyclic benzetheno adducts of dG, dA and dC (Gaskell et al., 2002; Jowa et al., 1986; Pongracz and Bodell, 1991; Pongracz et al., 1990). To study their biochemical and biological properties, we previously synthesized oligonucleotides containing these adducts at a single location (Chenna and Singer, 1995, 1997). Our site-directed mutagenesis experiments showed that these adducts are highly mutagenic in the cell (Xie et al., 2005) . We have also demonstrated that all three benzetheno adducts are substrates for exonuclease III (Exo III) and endonuclease IV (Endo IV) of *E. coli* and the major apurinic/apyrimidinic (AP) endonuclease (APE1) of humans (Chenna et al., 1995; Guliaev et al., 2004; Hang et al., 1996; Hang et al., 1998). These enzymes hydrolyze the phosphodiester bond immediate 5' to the adduct, leaving the benzetheno derivative on the 5'-terminal of the 3' fragment as a "dangling base" (Hang et al., 1996), a mechanism which was later referred as nucleotide incision repair (NIR) (Ischenko and Saparbaev, 2002).

Benzene-derived DNA adducts have also been reported in certain tissues or cultured cells after exposure. By ^{32}P-postlabeling, Bauer *et al* showed the formation

of DNA adducts in liver of rabbits treated with benzene (Bauer et al., 1989). Bodell and co-workers later detected several adducts in human promyelocytic leukemia HL60 cells (Levay et al., 1991; Pongracz and Bodell, 1996) and in the bone marrow cells of mice (Bodell et al., 1996) treated with benzene, *p*-BQ or HQ. The principal adduct detected in these cells/tissues by ^{32}P-postlabeling corresponded to N^2-4-HOPh-dG-3'-phosphate (Levay et al., 1991; Pongracz and Bodell, 1996) (see Figure 1 for adduct structure). The same adduct could be identified *in vitro* by allowing guanosine 3'-phosphate to react with *p*-BQ (Levay et al., 1991; Pongracz and Bodell, 1996). To date, to the best of our knowledge, both biochemical and biological properties of this adduct have not been studied. In light of the observation that induction of benzene toxicity in the bone marrow correlates with the adduct formation (Bodell et al., 1996) and that induced mutagenesis studies suggest a major role of the *p*-BQ- or HQ-induced dG adducts in benzene mutagenesis (Gaskell et al., 2004, 2005; Nakayama et al., 2004), it was of great interest for us to investigate the repair of N^2-4-HOPh-dG by cellular repair mechanisms.

Based on the chemical structure of N^2-4-HOPh-dG and known substrate specificity of respective repair pathways, we examined the specificity toward this adduct of enzymes from three different *E. coli* repair mechanisms, all of which have been well characterized and are highly conserved from bacteria to mammalian cells. Such work was greatly facilitated by our ability to synthesize the defined DNA oligonucleotides containing N^2-4-HOPh-dG at a specific position (Chenna et al., 2008). As stated above, AP endonucleases Exo III and Endo IV are able to recognize and repair benzene-derived benzetheno adducts which have gained two extra rings on

the nucleotide base. While the BER pathway primarily targets relatively small base modifications such as alkylated and oxidized bases, it is able to repair certain 5- or 6-membered exocyclic ring adducts, as shown by us and others (Hang, 2004). The NER pathway is the major mechanism for repair of bulky DNA adducts of various chemical structures (Friedberg et al., 2005; Reardon and Sancar, 2005), which was expected by us to be the primary mechanism for repair of N^2-4-HOPh-dG adduct. In *E. coli*, the initial steps of NER are carried out by the UvrABC proteins, which perform at least two sequential reactions. First, UvrA and UvrB are involved in recognition of chemical modification and structural distortion in duplex DNA, leading to the formation of the UvrB-DNA complex; Second, UvrC is recruited to the damaged site and makes incisions on the sides of the damage.

In this study, the results showed that N^2-4-HOPh-dG is specifically recognized and processed by the UvrABC complex, but not by the glycosylases and AP endonucleases tested. To gain a structural rationale for this novel substrate specificity, molecular dynamics (MD) simulations were performed to analyze the effect of N^2-4-HOPh-dG on local conformation of the DNA duplex. The significance and implications of these findings were discussed.

2. Materials and Methods

2.1. DNA substrates and repair proteins

The synthesis of the 5'-DMT-3'-phosphoramidite of N^2-4-HOPh-dG and its site-specific incorporation into defined oligonucleotides were previously described by Chenna *et al.* (Chenna et al., 2008). The unmodified controls, complementary strands

and DNA size markers were purchased from Operon Inc. (Alameda, CA). All oligomers were HPLC- and/or PAGE-purified before assayed (see Figs 3 and 5 for sequences used in this study).

To generate plasmid globally modified by *p*-BQ, pCMV vector (Invitrogen) (15 μg) was treated with several concentrations of *p*-BQ (Sigma, St. Louis, MO, stock: 500 mg/ml in methanol) or methanol only in 80 μl at 37^{o}C overnight. Both *p*-BQ-treated and untreated plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and dissolved in TE buffer (pH 8.0).

Purified recombinant UvrA, UvrB and UvrC proteins were purchased from BD Biosciences (San Jose, CA). These subunits were purified from *E. coli* DR153 overexpressing the uvrA, uvrB and uvrC genes, respectively. Exo III was purchased from Gibco BRL (Carlsbad, CA). Endo IV was from R&D Systems (Minneapolis, MN). Mismatch uracil-DNA glycosylase (Mug), endonuclease III (Nfo), endonucleae VIII (Endo VIII), formamidopyrimidine-DNA glycosylase (Fpg), and MutY were all purchased from Trevigen (Gaithersburg, MD).

2.2. Incision assays

UvrABC incision of *p*-BQ-modified plasmid DNA was measured at 30^{o}C with varying UvrABC concentrations or times in a total volume of 50 μl. The UvrABC subunits were diluted with a dilution buffer and premixed prior to the reaction. The reactions were carried out using 0.75 μg plasmid DNA in a UvrABC buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 5 mM DTT and 2mM ATP. All reactions, after terminated with 25 mM EDTA, were resolved on a

1% agarose gel with 0.3 μg/ml ethidium bromide and visualized with an IS-500 Digital Imaging System (Alpha Innotech). DNA nicking was determined by the conversion of supercoiled plasmid into the linear form.

To test for UvrABC activity toward N^2-4-HOPh-dG, both adduct-containing and unmodified 40-mers were 5'-terminally labeled with [γ-^{32}P] ATP (specific activity 6,000 Ci/mmol; 1 Ci = 37 GBq, Perkin Elmer, Boston, MA) and annealed to a complementary strand in a buffer containing 10 mM HEPES–KOH (pH 7.5) and 100 mM NaCl. The reaction mixtures contained 2 nM radiolabeled DNA substrate in a UvrABC buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 5 mM DTT, 2mM ATP, and varying amounts of UvrABC subunits as indicated in the figures. The reactions were terminated by adding an equal volume of F/E solution (90% formamide, 50 mM EDTA, and 0.2% bromophenol blue) followed by heating at 95-100 °C for 5 min. The reaction products were resolved on a 12% denaturing PAGE and the radioactivity was scanned with a Molecular Imager FX (Bio-Rad, Hercules, CA). For quantification of radioactivity of the bands, Quantity One software (version 4.0.1) was used.

The incision assay used to examine potential endonuclease activity of Exo III and Endo IV toward N^2-4-HOPh-dG was carried out as described by Hang *et al*. (Hang et al., 1998). The reactions were performed with varying amounts of enzyme at 37^{o}C in a total volume of 10 μl in 10 mM HEPES-KOH, pH 7.4, 100 mM KCl and 1 mM DTT. The reactions were terminated and electrophoresed as described above.

2.3. DNA glycosylase assays

The enzymatic assay used to test glycosylase-catalyzed excision of N^2-4-HOPh-dG from a 25-mer oligonucleotide was carried out essentially as previously described (Hang et al., 2002). Briefly, the reactions were performed with a purified glycosylase in a 10 μl volume for 1 h at 37 °C in a corresponding glycosylase buffer. For further cleavage of potential AP sites formed by the action of a glycosylase, an alkaline buffer (5 μl) containing 300 mM NaOH, 97% formamide and 0.2% bromophenol blue was added to the reactions, which were then heated at 95-100°C for 5 min and resolved by 12% denaturing PAGE.

2.4. Molecular dynamics (MD) simulations

MD simulations were performed using the AMBER 9.0 modeling package with Cornell *et al*. force field and the parm99.dat parameter set. A model of unmodified 25-mer was built in a standard B-DNA conformation. The N^2-4-HOPh-G adduct was placed opposite C in the 7th position of the 25-mer duplex (see Fig. 5 for sequence). The force field parameters for this adduct were developed consistent with the AMBER force field. A geometry optimized coordinates for N^2-4-HOPh-dG was obtained with *ab initio* Hartree-Fock calculations with the 6-31G* basis set using PC GAMESS 7.1 (Schmidt et al., 1993). The force constants for bonds and angles were assigned by analogy with chemically similar atom types in the AMBER force field. The partial charges for the modified nucleotide were fit with the RESP module of AMBER (Bayly et al., 1993) using RED III (Pigache et al., 2004). The equilibration and production runs were performed based on a previously described procedure (Guliaev et al., 2002, 2004). All calculations used periodic boundary conditions with

explicit solvent. The constructed 25-mer duplex was solvated in a truncated octahedral box of TIP3P water molecules. The Coulombic interactions were calculated with the particle-mesh Ewald method using 1 Å charge grid spacing with B-spline interpolation and a sum tolerance of 10^{-6} Å. A cutoff of 12 Å was applied to Lennard-Jones interactions and SHAKE algorithm was used for all X-H bonds with the 2 femtosecond (fs) time step. Overall, 12.5 nanosecond (ns) MD simulations were carried for the 25-mer DNA duplex containing N^2-4-HOPh-G_7:C_{44} pair and the corresponding unmodified duplex. The stability of the MD simulation was evaluated based on rsmd values for each snapshot in the trajectory relative to its initial structure. Structures generally fluctuate stably after 1.0 ns and the last 12 ns were used for the conformational analysis.

Coordinates, velocities, and energies for each simulation were collected every 0.4 picosecond (ps). Each trajectory was analyzed and processed using PTRAJ and ANAL modules of the AMBER suite. For hydrogen bond analysis, the percent occupancy was calculated based on a heavy atom-heavy atom distance cutoff of ≤ 3.3 Å and a heavy atom-hydrogen-heavy atom angle of $\leq 130°$. The stacking interactions were estimated by computing the van der Waals interactions energies between adjacent bases, including the modified G and the opposite C. All calculations were performed on a 16 core Linux cluster.

3. Results

3.1. UvrABC incision of p-BQ-treated plasmid DNA

To determine whether *p*-BQ globally modified DNA contains lesions that are substrates for NER, plasmid pCMV was treated with *p*-BQ and then incubated with the assembled UvrABC complex. We observed that, under an excess of UvrABC, the percentage of incised plasmid (from supercoiled to open circular form) correlated with the *p*-BQ concentrations used (from 0 to 80 mg/ml) (data not shown). Based on the extent of incision and the level of background cutting, the plasmid DNA treated with 20 mg/ml of *p*-BQ was used for subsequent reactions shown in Fig. 2, in which UvrABC incised the *p*-BQ-modified plasmid DNA in a protein-dependent manner. Although the unmodified plasmid DNA exhibited low levels of non-specific cutting with increasing amounts of UvrABC, the modified plasmid DNA was efficiently incised by the nuclease, reaching almost 100% completion at the highest protein concentration used (Fig. 2, top right panel). Although the exact nature of the DNA lesion(s) incised by UvrABC is unknown in using this type of assay, these results suggest the involvement of NER in the repair of *p*-BQ-derived DNA damage, primary or secondary.

3.2. UvrABC incision of the oligonucleotide containing N^2-4-HOPh-dG

The UvrABC complex was further tested for its activity toward a single adduct N^2-4-HOPh-dG in DNA using an incision assay. As shown in Fig. 3 top, the 40-mer duplex substrate contained the adduct in the middle of the top strand (20th position) which was 5' end-labeled with ^{32}P. The representative gel image in Fig. 3 demonstrates that UvrABC was capable of incising this adduct-containing substrate, with the major incision made at the 8th phosphodiester bond 5' to the adduct, as

indicated by the 12-mer marker. Such an incision pattern (site) is in agreement with those reported earlier for other DNA lesions (Reardon and Sancar, 2005). In lane 4 of Fig. 3, several minor incisions were also observed on the 5' side of the major incision, which are further away from the adduct. Similar 5' extra cuts by UvrABC have also been described previously for other DNA adducts (Moolenaar et al., 1998; Zou et al., 2003). No specific incision(s) was detected for the unmodified 40-mer under the same UvrABC concentration used (Fig. 3, lane 2). Further characterization of kinetics of UvrABC incision against N^2-4-HOPh-dG is shown in Fig. 4. An initial rate of UvrABC incision can be demonstrated by a near linear increase in the total amount of both the major and extra incision products during the 30 min reaction time. These results clearly indicate that N^2-4-HOPh-dG is specifically recognized and repaired by the UvrABC-mediated NER pathway.

We previously reported that certain AP endonucleases, including *E. coli* Exo III and Endo IV and human APE1, are able to incise *p*-BQ-derived benzetheno adducts at the 5' phosphodiester bond adjacent to the adduct (Hang et al., 1996; Hang et al., 1998). Using the same *in vitro* assay, we tested these enzymes for their potential activity for N^2-4-HOPh-dG. There was no specific incision of the adduct-containing 40-mer observed when Exo III and Endo IV were tested in this study (data not shown).

We also examined whether BER might be involved in the removal of this adduct. A 25-mer oligomer substrate containing N^2-4-HOPh-dG at 7th position (Fig. 5 top) was used to react with a battery of available DNA glycosylases from *E. coli*. The reasons for using a 25-mer substrate were that, unlike the 40-mer substrate described above, the 25-mer was not incised by UvrABC due to its short size; the same 25-mer sequence but

with different adducts located in it has been used successfully for studying BER in our past research. It has been shown that alkylated bases as well as certain 5- and 6-membered exocyclic adducts are excised by specific DNA glycosylases. In *E. coli*, these enzymes are primarily the AlkA and Mug proteins (Hang, 2004; Singer and Hang, 1997). Here we did not detect any significant excision of N^2-4-HOPh-G by the glycosylases tested in Fig. 5, under the assay conditions utilized previously for their primary known substrates.

3.3. Conformation of DNA containing a N^2-4-HOPh-dG adduct

To analyze the impact of N^2-4-HOPh-dG on local DNA conformation, MD simulations using the AMBER 9.0 modeling package with the Cornell et al. force field were performed. The simulation stability for the N^2-4-HOPh-G_7:C_{44} pair-containing DNA duplex was evaluated by calculating rmsd values for each 0.4 ps relative to the starting structure. The system reached conformational equilibrium after 800 ps and the representative conformation was calculated by averaging the last 11.5 ns of the 12.5 ns MD simulation. N^2-4-HOPh-G_7 and C_{44} formed a stable base pair with the hydroxyphenyl (HOPh) substituent being located in the minor groove of the DNA. The overall conformation of the adduct-containing base pair was similar to that of the unmodified G:C pair and was stabilized by the three Watson-Crick hydrogen bonds N^2-4-HOPh-G_7N2---C_{44}O2, N^2-4-HOPh-G_7N1---C_{44}N3 and N^2-4-HOPh-G_7O6---C_{44}N4, which were occupied 95.3, 95.5 and 94.3 %, respectively, during the entire course of simulation (Table 1). The hydrogen bonding criteria were 3.3Å distance between heavy atoms with the hydrogen bonding angle for donor-hydrogen-acceptor of 130º.

The top and side views of the three base pair motifs, A_6-N^2-4-HOPh-G_7-C_8/T_{45}-C_{44}-G_{43}, and the corresponding control, are shown in Fig. 6. The dynamic analysis of the N^2-4-HOPh-dG torsion angles between the HOPh substituent and the guanine base, *i.e.*, α N1-C2-N^2-C6(HOPh) and β C2-N^2-C6(HOPh)-C5(HOPh), showed certain conformational flexibility with the overall distribution between 50° and -50°. The mean values and standard deviations (in parenthesis) are -4.6º (11°) and -4.6º (14°) for α and β, respectively. Thus, the average structure obtained from the MD simulation showed an almost planar position of the HOPh ring (bottom part of Fig. 6). No hydrogen bonding interactions were observed between the hydroxyl group of HOPh and the neighboring bases.

The van der Waals interaction energies computed over MD trajectory showed that the stacking interactions at the lesion site are approximately 3 kcal/mol lower than those at the corresponding site in the unmodified duplex (Fig. 7). The ability to generate more stable stacking interactions between the adducted G and its adjacent bases can be attributed to additional stacking contributions from the HOPh ring.

4. Discussion

One critical mechanism in prevention of mutagenic carcinogen-induced genotoxicity is to remove modified bases from the genomic DNA. Benzene and its major metabolites, such as *p*-BQ and HQ, have been shown to have the ability to modify DNA bases both chemically and in animal experiments. Our earlier work on *p*-BQ-induced benzetheno adducts has yielded valuable information as to their repair (Guliaev et al., 2004; Hang et al., 1996; Hang et al., 1997; Rothwell et al., 2000),

mutagenic potential (Xie et al., 2005), and structure-function relationships involved in these processes. However, for the *in vivo* adduct formed by *p*-BQ or HQ, N^2-4-HOPh-dG, neither its role in mutagenesis nor its repair has been revealed thus far. Given that guanine mutations are most commonly found in benzene-induced mutagenesis and that N^2-4-HOPh-dG is the major adduct formed *in vivo* by *p*-BQ/HQ, it is of importance to understand how such an adduct is dealt with by cellular processes and whether any repair mechanism is important for benzene-induced mutagenesis. In recent years, other mechanisms underlying benzene carcinogenicity have also been proposed, particularly in two areas (McDonald et al., 2001; Whysner et al., 2004). First, benzene-induced ROS formation gives rise to oxidative DNA damage and altered signaling pathways. Second, inhibition of topoisomerase II by benzene and its metabolites leads to chromosomal alterations that may play a role in the development of leukemia. However, the repair studies reported here continue to represent an important area of interest even though the exact biological role of these benzene-DNA adducts is not yet understood. Further studies will be needed, especially in mammalian systems, to determine whether multiple mechanisms are to contribute to benzene-induced carcinogenesis, or which mechanism plays the more significant role.

In the present study, we have studied and compared the repair specificity toward N^2-4-HOPh-dG of three *E. coli* repair mechanisms. By detecting the 5' incision, we revealed that this adduct is a novel substrate for *E. coli* UvrABC nuclease. Although NER is known to be the major mechanism for repair of a wide spectrum of genotoxic DNA adducts, especially bulky ones such as PAH- and enal-induced DNA adducts, there has been no direct evidence on its involvement in processing of specific

benzene-derived covalent adducts. Using the supF forward mutation assay, Gaskell et al (Gaskell et al., 2005) demonstrated that when *p*-BQ-treated plasmids were transfected into human NER-deficient XPA cells, an overall higher mutation frequency at guanine residues occurred compared with the NER-proficient cells, suggesting repair of *p*-BQ-induced dG adducts by NER. Using UvrABC as a model system, our data on UvrABC-mediated incision of *p*-BQ-modified plasmid DNA further support a role of NER in protection against benzene-induced DNA damage. Moreover, using defined oligonucleotide substrate, this work provides, for the first time, distinctive biochemical evidence on NER of a specific benzene-DNA adduct, *i.e.*, N^2-4-HOPh-dG. Further investigation will be undertaken to examine the excision repair of this adduct in mammalian cells. It should also be pointed out that it is still not known whether the exocyclic benzetheno adducts, which are larger in size than N^2-4-HOPh-dG, are repaired by the NER pathway, in addition to their AP endonuclease-mediated repair. In Fig. 2, the *p*-BQ-induced lesions in plasmid DNA that were incised by UvrABC may likely include those other than N^2-4-HOPh-dG, for instance, the benzetheno bases.

The UvrABC activity found for N^2-4-HOPh-dG has another important implication, that is, enabling one to use the UvrABC/ligation-mediated PCR (UvrABC/LMPCR) approach (Denissenko et al., 1996; Feng et al., 2006) to detect and quantify the formation of this adduct in critical cellular genes at nucleotide resolution.

In our data, the lack of repair of N^2-4-HOPh-dG by damage-specific AP endonucleases and DNA glycosylases suggests that the structural features of this

adduct and/or local DNA conformation do not support recognition by these enzymes. We previously discovered that *E. coli* Exo III and Endo IV and human APE1 are able to recognize and incise oligonucleotides containing a benzetheno adduct of dA, dC or dG (Chenna et al., 1995; Hang et al., 1996; Hang et al., 1998). To understand the structural basis for such specificity, we also studied the conformation of adducted DNA duplexes using MD simulations (Guliaev et al., 2004; Xie et al., 2005) which showed that the benzetheno adducts do not pair with the opposite base and cannot be easily accommodated into DNA duplexes due to their unusually large van der Waals surface. There is either a significant displacement of the adduct toward the major groove (for both A and C adducts), or the opposite base has to rotate out of the duplex (for G adduct), leading to an extrahelical conformation. Many previous structural studies suggest that DNA glycosylases and AP endonucleases, including Exo III and Endo IV, use a flip-out mechanism for base excision or nucleotide incision that flip the modified base out of the DNA groove (Friedberg et al., 2005). It has been shown that disruption of or compromised base pairing at a damage site could aid in flipping out the damaged base by human alkyladenine-DNA glycosylase (AAG), thus affecting specificity and enzymatic efficiency (O'Brien and Ellenberger, 2004). Therefore, based on our biochemical and MD simulation results, it is reasonable to suggest that N^2-4-HOPh-G, which forms both stable base pairing with opposite C through three hydrogen bonds (Fig. 6) and more favorable stacking interactions with its neighboring bases than an unmodified G ((~3 kcal mol^{-1} lower, see Fig. 7), is less susceptible to the flip-out mechanisms employed by the AP endonucleases or glycosylases tested, which may explain in part the inability of these enzymes to

remove this adduct. In the case of UvrABC activity for N^2-4-HOPh-G, a mechanism for its specificity could be hypothesized based on the recently proposed functions of base flipping in UvrB/UvrC interactions with damaged DNA (Malta et al., 2006; Malta et al., 2008). In the latter studies Malta *et al.* showed that base flipping takes place upon UvrB binding to damaged DNA, which, however, appears not to involve the damaged base itself, but the neighboring/opposite bases, such as the base 3' adjacent to a damaged base. The conformational bases for this putative mechanism to occur in UvrABC recognition and processing of N^2-4-HOPh-G are (1) the strong hydrogen bonding and stacking interactions observed for the adducted base; and (2) the lower stacking interactions for the neighboring bases of the adduct as compared to those of the adduct itself. Both could then facilitate base flipping that is thought to be important for UvrABC-mediated damage recognition and incision.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Tables

Table 1. Hydrogen bonding values for N^2-4-HOPh-G_7:C_{44} pair calculated based on a 10 MD trajectory.

Figure legends

Figure 1. Chemical structure of N^2-(4-hydroxyphenyl)-2'-deoxyguanosine (N^2-4-HOPh-dG).

Figure 2. UvrABC incision of the plasmid DNA treated with 20 mM *p*-BQ. Both the unmodified and modified pCMV plasmid (0.75 μg per sample) were incubated with increasing amounts of UvrABC (1x: 1.4 nM UvrA, 1.4 nM UvrB, and 1 nM UvrC) at 30^{o}C for 1 hr. Lanes 1 and 5 contained buffer only. The reactions were electrophoresed on 1% agarose gel with ethidium bromide.

Figure 3. UvrABC incision of the 40-mer substrate containing N^2-4-HOPh-dG. Oligonucleotide sequences used in this study are shown on the top, including both the 40-mer containing the adduct (X) at position 20th (from the 5'-end) and three oligonucleotide markers with different sizes. The gel image shows the detection of UvrABC incision of the oligonucleotide on the 5' side of the adduct. UvrABC (140 nM UvrA, 140 nM UvrB and 100 nM UvrC) was incubated with 20 fmol of 5' end-labeled unmodified (lane 2) and adduct-containing 40-mer (lane 4) in an UvrABC buffer (see *2.2. Incision assays*) at 37^{o}C for 1hr. The 5' incision sites are indicated by the mobility of the co-running size markers.

Figure 4. Kinetic studies of UvrABC incision of the 40-mer containing N^2-4-HOPh-dG. The right graph shows the rate of UvrABC incision as a function of reaction time.

UvrABC (140 nM UvrA, 140 nM UvrB and 100 nM UvrC) was incubated with 20 fmol of 5' end-labeled adduct-containing 40-mer (all lanes) for the time points indicated. All of the four 5' incision bands detected in the left gel image were scanned and calculated as incision products. Lane 7 contained the 12-mer marker which indicates the major and first 5' incision band.

Figure 5. Determination of potential DNA glycosylase activity toward N^2-4-HOPh-G. The 5'-end labeled 25-mer oligonucleotide containing the adduct at 7th position was used as a substrate. The incubation time was 1 hr at 37^{o}C for all the *E. coli* glycosylases tested. There was no detectable product(s) observed at the expected cleavage site (arrow) by these enzymes under the reaction conditions used.

Figure 6. Top (A) and side (looking at the minor groove) (B) views of the 3 base pair motifs containing G:C and N^2-4-HOPh-G:C pairs (A_6-G_7-C_8/T_{45}-C_{44}-G_{43} and A_6-N^2-4-HOPh-G_7-C_8/T_{45}-C_{44}-G_{43}). The structures shown are the trajectory-averaged structures for the last 9 ns of the MD simulations. Hydrogen bonds are shown in yellow and G:C and N^2-4-HOPh-G:C pairs are colored by atoms.

Figure 7. Population distribution of the van der Waals interaction energies between adjacent bases at the adduct site A_6-N^2-4-HOPh-G_7-C_8 and corresponding bases A_6-G_7-C_8 in unmodified oligonucleotide. The bases are labeled mod and unmod, respectively. The lower van der Waals interactions indicate lower perturbation in the stacking interactions. The N^2-4-HOPh-G_7 (blue) has a tendency to generate more stable stacking in B-DNA than a normal guanine G_7 in unmodified duplex (red). The analysis includes 4800 structures from a 12.5 ns trajectory.

Table 1. Hydrogen bonding values for N^2-4-HOPh-G_7:C_{44} pair calculated based on a 10 ns MD trajectory.

H-bond Donor	**H-bond Acceptor**	**% Occupied**	**Average Distance (Å)**	**Average Angle (^{o})**
4-HOPh-G_7N2	C_{44}O2	95.30	2.915 ± 0.14	16.62 ± 9.54
4-HOPhG_7N1	C_{44}N3	95.50	2.987 ± 0.11	17.46 ± 8.70
C_{44}N4	4-HOPh-G_7O6	94.30	2.898 ± 0.14	16.41 ± 9.29

The percent occupancy was calculated based on a heavy atom–heavy atom distance cutoff of ≤ 3.3 Å and a heavy atom-hydrogen-heavy atom angle of ≤ 130°.

Figure 1

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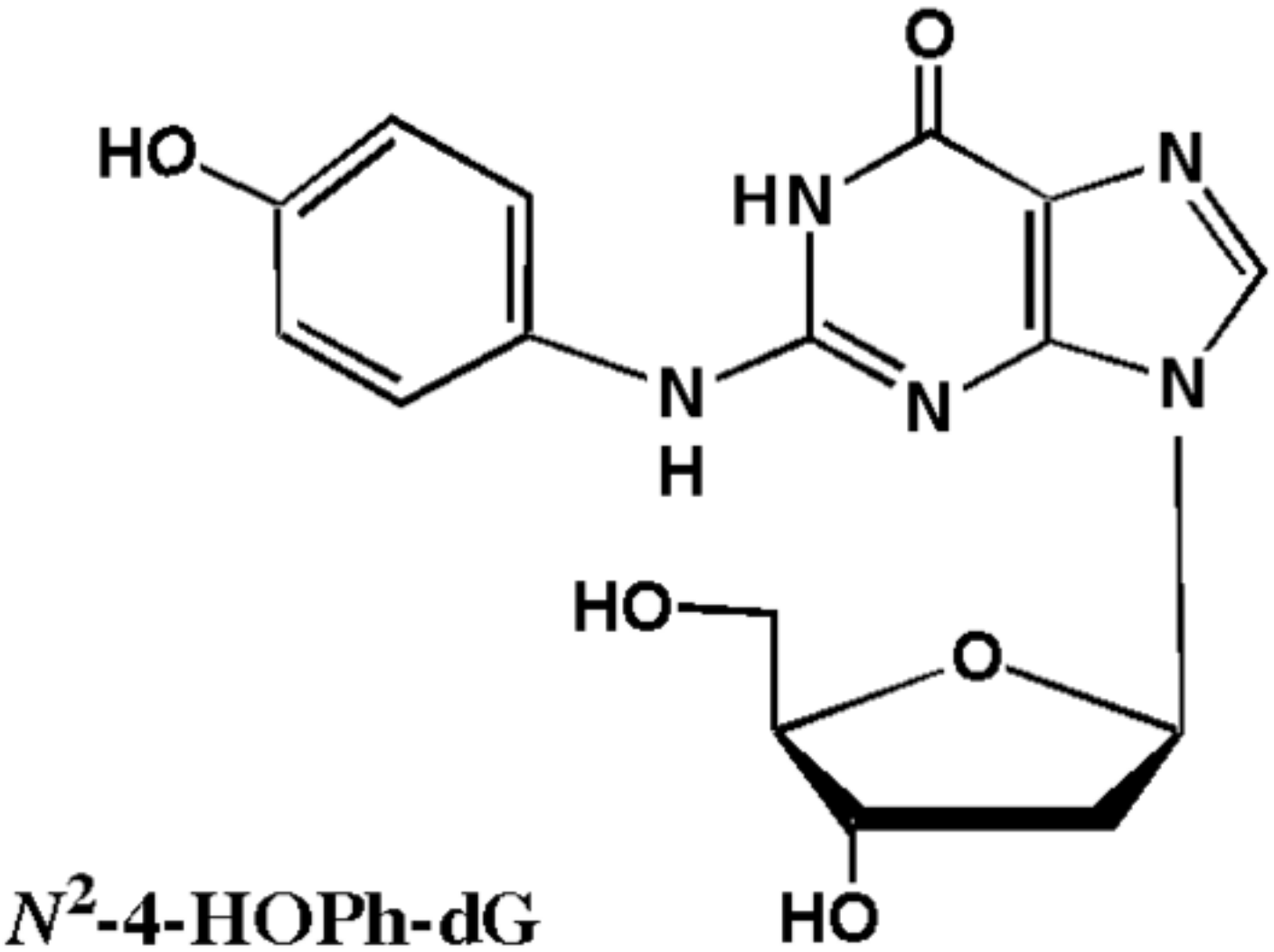

N^2-4-HOPh-dG

Figure 2

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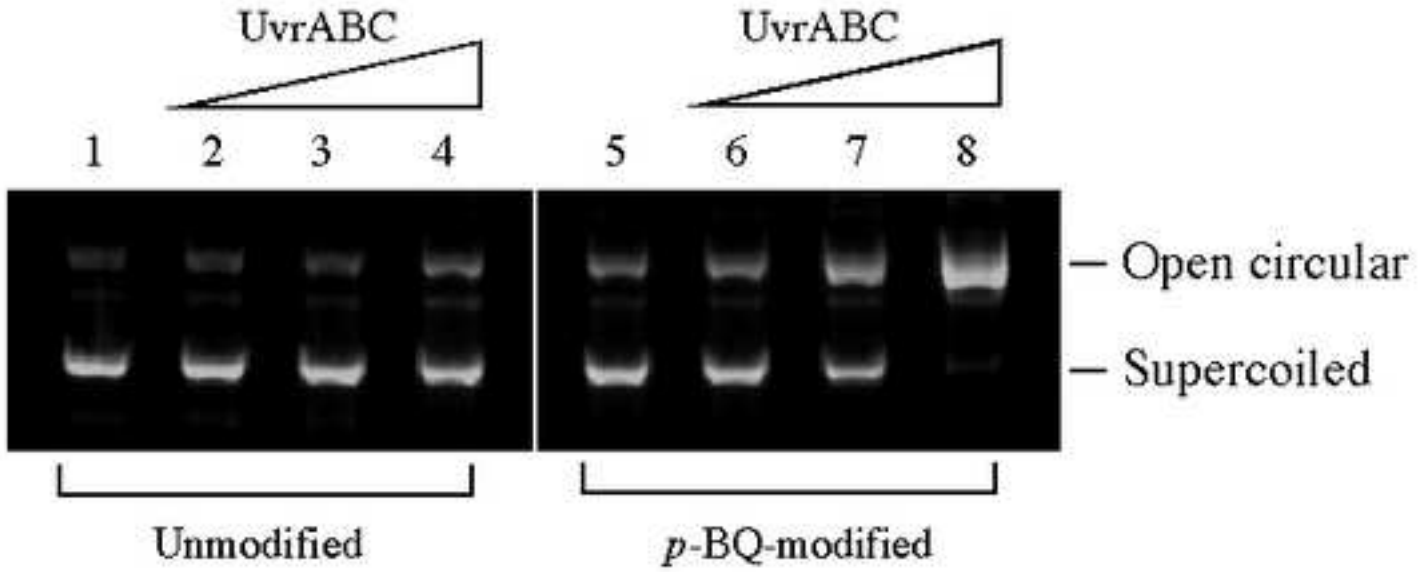

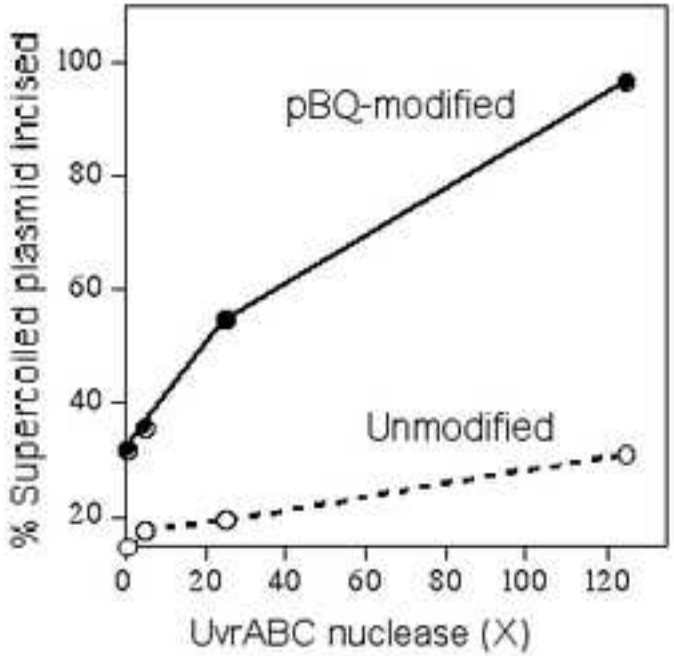

Figure 3

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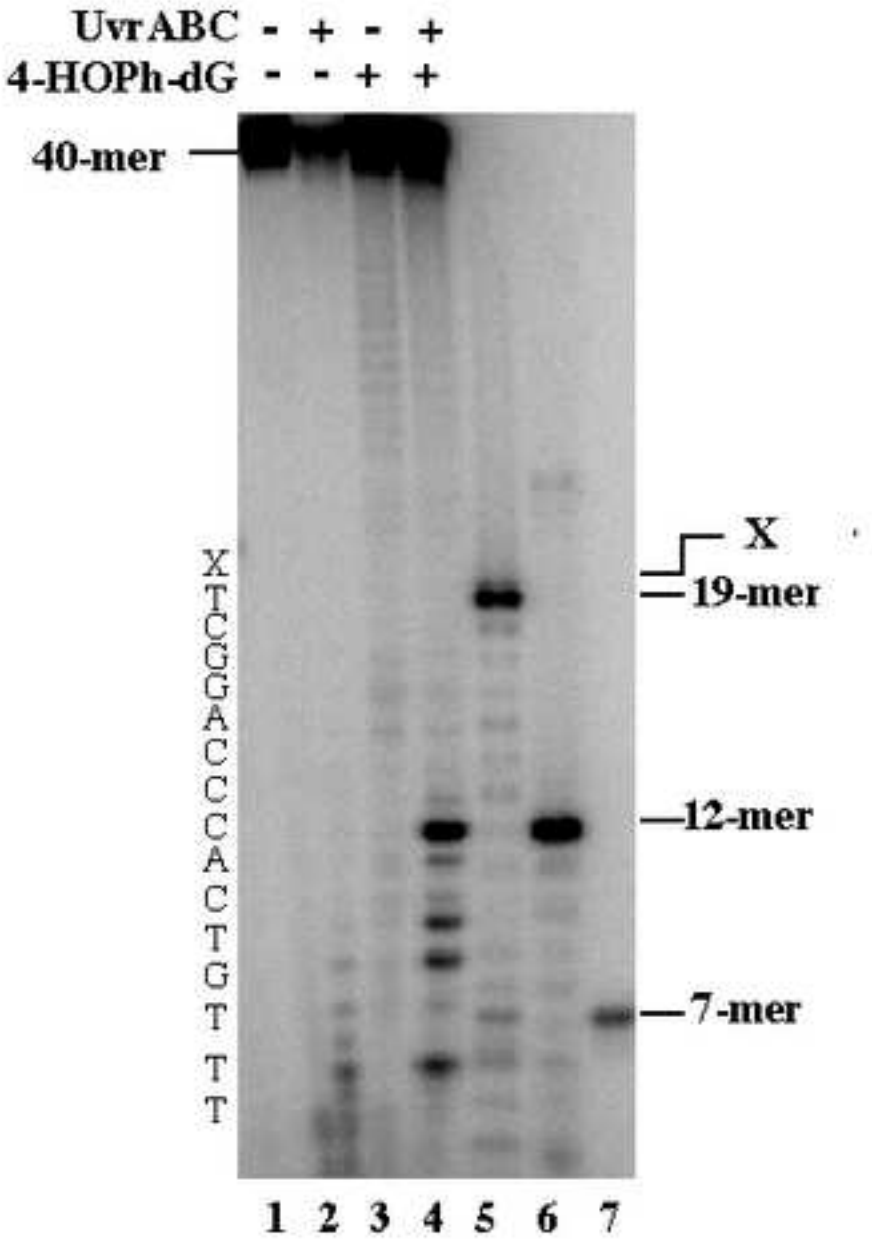
5'-TTGCTTTGTCACCCAGGCTXGACTGCAGTGGTACAATCAT (40-mer)
5'-TTGCTTTGTCACCCAGGCT (19-mer)
5'-TTGCTTTGTCAC (12-mer)
5'-TTGCTTT (7-mer)

X= N^2-4-HOPh-dG



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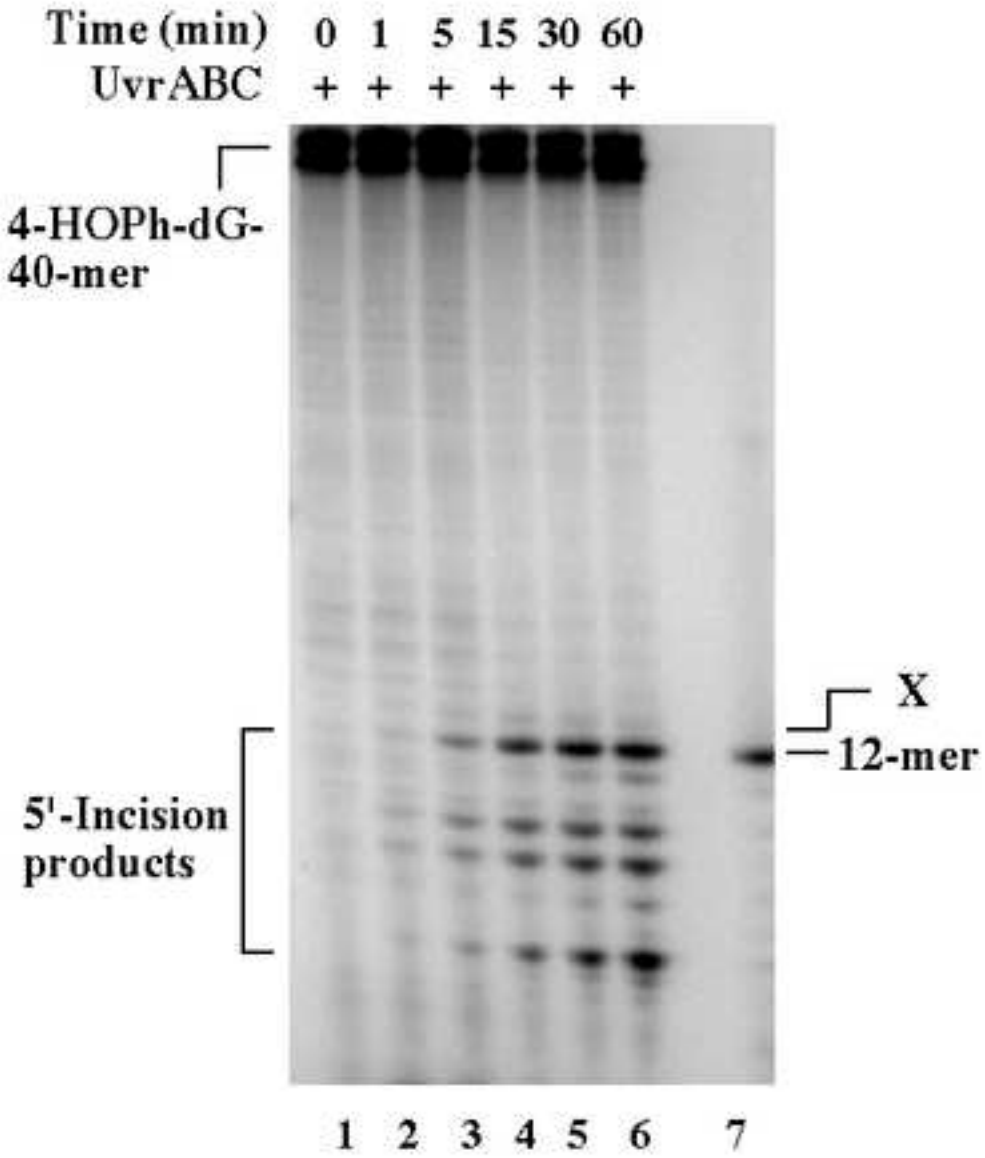

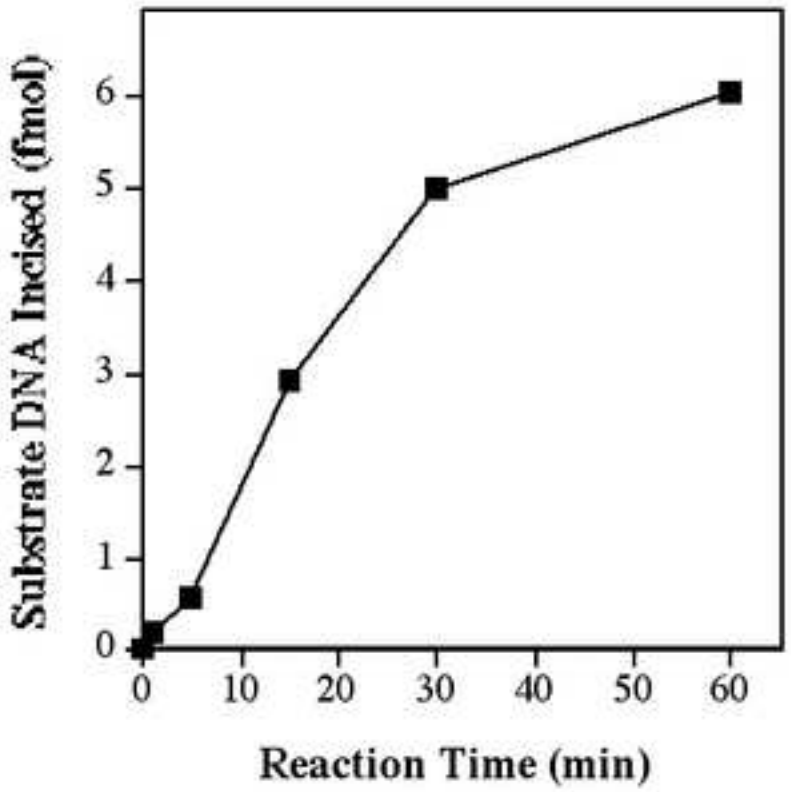

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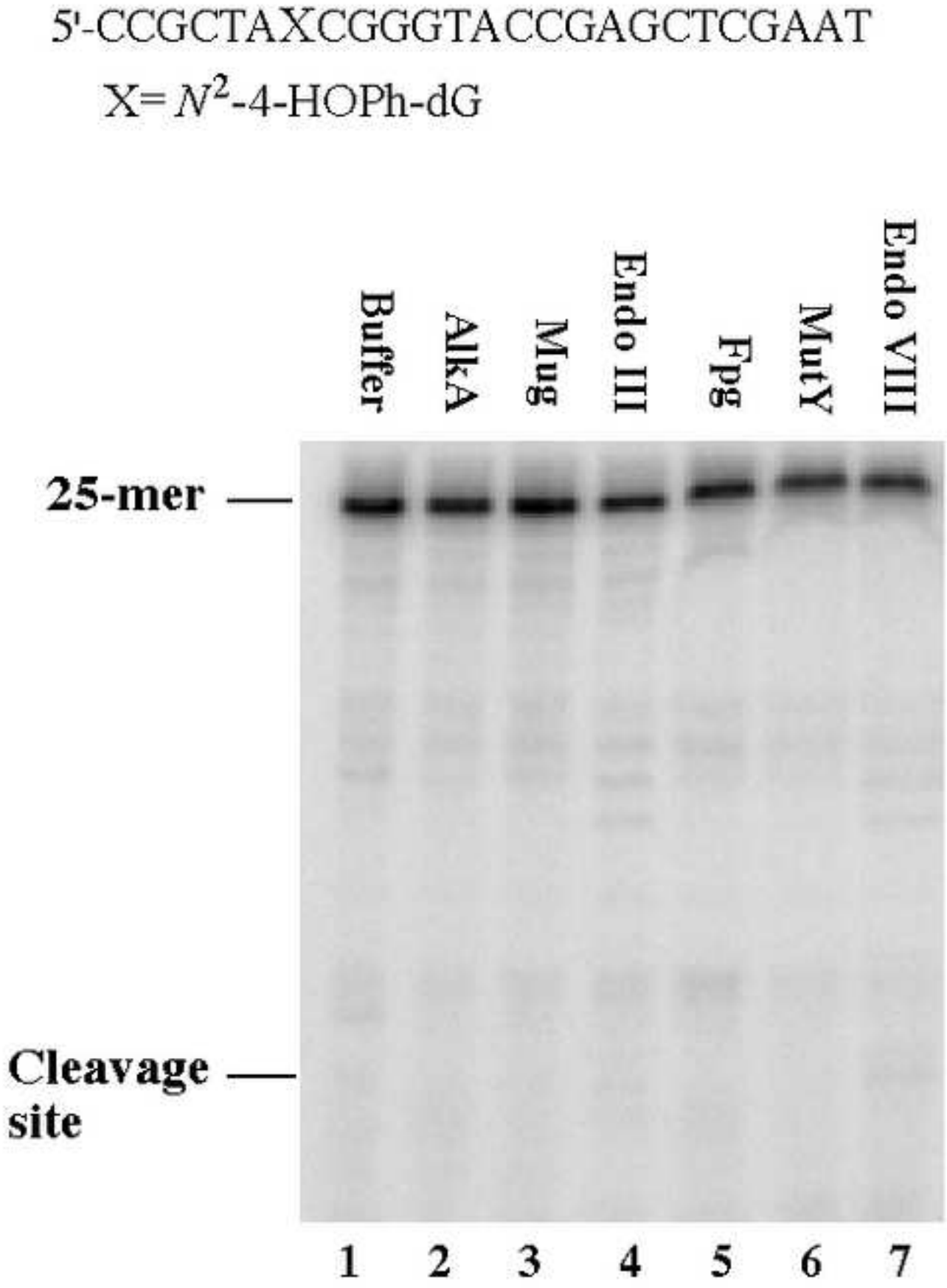

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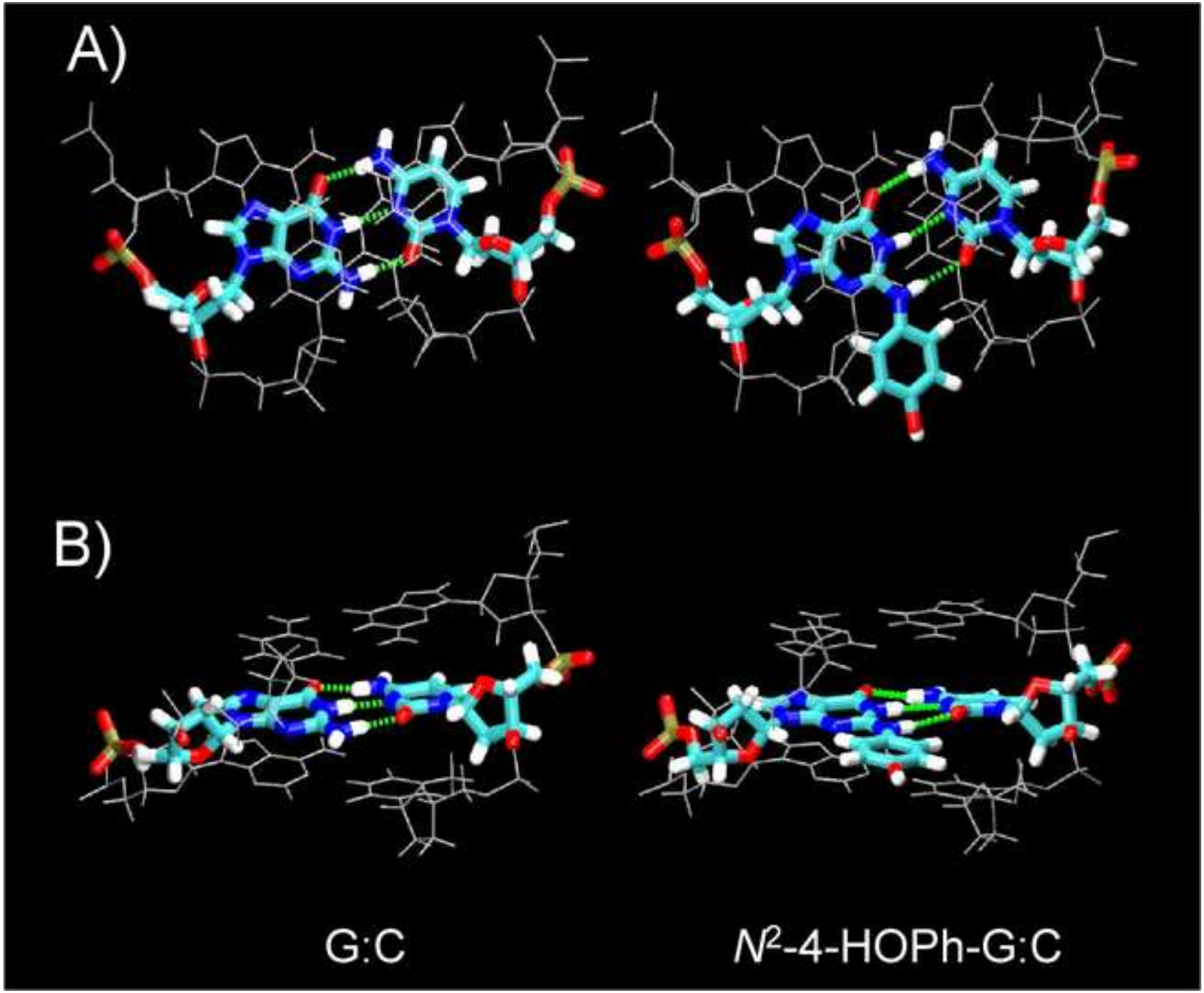

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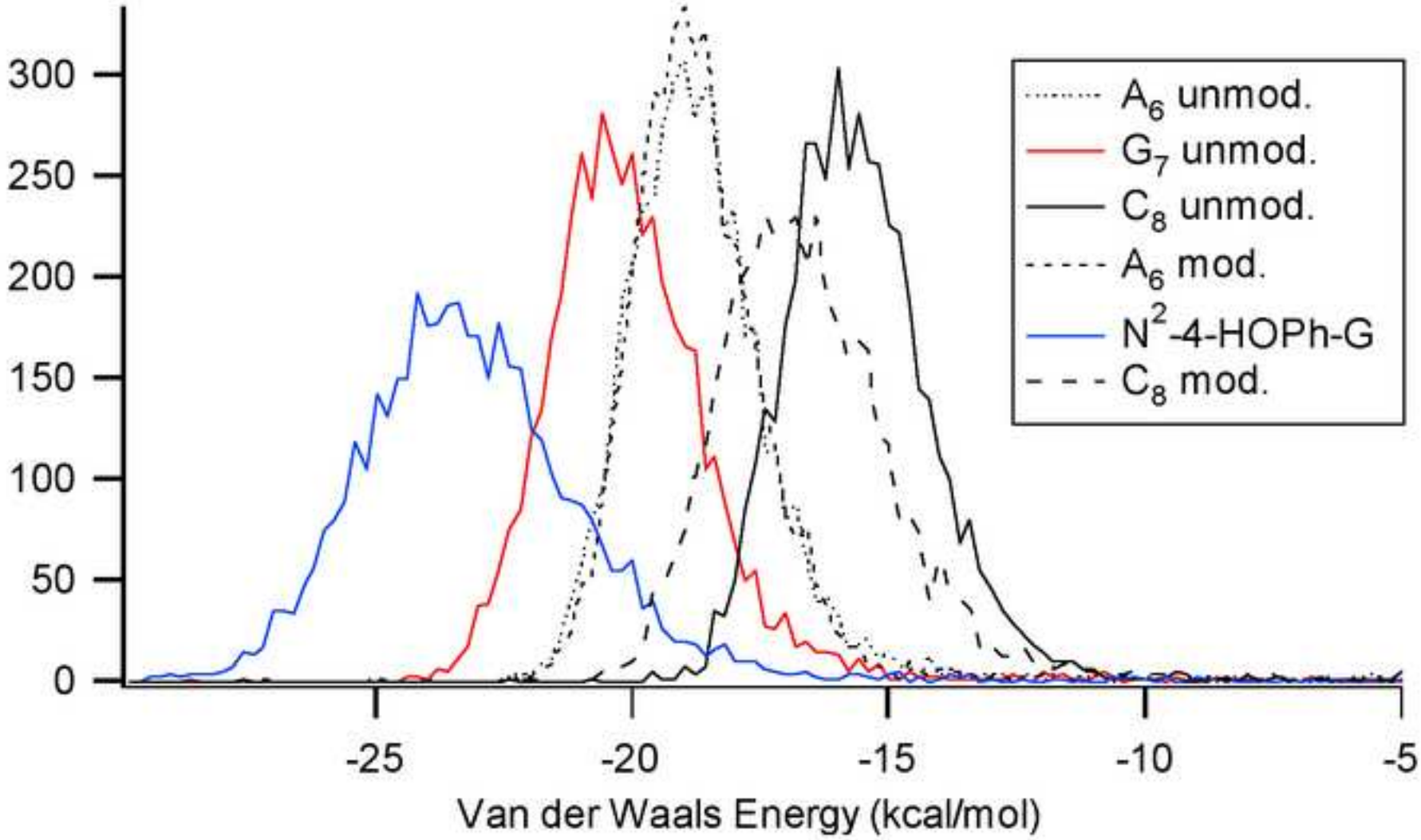

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