J. Exp. Biomed. Sci. 11 (2005) 103-108

Cellular DNA Repair of Oxidative Deoxyribose Damage by Mammalian Long-Patch Base Excision Repair

Jung-Suk Sung† and Mi-Young Son

Department of Biology, Dongguk University, Seoul 100-715, Korea

2-Deoxyribonolactone (dL) arises as a major DNA damage induced by a variety of agents, involving free radical attack and oxidation of C1'-deoxyribose in DNA. We investigated whether dL lesions can be repaired in mammalian cells and the mechanisms underlying the role of DNA polymerase β in processing of dL lesions. Pol β appeared to be trapped by dL residues, resulting in stable DNA-protein cross-links. However, repair DNA synthesis at site-specific dL sites occurred effectively in cell-free extracts, but predominantly accompanied by long-patch base excision repair (BER) pathway. Reconstitution of long-patch BER demonstrated that FEN1 was capable of removing the displaced flap DNA containing a 5'-dL residue. Cellular repair of dL lesions was largely dependent on the DNA polymerase activity of Pol β. Our observations reveal repair mechanisms of dL and define how mammalian cells prevent cytotoxic effects of oxidative DNA lesions that may threaten the genetic integrity of DNA.

Key Words: Oxidative DNA damage, DNA repair, DNA polymerase β, DNA-protein cross-link

INTRODUCTION

Cellular DNA is constantly damaged by endogenous and exogenous reactive species. The outcome of DNA damages is generally adverse, contributing to degenerative processes such as aging and cancer (Friedberg, 2003; Hoeijmakers, 2001). Abasic (AP) sites are expected to be the most frequent lesion in DNA, which can be formed by spontaneous hydrolysis of *N*-glycosylic bond or as a consequence of the removal of inappropriate bases by DNA glycosylases (Atamna et al., 2000; Krokan et al., 1997). In either case, the resulting AP sites are repaired by base excision repair (BER) pathway. Consistent with its importance in maintaining genomic stability and cell viability, BER pathway is highly conserved, and most of the central BER enzymes are essential at the whole animal and cell level (Wilson and Thompson, 1997).

Oxidative damage to DNA, mediated by free-radicals and reactive oxygen species, produces structurally distinct AP sites that modulate biochemical reactivity of the lesion. These lesions include C1'-oxidized AP site, 2-deoxyribonolactone (dL), which is the earliest-identified X-ray damage in DNA (von Sonntag, 1991). The dL damage is also known to arise in DNA by numerous genotoxic agents including UV and γ-irradiation, chromium (V) carcinogens, and the anticancer antibiotic neocarzinostatin that belongs to enediyne family (Hashimoto et al., 2001; Pratviel et al., 1991; Sigman et al., 1993). Under simulated physiological condition, the half-life of DNA cleavage at dL sites in duplex DNA was measured as ~50 h (Zheng and Sheppard, 2004) implying that the lesion is rather stable and may encounter with the cellular repair machinery that normally operate to repair regular AP sites. However, very little is known about the fate of dL in the cells due to the lack of the method to study dL lesion specifically. In the present study, we have employed DNA substrates containing defined site-specific dL residues to monitor which cellular repair pathway involves in the repair of dL lesions. The results show here that the repair of dL occurs via long-patch BER pathway, and its preventive effects on the formation of deleterious DNA-protein cross-links are discussed.

*Received: May 9, 2005
Accepted after revision: May 28, 2005
†Corresponding author: Jung-Suk Sung, Department of Biology, Dongguk University, Seoul 100-715, Korea.
Tel: 02-2260-3322, Fax: 02-2275-8294
e-mail: sungjs@dongguk.edu

MATERIALS AND METHODS

1. Materials

All reagents were from Sigma/Aldrich (St Louis, USA). Radionuclides were obtained from PerkinElmer Life Science (Wellesley, USA). Neocarzinostatin was gift from Dr. Peter Dedon of Massachusetts Institute of Technology, USA. DNA oligonucleotides containing dL precursor residues were provided by Dr. Bruce Demple of Harvard University, USA. Recombinant Pol β, FEN1 nuclease, and Ape1 were purified as described previously (Masuda et al., 1998; Prasad et al., 2000). All other enzymes were obtained from New England BioLabs (Beverly, USA). HeLa and DNA Pol β proficient or deficient SV-40 immortalized mouse embryonic fibroblast (MEF) cell lines were obtained from American Type Culture Collection (Manassas, USA).

2. Preparation of DNA repair substrates

Oligonucleotide 30-mer (GTCACGTGCTGCAXACG-ACGTGCTGAGCCT) containing a site-specific dL precursor residue (X; 1'-*t*-butylcarbonyl-uridylate) was hybridized to the 31-mer complementary strand and labeled at the 3'-end by incorporation of [α-^{32}P] dCTP using exonuclease-free Klenow fragment of DNA polymerase I. ^{32}P-labeled plasmid pGEM DNA substrate were constructed as described previously (Sung and Mosbaugh, 2003), except that the oligonucleotide primer containing a site specific dL precursor residue was ^{32}P-labeled at the 3'-end and annealed to single-stranded pGEM DNA. To generate a site-specific dL lesion in DNA substrates, 10 pmol of duplex DNA containing a dL precursor residue was subjected to photolysis in a Photochemical Reactor (Rayonet Corp., Branford, USA) at UV 350 nm for 90 min as previously described (DeMott et al., 2002).

3. Preparation of cell-free extracts

HeLa and MEF cell-free extracts were prepared from confluent cells as previously described (Bennett et al., 2001), and dialyzed extensively against 20 mM Hepes-KOH (pH 7.6), 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM PMSF, 10% (v/v) glycerol, and 1x protease inhibitor cocktail. The protein concentration of cell-free extracts was determined using Bio-Rad Protein Assay reagent (Hercules, USA).

4. DNA repair assay

Standard DNA repair reaction mixtures (100 μl) contained 100 mM Hepes-KOH (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 2 mM ATP, 0.5 mM β-NAD, 20 μM each of dNTP, 5 mM phosphocreatine, 200 units/ml phosphocreatine kinase, 10 nM of DNA substrate, and the indicated amounts of cell-free extracts or purified enzymes. Following incubation at 30℃ for the specified times, the reaction products were isolated as previously described (Sung et al., 2001), and digested with *Bam*HI and *Hind*III where indicated in the figure legends. To examine the formation of DPC, the DNA products were resolved by 1% agarose gel electrophoresis or by 8% SDS-PAGE. Repair processing of DNA and its associated repair patches were determined by the analysis of reaction products by 15% denaturing (8 M urea) polyacrylamide gel electrophoresis.

5. Cell viability assay

Cell viability was determined by MTT assay (Mosmann, 1983). After treatment of indicated amounts of neocarzinostatin for 2 h, the cells were cultured for 24 h and 100 ml of the MTT (5 mg/ml) was added to each well of 24- well plates. Supernatants were then removed and the formazan crystals were solubilized in 1 ml of dimethylsulfoxide. Optical density was determined at 540 nm using an ELISA reader.

RESULTS

1. Formation of DNA-protein cross-links mediated by dL lesions

The major oxidized form of AP site, a dL lesion, has been recently implicated in the formation of DNA-protein cross-link (DPC) by various bacterial repair enzymes (Greenberg et al., 2004). Utilizing the plasmid DNA substrate containing a site-specific dL residue, we examined whether the key mammalian DNA repair polymerase, Pol β, involves the repair of dL residue (Fig. 1). Incubation of dL-containing DNA with purified Pol β, and following electrophoretic analysis on an agarose gel revealed the formation of new DNA species that appeared to be distinct from free forms of closed (form I) and nicked (form II) plasmid DNA molecules (Fig. 1B). The covalent nature of the linkage

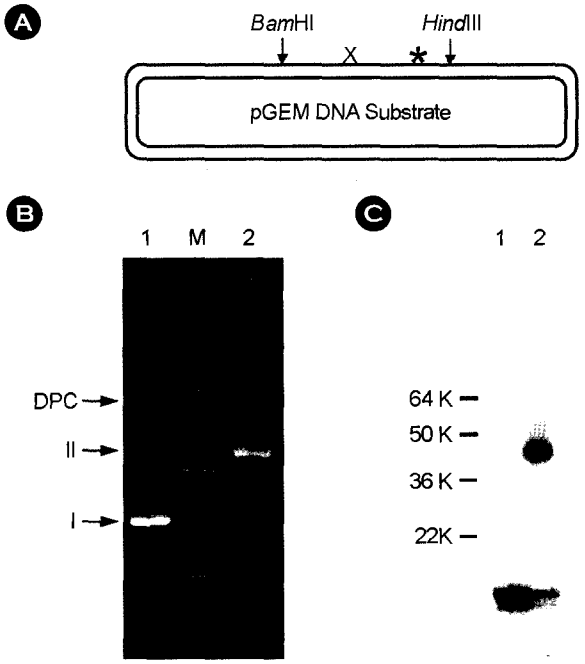

Fig. 1. Detection of the dL-specific DNA-protein cross-link with Pol β. (**A**) A diagram of the plasmid pGEM DNA substrate, which contained the target dL lesion bracketed by *Bam*HI and *Hind*III restriction sites. The location of the 32P-labeled nucleoside is indicated by an asterisk. (**B**) Standard DNA repair reaction was carried out by incubation of ^{32}P-labeled pGEM DNA substrate (10 nM) containing a photochemically generated dL site without (lane 1) or with 20 nM Pol β (lane 2). The resulting DNA products were analyzed by agarose gel electrophoresis along with 1 kb DNA ladder (lane M). (**C**) DNA products isolated from the reaction conducted without (lane 1) or with 1 nM Ape1 and 20 nM Pol β (lane 2) were digested with *Bam*HI and *Hind*III. Samples were analyzed by SDS-PAGE and ^{32}P-labeled DNA bands were visualized by PhosphorImager. The band positions of the M_r markers are indicated in the left.

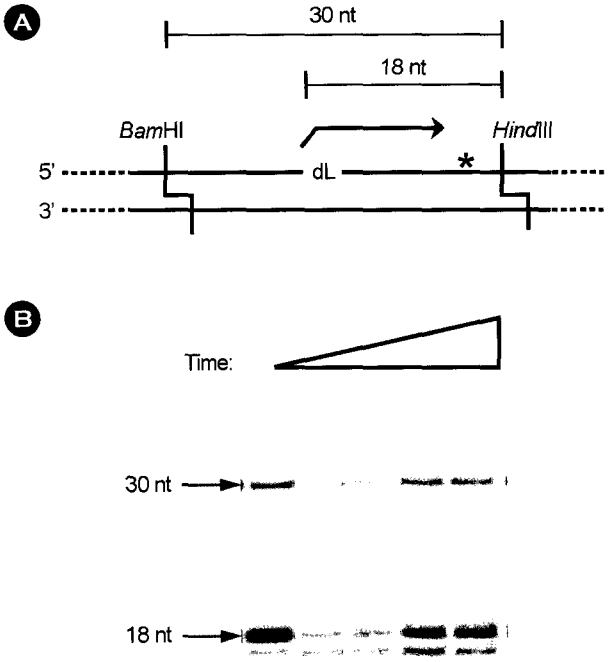

Fig. 2. DNA synthesis associated with the repair of dL lesion in HeLa cell-free extracts. (**A**) Schematic representation of the partial segment of pGEM DNA is depicted. The location of the ^{32}P-nucleotide residue is indicated by an asterisk. The repair DNA synthesis initiated at the target occurred in the direction of the arrow. (**B**) Standard repair reaction mixtures containing 10 nM of pGEM DNA substrate harboring a pre-incised dL site were incubated with 50 μg of HeLa cell-free extract for 0, 2, 5, 10, and 30 min. DNA products were isolated, treated with *Bam*HI and *Hind*III, and analyzed by DNA sequencing gel.

between Pol β and ^{32}P-labeled DNA was confirmed by SDS-PAGE analysis (Fig. 1C). The major radiolabeled species with electrophoretic mobility (Mr ~45,000) greatly shifted from the free DNA was consistent with the stable cross-linking between Pol β (Mr ~39,000) and 18-mer DNA fragment (Mr ~6,000) that was produced by the incision of dL site by Ape1. Similar result was observed previously but with the oligonucleotide DNA containing a dL lesion (DeMott et al., 2002). Therefore, these results suggest that Pol β cannot process dL lesion, but forms the DPC that is highly specific to the lesion rather than the architecture of DNA molecule.

2. Alteration of DNA repair mode due to inability of Pol β in removing dL lesion

Pol β is a key DNA polymerase that plays dual functions in BER by both removing a 5'-deoxyribophosphate (dRP) residue through its associated dRP lyase activity and initiating the repair synthesis by filling in one nucleotide gap (Matsumoto and Kim, 1995; Sobol et al., 1996). Consequently, Pol β is essential in the predominant mammalian BER pathway that results in a single-nucleotide repair patch; referred as short-patch BER. To examine whether the DPC formation of Pol β at dL site alters the repair pathway of this major oxidized AP lesion, we next determined repair patches produced during the repair of dL lesion. When dL-DNA substrates were incubated with HeLa cell-free extracts a time-dependent increase of DNA synthesis was observed from the target dL residue (Fig. 2). Interestingly, this repair

DNA synthesis at the dL lesion also produced patch sizes of 2~12 nucleotides (Fig. 2). This observation was consistent with the pattern of DNA synthesis shown in an alternative BER sub-pathway, long-patch BER, which has been previously identified according to the utilization of alternate DNA polymerase activities (Fortini et al., 1998). It has been shown that long-patch BER pathway involves the strand displacement repair synthesis of at least two or more nucleotides and the excision of 5'-dRP residue as a part of the flap oligonucleotide by FEN1 nuclease (Huggins et al., 2002). Taken together, our results indicated that the mode of conventional short-patch BER was altered to the long-patch BER due to the inability of Pol β to process dL residues.

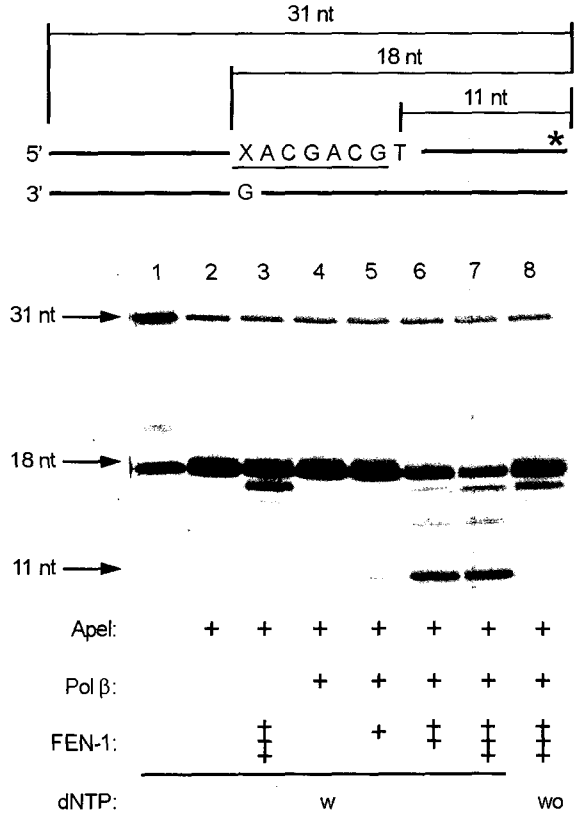

Fig. 3. Efficient processing of the flap DNA containing dL lesion by long-patch BER pathway. A duplex 3'-^{32}P-labeled 31-mer DNA substrate (10 nM) containing a site-specific dL site was incubated for 1 h at 30℃ with 1 nM Apel, 10 nM Pol β, and 1 nM FEN1, where indicated. The reactions were conducted in the absence (lanes 1~7) or the presence (lanes 8) of dNTPs, but excluding dTTP. Schematic representation of the substrate is depicted above the panel. The asterisk indicates the position of the radiolabel, X denotes a dL residue, and the underlined nucleotide sequence represents the DNA segment that is displaced by the limited DNA synthesis of 7 nucleotides. Each reaction product was analyzed by DNA sequencing gel.

3. Repair of dL lesion by an alternative long-patch BER

We next determined whether the processing of dL residue indeed occurred by long-patch BER. The profiles of DNA processing at the target dL were monitored in the reactions reconstituted with various enzymes involved in long-patch BER. The treatment of 3'-end ^{32}P-labeled DNA substrates with Apel converts majority of DNA substrates to discernable 18-mer DNA products (Fig. 3, lane 2), consistent with the incision at the 5' site of dL residues by Apel (DeMott et al., 2002; Xu et al., 2003). Additional treatments with either Pol β or FEN1 alone did not mediate further processing of DNA, except that the residual nuclease activity of FEN1 mediated nonspecific DNA degradation (Fig. 3, lanes 3 and 4). On the other hand, the reactions containing both Pol β and FEN1, in addition to Apel, produced discernable DNA products of 11 nucleotides, but only in the presence of dNTP (Fig. 3, lanes 4~7). The generation of 11-mer DNA products was indicative of the strand displacement DNA synthesis of 7 nucleotides by Pol β and the following removal of flap DNA by FEN1. This result first demonstrates the repair of dL lesions through combined actions of long-patch BER enzymes, Apel, Pol β, and FEN1, which are involved in the incision of DNA at the lesion, DNA synthesis of displacing 5'-dL lesion, and the cleavage of the resulting 5'-dL flap DNA, respectively.

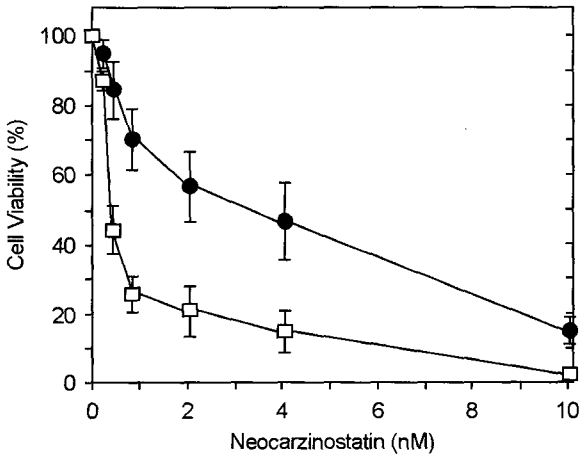

Fig. 4. Pol β plays a key role in the cellular repair of dL lesions induced by neocarzinostatin. Shown are cell viability curves after treatment with 0, 0.2, 0.4, 0.8, 2, 4, and 10 nM of neocarzinostatin of wild type MEF cells (closed circle) and Pol β knock-out MEF cells (opened square). Mean values and S.D. of three experiments are indicated.

4. Pol β plays an important role in dL repair through its DNA polymerase activity

In long-patch BER, the involvement of other DNA polymerases, such as Pol δ and Pol ε, has been suggested (Fortini et al., 1998). Pol β, however, has been also implicated in initiating DNA strand displacement of long-patch BER by incorporation of the first nucleotide (Dianov et al., 1999). Thus, we next examined whether Pol β plays such roles in dL repair by using MEF cells proficient (+/+) or deficient (−/−) of Pol β (Fig. 4). To introduce dL lesions into genomic DNA, we utilized neocarzinostatin, which is an antitumor antibiotic that exhibits cytotoxic action through DNA damage via hydrogen abstraction, mainly forming dL lesions (Urbaniak et al., 2004). After treatments with neocarzinostatin, Pol β knock-out cells exhibited significantly decreased cell survival compared to the wild-type cells (Fig. 4). This result suggests that DNA polymerase activity of Pol β is required to mediate long-patch BER and, thereby, remove dL lesions rapidly before the action of dRP lyase of Pol β to form deleterious DPCs in DNA.

DISCUSSION

In general, a long-patch BER has been considered to be a minor pathway relative to the predominant short-patch BER in the repair of various DNA damages. Until now, it is not known which factor(s) are involved in the selection of either short- or long-patch BER mode. One study suggested that the removal of 5'-dRP, which appeared to be a late-limiting step in BER, is critical to determine the mode of BER (Srivastava et al., 1998). Similarly, the dL lesion itself may be also important in the selection of BER modes, since the 5'-dL residue in DNA is refractory to the dRP lyase activity of Pol β and rather forming DPC (Fig. 1). This may induce 'switch over' the repair pathway toward the strand displacement DNA synthesis associated with long-patch BER. Overall, this notion indicates that long-patch BER may evolve in repairing DNA lesions that cannot be repaired by short-patch BER.

Pol β and FEN1 interact functionally, revealed by the stimulatory effects on each of the catalytic activity by the other enzyme (Prasad et al., 2000). Recently, it has been shown that the substrate mimicked the initial transient long-patch BER intermediate constituted a nick with 5'-tetrahydrofuran, which is resistant to Pol β excision, is poor substrate for Pol β -mediated DNA synthesis; but, DNA synthesis is strongly stimulated by FEN1, suggesting that FEN1 removes a barrier to Pol β DNA synthesis (Liu et al., 2005). Therefore, in the case of dL repair, FEN1 appears to provide at least two major contributions; (i) stimulating Pol β polymerase activity to displace the 5'-dL residue from its DNA template and (ii) removing the lesion from DNA by its efficient catalytic activity on the displaced flap DNA.

Recently, adenomatous polyposis coli (APC), the tumor suppressor protein, has been implicated in preventing Pol β-mediated strand displacement synthesis by masking the domain of Pol β which interacts with PCNA, thereby decreasing the long-patch BER, but not short-patch BER (Narayan et al., 2005). While the critical role of APC has been suggested in the susceptibility of cells to carcinogenic and chemotherapeutic agents (Narayan et al., 2005), it would be also interesting to determine whether the expression of high level APC affects the repair of dL or the formation of dL-mediated DPC. In spite of the efficient repair of dL lesion by long-patch BER, considering that the dRP lyase activity of Pol β mediates at least ~30% of AP site repair (Bennett et al., 2001), we can not rule out the possibility of cellular DPC formation by dL lesions. The potential biological and clinical significance of such DPCs remains to be explored. Furthermore, the development of the methodology to measure cellular dL lesions quantitatively would be helpful in understanding the biological significance of this oxidative lesion, and also providing useful information in developing potential chemotherapeutic agents.

Acknowledgments

We thank Professor Bruce Demple at Harvard University for his kind providence of DNA oligonucleotides. This work was supported by Dongguk University Research Fund.

REFERENCES

Atamna H, Cheung I, Ames BN. A method for detecting abasic sites in living cells: age-dependent changes in base excision repair. Proc Natl Acad Sci USA. 2000. 97: 686-691.

Bennett SE, Sung JS, Mosbaugh DW. Fidelity of uracil-initiated base excision DNA repair in DNA polymerase β-proficient and -deficient mouse embryonic fibroblast cell extracts. J Biol Chem. 2001. 276: 42588-42600.

DeMott MS, Beyret E, Wong D, Bales BC, Hwang JT, Greenberg MM, Demple B. Covalent trapping of human DNA polymerase β by the oxidative DNA lesion 2-deoxyribonolactone. J Biol Chem. 2002. 277: 7637-7640.

Dianov GL, Prasad R, Wilson SH, Bohr VA. Role of DNA polymerase β in the excision step of long patch mammalian base excision repair. J Biol Chem. 1999. 274: 13741-13743.

Fortini P, Pascucci B, Parlanti E, Sobol RW, Wilson SH, Dogliotti E. Different DNA polymerases are involved in the short- and long-patch base excision repair in mammalian cells. Biochemistry 1998. 37: 3575-3580.

Friedberg EC. DNA damage and repair. Nature 2003. 421: 436 -440.

Greenberg MM, Weledji YN, Kim J, Bales BC. Repair of oxidized abasic sites by exonuclease III, endonuclease IV, and endonuclease III. Biochemistry 2004. 43: 8178-8183.

Hashimoto M, Greenberg MM, Kow YW, Hwang JT, Cunningham RP. The 2-deoxyribonolactone lesion produced in DNA by neocarzinostatin and other damaging agents forms cross-links with the base-excision repair enzyme endonuclease III. J Am Chem Soc. 2001. 123: 3161-3162.

Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. Nature 2001. 411: 366-374.

Huggins CF, Chafin DR, Aoyagi S, Henricksen LA, Bambara RA, Hayes JJ. Flap endonuclease 1 efficiently cleaves base excision repair and DNA replication intermediates assembled into nucleosomes. Mol Cell. 2002. 10: 1201-1211.

Krokan HE, Standal R, Slupphaug G. DNA glycosylases in the base excision repair of DNA. Biochem J. 1997. 325: 1-16.

Liu Y, Beard WA, Shock DD, Prasad R, Hou EW, Wilson SH. DNA polymerase β and flap endonuclease 1 enzymatic specificities sustain DNA synthesis for long patch base excision repair. J Biol Chem. 2005. 280: 3665-3674.

Masuda Y, Bennett RA, Demple B. Dynamics of the interaction of human apurinic endonuclease (Ape1) with its substrate and product. J Biol Chem. 1998. 273: 30352-30359.

Matsumoto Y, Kim K. Excision of deoxyribose phosphate residues by DNA polymerase β during DNA repair. Science 1995. 269: 699-702.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983. 65: 55-63.

Narayan S, Jaiswal AS, Balusu R. Tumor suppressor APC blocks DNA polymerase β-dependent strand displacement synthesis during long patch but not short patch base excision repair and increases sensitivity to methylmethane sulfonate. J Biol Chem. 2005. 280: 6942-6949.

Prasad R, Dianov GL, Bohr VA, Wilson SH. FEN1 stimulation of DNA polymerase β mediates an excision step in mammalian long patch base excision repair. J Biol Chem. 2000. 275: 4460-4466.

Pratviel G, Pitie M, Bernadou J, Meunier B. Mechanism of DNA cleavage by cationic manganese porphyrins: hydroxylations at the 1'-carbon and 5'-carbon atoms of deoxyriboses as initial damages. Nucleic Acids Res. 1991. 19: 6283-6288.

Srivastava DK, Berg BJ, Prasad R, Molina JT, Beard WA, Tomkinson AE, Wilson SH. Mammalian abasic site base excision repair. identification of the reaction sequence and rate-determining steps. J Biol Chem. 1998. 273: 21203-21209.

Sigman DS, Mazumder A, Perrin DM. Chemical nucleases. Chem Rev. 1993. 93: 2295-2316.

Sobol RW, Horton JK, Kuhn R, Gu H, Singhal RK, Prasad R, Rajewsky K, Wilson SH. Requirement of mammalian DNA polymerase β in base-excision repair. Nature 1996. 379: 183 -186.

Sung JS, Bennett SE, Mosbaugh DW. Fidelity of uracil-initiated base excision DNA repair in *Escherichia coli* cell extracts. J Biol Chem. 2001. 276: 2276-2285.

Sung JS, Mosbaugh DW. *Escherichia coli* uracil- and ethenocytosine-initiated base excision DNA repair: rate-limiting step and patch size distribution. Biochemistry 2003. 42: 4613 -4625.

Urbaniak MD, Bingham JP, Hartley JA, Woolfson DN, Caddick S. Design and synthesis of a nitrogen mustard derivative stabilized by apo-neocarzinostatin. J Med Chem. 2004. 47: 4710 -4715.

von Sonntag C. The chemistry of free-radical-mediated DNA damage. Basic Life Sci. 1991. 58: 287-317.

Wilson DM 3rd, Thompson LH. Life without DNA repair. Proc Natl Acad Sci USA. 1997. 94: 12754-12757.

Xu YJ, DeMott MS, Hwang JT, Greenberg MM, Demple B. Action of human apurinic endonuclease (Ape1) on C1'-oxidized deoxyribose damage in DNA. DNA Repair. 2003. 2: 175-185.

Zheng Y, Sheppard TL. Half-life and DNA strand scission products of 2-deoxyribonolactone oxidative DNA damage lesions. Chem Res Toxicol. 2004. 17: 197-207.