Mutat. Res.

Miscoding properties of 1,N^6-ethanoadenine, a DNA adduct derived from reaction with the antitumor agent 1,3-bis(2-chloroethyl)-1-nitrosourea

Bo Hang, Anton B. Guliaev, Ahmed Chenna[1], and B. Singer*

Life Sciences Division,
Lawrence Berkeley National Laboratory,
University of California,
Berkeley, California 94720
USA

*Corresponding author: Tel: 510-642-0637
Fax: 510-486-6488

[1]Present address: Aclara BioSciences
Mountain View, CA 94043
USA

Abbreviations: EA, 1,*N*^{6}-ethanoadenine; εA, 1,*N*^{6}-ethenoadenine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; polymerases α, β, η and ι: pols α, β, η and ι.

Abstract

1,N^6-Ethanoadenine (EA) is an exocyclic adduct formed from DNA reaction with the antitumor agent, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). To understand the role of this adduct in the mechanism of mutagenicity or carcinogenicity by BCNU, an oligonucleotide with a site-specific EA was synthesized using phosphoramidite chemistry. We now report the *in vitro* miscoding properties of EA in translesion DNA synthesis catalyzed by mammalian DNA polymerases (pols) α, β, η and ι. These data were also compared with those obtained for the structurally related exocyclic adduct, 1,N^6-ethenoadenine (εA). Using a primer extension assay, both pol α and pol β were primarily blocked by EA or εA with very minor extension. Pol η, a member of the Y family of polymerases, was capable of catalyzing a significant amount of bypass across both adducts. Pol η incorporated all four nucleotides opposite EA and εA, but with differential preferences and mainly in an error-prone manner. Human pol ι, a paralog of human pol η , was blocked by both adducts with a very small amount of synthesis past εA. It incorporated C and, to a much lesser extent, T, opposite either adduct. In addition, the presence of an A adduct, *e.g.* εA, could affect the specificity of pol ι toward the template T immediately 3' to the adduct. In conclusion, the four polymerases assayed on templates containing an EA or εA showed differential bypass capacity and nucleotide incorporation specificity, with the two adducts not completely identical in influencing these properties. Although there was a measurable extent of error-free nucleotide incorporation, all these polymerases primarily misincorporated opposite EA, indicating that the adduct, similar to εA, is a miscoding lesion.

Keywords: BCNU; Ethano adduct; Etheno adduct; Polymerase; Translesion DNA synthesis

1. Introduction

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) is an effective chemotherapeutic agent which is commonly used in the treatment of certain types of tumors, particularly intracranial tumors [1-3]. However, it is also classified as an animal carcinogen [2,4] and is associated with human acute nonlymphocytic leukemia in the treatment of previously existing malignancy with other anticancer therapies [2]. Both *in vitro* and *in vivo* studies have demonstrated that BCNU is genotoxic as measured by point mutation and chromosomal damage [5]. The mutational specificity studied in mammalian cells [6,7] showed that the BCNU-induced mutations were predominantly base-pair substitutions.

BCNU is a synthetic compound that belongs to a family of haloethylnitrosoureas. These bifunctional alkylating agents are highly reactive and directly acting [5,8,9]. Reaction with DNA by BCNU forms several exocyclic DNA adducts, including the saturated ethano adducts of adenine, cytosine, and guanine (1,N^6-ethanoA, 3,N^4-ethanoC, N^2,3-ethanoG, and 1,O^6-ethanoG) [8,10]. In addition, BCNU can produce monosubstituted purine bases (either a hydroxyethyl or a haloethyl group) and a dC-dG cross-link, 1-(3-cytosinyl)-2-(1-guanyl)ethane, in DNA [8]. The exact biological roles of these lesions are largely unknown thus far, except that the dC-dG cross-link is cytotoxic [11] and its cellular formation is related to tumor cell resistance to the nitrosoureas used [8,12,13]. The exocyclic ethano adducts have also been proposed to be important with respect to the biological functions of BCNU due to the potential of its structural interference with base pairing [14].

The ethano adducts, formed by BCNU, structurally resemble the etheno derivatives produced by several environmental chemical carcinogens such as vinyl chloride and urethane [15,16]. These two types of adducts differ in saturation of a C7-C8 double bond

in the 5-membered exocyclic ring. The etheno adducts have been synthesized and extensively studied for their structures as well as biochemical effects on DNA replication and repair [16,17]. However, much less information on the same aspects is known with the related ethano adducts. The first synthesis of an ethano derivative, 3,N^4-ethanodeoxycytidine, and its incorporation into a site-specific oligonucleotide was described by Zhang *et al* [18], which led them to investigate how the ethano C adduct affects translesion DNA synthesis. From their *in vitro* studies, replication by the exo^- or exo^+ Klenow fragment of DNA pol I was primarily blocked by the adduct which also miscodes [19]. Recent synthesis in our laboratory of a defined oligonucleotide containing a site-directed 1,N^6-ethanoadenine (EA) [20] has greatly facilitated our studies on how the EA adduct is processed by enzymes involved in DNA repair [21] and replication (reported in this work).

During last few years, a new class of DNA polymerases, the Y family, has been identified from both prokaryotic and eukaryotic sources [22-25]. *In vitro,* these enzymes are known to have the ability to bypass DNA lesions and also to have low processivity and extraordinarily low fidelity when replicating normal DNA. The *in vivo* functions of many of these enzymes are unknown except in a few cases. For example, pol η, encoded by yeast *RAD30A* or human XPV, can faithfully and efficiently replicate across the *cis-syn* T-T dimer [26,27] and mutations in the XPV gene cause the variant form of xeroderma pigmentosum. Studies using *in vitro* replication systems have revealed that the three related Y family enzymes, pols η (Rad30A), ι (Rad30B) and κ (DinB1), are capable of bypassing various DNA lesions [*e.g.* 28-36] that are normally blockers of classical DNA polymerases such as pol α and pol β. However, such translesion syntheses can be error-free or error-prone. Nevertheless, these studies have provided biochemical basis for what could occur during cellular replication of the lesion-containing genomic DNA.

.

In this work, we examined the effect of the BCNU-derived EA adduct on *in vitro* replication catalyzed by four mammalian DNA polymerases which are from different families, pol α (B family), β (X family), and pol η and pol ι (Y family). The ability to bypass the EA adduct by these enzymes, as well as their specificity of nucleotide incorporation opposite the adduct, was assayed using *in vitro* replication assays on the oligonucleotide templates containing a site-directed adduct. The data obtained with the EA adduct were compared in parallel to those for the analogous εA adduct, in order to evaluate how the adduct structure influences replication specificity of the DNA polymerases.

2. Materials and methods

2.1. Enzymes

Calf thymus DNA pol α was a generous gift from Dr. Fred Perrino (Wake Forest University, Winston-Salem, NC). The concentration of pol α was 0.05 units/μL (one unit is defined as the amount of enzyme that incorporates 1 nmol of ^{32}P-α-dTMP in 60 min at 37 ^{o}C on activated calf thymus DNA). Human pol β was purchased from Trevigen (Gaithersburg, MD) and had a concentration of 4 units/μL (one unit is defined as the amount of enzyme required to incorporate 1 nmol of total nucleotide into acid-insoluble form in 60 min at 37 ^{o}C). Purified human pol η and pol ι were obtained from Enzymax (University of Kentucky, Lexington). T4 polynucleotide kinase was purchased from Amersham Pharmacia Biotech (Cleveland, OH).

2.2. Oligodeoxynucleotides

1,N^6-Ethano-2'-deoxyadenosine and its phosphoramidite were synthesized as previously described by Marenda *et al* [20]. 1,N^6-Etheno-2'-deoxyadenosine was purchased from Glen Research (Sterling, VA). 25-mer oligodeoxynucleotides containing a single EA or εA at the 6th position (Figure 1) were synthesized in this laboratory using an Applied Biosystems Model 394 automated DNA synthesizer or by Operon Technologies (Alameda, CA), respectively. All adduct-containing oligomers were HPLC-purified and, on enzyme digestion with subsequent HPLC analysis, found to contain the correct modified base. Both 18-mer and 20-mer primers (Figure 1) were synthesized and purified through HPLC and polyacrylamide gel electrophoresis (PAGE) by Operon Technologies.

2.3. DNA replication assays

2.3.1. Preparation of DNA primer-templates (P/T)

In all the replication assays a short oligonucleotide primer (18- or 20-mer) was annealed to a longer 25-mer template to create a polymerase substrate. The primers were 5' end-labeled with [γ-^{32}P] ATP (specific activity > 6000 Ci/mmol, Amersham Pharmacia Biotech) as previously described [37,38] and then mixed in 1:1.5 molar ratio with a template in a buffer containing 70 mM Tris-HCl (pH 7.8) and 10 mM $MgCl_2$. The mixtures were heated for 2 min at 95-100 ^{o}C and annealed by slow cooling.

2.3.2. Full replication assays

Each polymerization reaction contained 2 nM of an annealed P/T, 200 μM of each of all four dNTPs (Amersham Pharmacia Biotech) and a DNA polymerase (concentrations specified in Figure legends). Reactions were incubated at 37 ^{o}C for various times in a buffer containing 25 mM KH_2PO_4 (pH 7.0), 5 mM $MgCl_2$, 5 mM DTT, 100 μg/ml bovine

serum albumin, and 10% glycerol. Reactions were terminated by adding 2 vol of a F/E solution (90% formamide and 50 mM EDTA). The samples were then heated for 3 min at 90-100 ^{o}C and then chilled on ice. Aliquots of 5 μL were loaded onto a 16% PAGE containing 8M urea. Following electrophoresis, the gels were dried and visualized by X-ray film autoradiography. For quantitation, the gels were phosphorimaged on a Bio-Rad FX Molecular Imager and the band intensity quantitated using the Quantity One software.

2.3.3. Single base insertion assays

In these reactions, each sample contained 2 nM of P/T and 200 μM of a single dNTP in order to identify the specific dNTP(s) to be incorporated opposite the adduct by the polymerase added to the reaction. For running-start reactions, the mixtures also contained 5 μM of dCTP which is complementary to the guanine base prior to the adduct (see Figure 2B, top scheme). Reactions were incubated at 37 ^{o}C for 30 min and terminated as described above. After the gels were scanned by a phosphorimager (see 2.3.2), the percent incorporation of each dNTP was calculated as the percent of primer in each lane extended to the adduct site and beyond.

3. Results

3.1. Replication of templates containing an EA or εA with pol α

Pol α is a high fidelity polymerase which is primarily involved in normal DNA replication [39]. In this work, the complete and accurate replication by the DNA pol α from calf thymus was found with the control template using a running start assay (Figure 2A, left). However, on both EA- and εA-containing templates, such replication syntheses were primarily blocked one base prior to the adduct with a very small percentage of full

replication (Figure 2A, center and right), indicating that pol α is unable to readily insert a nucleotide opposite either adduct. Using a single base insertion assay, it was clear that EA paired with both T and A, while εA primarily paired with A when small amount of pol α-catalyzed replication reached the EA or εA site (Figure 2B).

3.2. Replication of templates containing an EA or εA with human pol β

As one of the major enzymes in base excision repair (BER) pathway, Pol β catalyzes the accurate template-directed repair synthesis which fills the gap generated by base excision [39,40]. As shown in Figure 3A (center), under the conditions used, human pol β could not catalyze detectable translesion DNA synthesis across the EA adduct. For εA, a minor product was observed near the top of the gel (Figure 3A, right), indicating a small amount of bypass by the enzyme. Similar to pol α, the replication on both EA- and εA-templates was primarily blocked one base before the adduct. Also similar to pol α, pol β could incorporate small amounts of A and T opposite EA and mainly A opposite εA (Figure 3B). On the unmodified template with the identical sequence, pol β carried out efficient synthesis with A:T base pairing at the A site.

3.3. Significant bypass of EA and εA by human pol η with both error-free and error-prone nucleotide incorporation

Pol η has been known for its ability to catalyze translesion DNA synthesis across various types of DNA damage in either error-free or error-prone manner or in both. In this work, we found that the human pol η also catalyzed significant amounts of translesion synthesis past EA or εA (Figure 4A, center and right). With time, the amounts of extension products gradually increased to near full-length replication for both adducts. The position of the top bands in Figure 4A (center and right) also suggests the co-existence of full-length

and one base deletion, the latter of which remains to be determined. Such a deletion was frequently observed with pol η in replicating an εA-template as previously described [33]. It was also shown that for EA, the majority of the replication was blocked at or one base before the adduct, while εA caused a block to pol η mainly one base before the adduct (Figure 4A, center and right). On the 25-mer unmodified template, Fully extended products were observed with pol η reactions (Figure 4A, right).

In single nucleotide insertion assays, pol η could insert all four nucleotides opposite either EA or εA (Figure 4B). The relative percent incorporation of each dNTP was calculated as the percent of synthesis in each lane at the adduct site and beyond (see Materials and Methods). Opposite EA, pol η inserted 35.0% A, 13.1% C, 20.5% G and 31.5% T. In comparison, the enzyme inserted 46.7% A, 12.4% C, 22.3% G and 18.6% T opposite εA. In both cases, the amounts of error-free incorporation opposite the adduct were less than one-third of total incorporation. In conclusion, pol η is able to perform significant amount of translesion synthesis cross either EA or εA, but such process is mainly error-prone as seen from the relative misinsertion percent.

3.4. Error-prone nucleotide incorporation opposite EA or εA by human pol ι

Human pol ι has been reported to readily incorporate nucleotides opposite a number of DNA lesions, but mostly in an error-prone manner [*e.g.* 29,34,41-43]. A unique feature of this enzyme is that it preferentially incorporates G, rather than A, opposite the template T, leading to a T:G mismatch [29,43,44]. In contrast, the extension is generally most efficient and accurate when A is the template base. The enzyme frequently stops at a T base, as shown in Figure 5A (left), a phenomenon which was described previously as the “T stop” [43]. In replicating modified DNA, translesion synthesis was blocked by EA or εA with

very small amount of bypass with εA which stopped at the next T (Figure 5A). In order to rule out the possibility that the presence of this T may affect the lesion bypass, we replaced the T with A in the εA-containing 25-mer template (Figure 5B, scheme). Under such change, there was no measurable bypass observed with εA adduct (Figure 5B, right) and pol ι was able to extend the primer further beyond the normal template A (Figure 5B, left).

The base insertion specificities for EA and εA are similar, with pol ι primarily incorporating C with much less T opposite the adduct (Figure 5C, center and right), indicating that pol ι mainly carry out error-prone nucleotide incorporation opposite either adduct. On the control template, pol ι correctly inserted T opposite the template A (Figure 5C, left).

3.5. Effect of εA on pol ι insertion specificity toward template T downstream from εA

Since the local sequence context of the template or template-primer plays a role in determining the nucleotide insertion specificity of pol ι [45,46], we asked whether the presence of an exocyclic adduct would pose any impact on such specificity. Two 20-mer primers with either A or C at the 3' terminus were synthesized and 5'-end labeled with ^{32}P. Each was then annealed to the εA- or A-containing 25-mer template to create an εA(A):T base pair or εA(A):C mismatch at the replication initiating site (see schemes in Figure 6). The selection of C and T opposite the template εA or A was based on the insertion specificity of pol ι toward εA shown in Figure 5C. In the presence of the A:T pair (Figure 6B, left), pol ι inserted A, T and G opposite the template T. In contrast, it uniquely inserted T opposite the template T when the oligomer substrate had an εA:T at the terminus (Figure 6B, right). Similar insertion pattern was seen with A:C mispair but the amounts of insertion were much less than those for the A:T pair (Figure 6A and B, left), suggesting that T:C mispair is a poor substrate for primer extension by pol ι. When εA paired with C, pol ι also

only incorporated T opposite the template T (Figure 6A, right). In all cases, pol ι did not insert C opposite the template T regardless of the base-pair at the terminus. These results clearly showed that the presence of an εA base at the 5' side can change the base insertion specificity of pol ι toward the next template base T.

4. Discussion

One approach to a better understanding of the mechanism of BCNU mutagenicity or carcinogenicity is to relate specific DNA lesions produced by this agent to its biological consequences. BCNU is known to mainly cause G:C→T:A transversions and G:C→A:T transitions in mammalian (CHO) cells [6]. However, the frequency of both types of base-pair substitutions could be greatly reduced in the human O^6-alkylguanine-DNA alkyltransferase-transformed CHO cells, suggesting that both BCNU-initiated mutations were O^6-alkylguanine-related [7]. Consequently, in such transformed cells, T:A→G:C transversions emerged as the most common base substitution [7], indicating the biological relevance of A:T base pair modifications by BCNU.

In this work, we investigated the *in vitro* mutagenic properties of 1,N^6-ethanoadenine (EA), one of the base modifications by BCNU, using a primer extension system with four mammalian DNA replicating enzymes. Under the conditions used in our system, EA mainly blocks DNA synthesis catalyzed by pols α, β and ι. These three enzymes predominantly carried out error-prone nucleotide incorporation opposite the adduct, but with different specificities. In contrast, human pol η was capable of performing a significant amount of synthesis across EA. The enzyme primarily misincorporated opposite the adduct, even though a significant amount of error-free base pairing, EA:T, was observed. Of the Y family polymerases, pol η has probably been the most widely tested for

similar lesion bypass capacities. This enzyme catalyzes the error-free translesion DNA synthesis across *cis-syn* T-T dimer [26,27] and 7,8-dihydro-8-oxoguanine (8-oxoG) [28]. It also incorporates, with relatively high fidelity, nucleotides opposite several lesions including acetylaminofluorene-dG and cisplatin G-G intrastrand cross-link [Masutani00; Yuan00]. On the other hand, pol η catalyzes significant error-prone nucleotide incorporation opposite a variety of different types of DNA modifications such as (+)-*trans*-*anti*-benzo[a]pyrene-N_2-dG and an AP site [47]. Interestingly, pol η is able, with relative efficiency, to bypass all the exocyclic adducts tested so far. This includes εA, EA, εC and 8-hydroxymethyl-εC [33,35, this work]. These results raise the questions as to whether pol η is specific for this group of structurally related adducts [48-50] and whether this enzyme has any cellular function toward these adducts. As for other polymerases studies in this work (pols α, β and ι), EA was primarily a blocker. The lesion bypass through combined actions of two polymerases is being studied in order to examine whether such terminated synthesis at the adduct site can be further extended by another polymerase. Two of the Y family DNA polymerases, pol ξ (Rev3 and Rev7) and pol κ, are known to have such elongating function [29, 47, 51].

εA is a highly mutagenic lesion in mammalian cells [52,53]. Using a shuttle plasmid vector containing a single εA, Levine *et al* [53] described the predominant induction of εA→T, together with εA→G and εA→C, in human cells. Such efficient induction of εA→T transversions is consistent with the finding that the tumors in humans and animals exposed to vinyl compounds showed A:T→T:A transversions which could be due to the misinsertion of dAMP opposite εA by a polymerase(s) [53]. In search for enzymes which could be responsible for such misinsertion and lesion bypass, various translestional DNA polymerases were tested. Previous *in vitro* primer extension studies showed that both pol η and pol κ catalyze error-free and error-prone synthesis past εA with pol η being much more efficient than pol κ in such bypass [33]. Our *in vitro* results reported here also

support a possible role of pol η in cellular translesion synthesis across εA as based on the relatively efficient bypass and ~ 50% of A misinsertion opposite εA, which would lead to an εA→T transversion. Human pol ι [this work] and human REV1 [54] appear to be unable to efficiently bypass εA *in vitro,* but the two enzymes misinsert. The final verification of these *in vitro* findings need data from genetic studies such as a gene knockout.

In general, the results obtained from the comparative experiments on EA and εA in this study differ only in minor aspects. The structural differences between these two exocyclic adducts were earlier [21]. The saturation of the double bond in the imidazole ring of EA partially reduces its stacking ability and results in a non-planar conformation of the extra ring. The two additional hydrogens of EA, as compared to εA, contribute an extra surface area for the adduct. However, as shown by molecular modeling, these changes in adduct structure do not affect the conformation of the lesion in the DNA duplex [21]. Both lesions form a sheared base pair with the adduct displaced toward the major groove. No Watson–Crick base pairing is observed between the adduct and the opposite base. This may explain the observed similar patterns of bypass and insertion specificity toward these two adducts by the polymerases used in this study.

Human cells possess two Rad30 paralogs, Rad30A (pol η) and Rad30B (pol ι). However, pol ι differs from pol η greatly in enzymatic properties and its cellular functions are still speculative. Pol ι preferentially inserts G, rather than the Watson-Crick base A, opposite the template T [29,43,44]. In addition, the enzyme forms a T:T base pair almost as frequently as forming an A:T pair. The insertion specificity of pol ι also depends on the neighbor base sequence [45,46]. In this study, pol ι could not replicate past the EA adduct and bypassed εA to a very small extent. The enzyme primarily performed error-prone nucleotide incorporation opposite both adducts (EA:C and εA:C). Interestingly, the

presence of εA upstream from the template T clearly had an impact on the insertion specificity of pol ι toward the T. Instead of the incorporation of three of the nucleotides, T, A and G, opposite template T following template A, pol ι uniquely inserts a T opposite the template T following εA (Figure 6).

Recent crystallographic studies provided some insight into structural differences between the classical and translesional (Y family) polymerases. Pol α and pol β normally possess a tight active site that can accommodate only a single base [39,55-57]. In contrast, the absence of the "O" and "O1" helices in the fingers domain of *S. cerevisiae* pol η makes its active site more open, thus enabling the enzyme to replicate T-T dimers and 8-oxo-G [58]. The ability to place two template bases in the active site was also observed for the *S. solfataricus* polymerase Dpo4 which is a DinB homolog [59]. Such "openness" of the active site could be conserved across the whole Y family and be a general feature for the polymerases in this family. In relevance to our work, the presence of the exocyclic etheno or ethano ring on A increases the van der Waals space by almost 30%. Therefore, we propose that the adduct occupies extra space and could also be constrained at the active site of pol ι, imposing steric limits, thus resulting in the incorporation of a smaller pyrimidine base, T, rather than G or A, opposite the next template T. The structures of the polymerases together with a bound template should provide further mechanistic details.

Acknowledgements

The authors thank Dr. Fred Perrino (Wake Forest University) for calf thymus DNA pol α and Dr. Zhigang Wang (University of Kentucky) for partial providing of human pol η and ι. The authors also acknowledge the technical assistance of Vivian Bui. This work was supported by NIH grants CA47723 (to B.S) and CA72079 (to B.H.) and was administrated

by the Lawrence Berkeley National Laboratory under Department of Energy contract DE-AC03-76SF00098.

References

[1] D.B. Ludlum, The chloroethylnitrosoureas: sensitivity and resistance to cancer chemotherapy at the molecular level, Cancer Invest. 15 (1997) 588-598.

[2] U.S. Department of Health and Human Services, NTP, 8th Report on Carcinogens (1), III (1998) 667-669.

[3] H.A. Fine, The basis for current treatment recommendations for malignant gliomas, J. Neurooncol. 20 (1994) 111–120.

[4] International Agency for Research on Cancer, IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans (Supplement 7). IARC Scientific Publications, Lyon, France, 1987.

[5] J.K. Wiencke, J. Wiemels, Genotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), Mutat. Res. 339 (1995) 91-119.

[6] D.T. Minnick, M.L. Veigl, W.D. Sedwick, Mutational specificity of 1,3-bis-(2-chloroethyl)-1-nitrosourea in a Chinese hamster ovary cell line, Cancer Res. 52 (1992) 4688-4695.

[7] D.T. Minnick, S.L. Gerson, L.L. Dumenco, M.L. Veigl, W.D. Sedwick, Specificity of bischloroethylnitrosourea-induced mutation in a Chinese hamster ovary cell line transformed to express human O^6-alkylguanine-DNA alkyltransferase, Cancer Res. 53 (1993) 997-1003.

[8] D.B. Ludlum, DNA alkylation by the haloethylnitrosoureas: nature of modifications produced and their enzymatic repair or removal, Mutat. Res. 233 (1990) 117-126.

[9] Ludlum,D.B. The chloroethylnitrosoureas: sensitivity and resistance to cancer chemotherapy at the molecular level, Cancer Invest. 15 (1997) 588-598.

[10] D.B. Ludlum, Formation of cyclic adducts in nucleic acids by the haloethylnitrosoureas, in: B. Singer and H. Bartsch (Eds.), The role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis, IARC Scientific Publications, Lyon, France, 1986, No. 70, pp. 137-146.

[11] W.P. Tong, M.C. Kirk, D.B. Ludlum, Formation of the cross-link 1-[N^3-deoxy-cytidyl],2-[N^1-deoxyguanosinyl]ethane in DNA treated with N,N'-bis(2-chloroethyl)-N-nitrosourea), Cancer Res. 42 (1982) 3102-3105.

[12] L.C. Erickson, G. Laurent, N.A. Sharkey, K.W. Kohn, DNA cross-linking and monoadduct repair in nitrosourea-treated human tumor cells, Nature 288 (1980) 727-729.

[13] D.B. Ludlum, Q. Li, Z. Matijasevic, Role of base excision repair in protecting cells from the toxicity of chloroethylnitrosoureas, in: B. Singer and H. Bartsch (Eds.), Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis, IARC Scientific Publications, Lyon, France, 1999, No. 150, pp. 271-278.

[14] Y. Habraken, C.A. Carter, M.C. Kirk, J.M. Riordan, D.B. Ludlum, Formation of N^2,3-ethanoguanine in DNA after *in vitro* treatment with the therapeutic agent, N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea, Carcinogenesis 11 (1990) 223-228.

[15] N.J. Leonard, Etheno-bridged nucleotides in structural diagnosis and carcinogenesis, Biochem. Mol. Biol. Chemtracts 3 (1992) 273-297.

[16] H. Bartsch, Exocyclic adducts as new risk markers for DNA damage in man, in: B. Singer and H. Bartsch (Eds.), Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis, IARC Scientific Publications, Lyon, France, 1999, No. 150, pp. 1-16.

[17] A. Barbin, Etheno-adduct-forming chemicals: from mutagenicity testing to tumor mutation spectra. Mutat. Res. 462 (2000) 55-69.

[18] W. Zhang, R. Rieger, C. Iden, F. Johnson, Synthesis of 3,N^4-etheno, 3,N^4-ethano, and 3-(2-hydroxyethyl) derivatives of 2'-deoxycytidine and their incorporation into oligomeric DNA, Chem. Res. Toxicol. 8 (1995) 148-156.

[19] R.R. Bonala, R.A. Rieger, S. Shibutani, A.P. Grollman, C.R. Iden, F. Johnson, 3,N^4-Ethano-2'-deoxycytidine: chemistry of incorporation into oligomeric DNA and reassessment of miscoding potential, Nucleic Acids Res. 27 (1999) 4725-4733.

[20] H. Maruenda, A. Chenna, L.K. Liem, B. Singer, Synthesis of 1,N^6-ethano-2'-deoxyadenosine, a metabolic product of 1,3-bis(2-chloroethyl)nitrosourea, and its incorporation into oligomeric DNA, J. Org. Chem. 63 (1998) 4385-4389.

[21] A.B. Guliaev, B. Hang, B. Singer, Structural insights by molecular dynamics simulations into differential repair efficiency for ethano-A versus etheno-A adducts by the human alkylpurine-DNA-N-glycosylase (APNG), Nucleic Acid Res. 30 (2002) 3778-3787.

[22] R.E. Johnson, M.T. Washington, S. Prakash, L. Prakash, Bridging the gap: a family of novel DNA polymerases that replicate faulty DNA, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 12224-12226.

[23] R. Woodgate, A plethora of lesion-replicating DNA polymerases, Genes Dev. 13 (1999) 2191-2195.

[24] H. Ohmori, E.C. Friedberg, R.P. Fuchs, M.F. Goodman, F. Hanaoka, D. Hinkle, T.A. Kunkel, C.W. Lawrence, Z. Livneh, T. Nohmi, L. Prakash, S. Prakash, T. Todo, G.C. Walker, Z. Wang, R. Woodgate, The Y-family of DNA polymerases, Mol. Cell 8 (2001) 7-8.

[25] Z.-G. Wang, Translesion synthesis by the UmuC family of DNA polymerases, Mutat. Res. 486 (2001) 59-70.

[26] R.E. Johnson, C.M. Kondratick, S. Prakash, L. Prakash, hRAD30 mutations in the variant form of xeroderma pigmentosum, Science 285 (1999) 263-265.

[27] C. Masutani, R. Kusumoto, A. Yamada, N. Dohmae, M. Yokoi, M. Yuasa, M. Araki, S. Iwai, K. Takio, F. Hanaoka, The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase η, Nature 399 (1999) 700-704.

[28] L. Haracska, S.L. Yu, R.E. Johnson, L. Prakash, S. Prakash, Efficient and accurate replication in the presence of 7,8-dihydro-8-oxoguanine by DNA polymerase η, Nature Genet. 25 (2000) 458-461.

[29] R.E. Johnson, M.T. Washington, L. Haracska, S. Prakash, L. Prakash, Eukaryotic polymerases ι and ζ act sequentially to bypass DNA lesions, Nature 406 (2000) 1015-1019.

[30] L. Haracska, S. Prakash, L. Prakash, Replication past O^6-methylguanine by yeast and human DNA polymerase η. Mol. Cell. Biol. 20 (2000) 8001-8007.

[31] Y. Zhang, F. Yuan, X. Wu, O. Rechkoblit, J.S. Taylor, N.E. Geacintov, Z. Wang, Error-prone lesion bypass by human DNA polymerase η. Nucleic Acids Res. 28 (2000) 4717-4724.

[32] C. Masutani, R. Kusumoto, S. Iwai, F. Hanaoka, Mechanisms of accurate translesion synthesis by human DNA polymerase η, EMBO J. 19 (2000) 3100-3109.

[33] R.L. Levine, H. Miller, A. Grollman, E. Ohashi, H. Ohmori, C. Masutani, F. Hanaoka, M. Moriya, Translesion DNA synthesis catalyzed by human pol η and pol κ across 1,N^6-ethenodeoxyadenosine, J. Biol. Chem. 276 (2001) 18717-18721.

[34] Y. Zhang, F. Yuan, X. Wu, J.-S. Taylor, Z. Wang, Response of human DNA polymerase ι to DNA lesions, Nucleic Acids Res. 29 (2001) 928-935.

[35] B. Singer, M. Medina, Y. Zhang, Z. Wang, A.B. Guliaev, B. Hang, 8-(Hydroxymethyl)-3,N^4-etheno-C, a potential carcinogenic glycidaldehyde product, miscodes *in vitro* using mammalian DNA polymerases, Biochemistry 41 (2002) 1778-1785.

[36] P.L. Fischhaber, V.L. Gerlach, W.J. Feaver, Z. Hatahet, S.S. Wallace, E.C. Friedberg, Human DNA polymerase kappa bypasses and extends beyond thymine glycols

during translesion synthesis *in vitro*, preferentially incorporating correct nucleotides, J. Biol. Chem. 277 (2002) 37604-37611.

[37] B. Singer, F. Chavez, S.J. Spengler, J.T. Kusmierek, L. Mendelman, M.F. Goodman, Comparison of polymerase insertion and extension kinetics of a series of O^2-alkyldeoxythymidine triphosphates and O^4-methyldeoxythymidine triphosphate, Biochemistry 28 (1989) 1478-1483.

[38] V. Litinski, A. Chenna, J. Sagi, B. Singer, Sequence context is an important determinant in the mutagenic potential of 1,N6-ethenodeoxyadenosine (εA): formation of εA basepairs and elongation in defined templates, Carcinogenesis 18 (1997) 1609-1615.

[39] T.A. Kunkel, K. Bebenek, DNA replication fidelity, Annu. Rev. Biochem. 69 (2000) 497-529.

[40] S.H. Wilson, Mammalian base excision repair and DNA polymerase β, Mutat. Res. 407 (1998) 203-215.

[41] A. Tissier, E.G. Frank, J.P. McDonald, S. Iwai, F. Hanaoka, R. Woodgate, Misinsertion and bypass of thymine–thymine dimers by human DNA polymerase ι, EMBO J. 19 (2000) 5259-5266.

[42] E.G. Frank, J.M. Sayer, H. Kroth, E. Ohashi, H. Ohmori, D.M. Jerina, R. Woodgate, Translesion replication of benzo[a]pyrene and benzo[c]phenanthrene diol epoxide adducts of deoxyadenosine and deoxyguanosine by human DNA polymerase ι, Nucleic. Acids Res. 30 (2002) 5284-5292.

[43] Y. Zhang, F. Yuan, X. Wu, Z. Wang, Preferential incorporation of G opposite template T by the low-fidelity human DNA polymerase ι, Mol. Cell. Biol. 20 (2000) 7099-7108.

[44] A. Tissier, J.P. McDonald, E.G. Frank, R. Woodgate, Pol ι, a remarkably error-prone human DNA polymerase, Genes Dev. 14 (2000) 1642-1650.

[45] A. Vaisman, A. Tissier, E.G. Frank, M.F. Goodman, R. Woodgate, Human DNA polymerase ι promiscuous mismatch extension, J. Biol. Chem. 276 (2001) 30615-30622.

[46] E.G. Frank, A. Tissier, J.P. McDonald, V. Rapic-Otrin, X. Zeng, P.J. Gearhart, R. Woodgate, Altered nucleotide misinsertion fidelity associated with polι-dependent replication at the end of a DNA template, EMBO J. 20 (2001) 2914-2922.

[47] F. Yuan, Y. Zhang, D.K. Rajpal, X. Wu, D. Guo, M. Wang, J.-S. Taylor, Z. Wang, Specificity of DNA Lesion Bypass by the Yeast DNA Polymerase, J. Biol. Chem. 275 (2000) 8233-8239.

[48] B. Singer, B. Hang, What structural features determine repair enzyme specificity and mechanism in chemically modified DNA? Chem. Res. Toxicol. 10 (1997) 713-732.

[49] B. Singer, H. Bartsch, Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis, IARC Scientific Publications, Lyon, France, 1999, No 150, pp. 361.

[50] L.J. Marnett, J.P. Plastaras, Endogenous DNA damage and mutation, Trends in Genetics 17 (2001) 214-221.

[51] L. Haracska, L. Prakash, S. Prakash, Role of human DNA polymerase kappa as an extender in translesion synthesis, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 16000-16005.

[52] G.A. Pandya, M. Moriya, 1,N^6-Ethenodeoxyadenosine, a DNA adduct highly mutagenic in mammalian cells, Biochemistry 35 (1996) 11487 –11492.

[53] R.L. Levine, I.-Y. Yang, M. Hossain, G.A. Pandya, A.P. Grollman, M. Moriya, Mutagenesis induced by a single 1,N^6-ethenodeoxyadenosine adduct in human cells, Cancer Res. 60 (2000) 4098-4104.

[54] Y. Zhang, X. Wu, O. Rechkoblit, N.E. Geacintov, J.-S. Taylor, Z. Wang, Response of human REV1 to different DNA damage: preferential dCMP insertion opposite the lesion, Nucleic Acids Res. 30 (2002) 1630-1638.

[55] M.R. Sawaya, R. Prasad, S.H. Wilson, J. Kraut, H. Pelletier, Crystal structures of human DNA polymerase β complexed with gapped and nicked DNA: evidence for an induced fit mechanism, Biochemistry 36 (1997) 11205-11215.

[56] C.A. Brautigam, T.A. Steitz, Structural and functional insights provided by crystal structures of DNA polymerases and their substrate complexes, Curr. Opin. Struct. Biol. 8 (1998) 54-63.

[57]. J.M. Krahn, W.A. Beard, H. Miller, A.P. Grollman, S.H. Wilson, Structure of DNA polymerase β with the mutagenic DNA lesion 8-oxodeoxyguanine reveals structural insights into its coding potential, Structure (Camb) 11 (2003) 121-127.

[58] J. Trincao, R.E. Johnson, C.R. Escalante, S. Prakash, L. Prakash, A.K. Aggarwal, Structure of the catalytic core of S. cerevisiae DNA polymerase eta: implications for translesion DNA synthesis, Mol. Cell 8 (2001) 417-426.

[59] H. Ling, F. Boudsocq, R. Woodgate, W. Yang, Crystal structure of a Y family DNA polymerase in action: a mechanism for error-prone and lesion-bypass replication, Cell 107 (2001) 91-102.

Figure legends

Figure 1. Top: Chemical structures of 1,N^6-ethanodenine (EA) and 1,N^6-ethenoadenine (εA). Bottom: DNA templates and primers used in this study.

Figure 2. A: Full replication catalyzed by calf thymus pol α on normal, EA- and εA-containing templates using 200 μM of each of all four dNTPs. The enzyme concentration used for this time course was 0.05 units. Standard reactions were performed as described in Materials and Methods. The template primer (P/T) used is shown on top with the 18-mer primer being ^{32}P-labeled at the 5' end, and the partial template sequence is shown vertically on the sides of the figure. B: Single base insertion opposite normal A, EA and εA using 200 μM of a single dNTP. In addition, 5 μM dCTP was added to initiate a running start one base before the A or modified A sites. All the nucleotide incorporation reactions contained 0.05 units of pol α and were incubated at 37 ^{o}C for 30 min.

Figure 3. A: Full replication catalyzed by human pol β on normal, EA- and εA-containing templates. Pol β concentration used for this time course was 0.6 units. The reaction conditions are the same as described in Figure 2. B: Single base insertion opposite normal A, EA and εA. Each individual reaction contained 1.6 units of pol β and were incubated under the same experimental conditions as described in Figure 2.

Figure 4. Translesion DNA synthesis cross EA and εA by human pol η. A: Time course of full replication with pol η (0.35 ng/μl) under the conditions specified in Figure 2. B: Standard nucleotide insertion assays using a single dNTP and 0.83 ng/μl of pol η. See details in Figure 2.

Figure 5. Human pol ι-catalyzed replication synthesis on templates containing A, EA or εA. A: Time course of full replication with human pol ι (6.25 ng/μl) under the experimental conditions specified in Figure 2. Note that the T in the control template (left panel) posed a block to the synthesis by pol ι. B: Full replication reactions using the templates which contained an A instead of T on the downstream side of template A or εA (see the scheme). Same concentration (6.25 ng/μl) of pol ι was used for these reactions. C: Standard nucleotide insertion reactions catalyzed by human pol ι. See details in Figure 2.

Figure 6. Standing-start assays for identifying the nucleotide incorporation specificity of human pol ι toward the template T 3' next to an εA adduct. As shown on top of A and B panels, a 20-mer primer with a C or T base at its 3' terminus was utilized to form a forced match with εA on the 25-mer template. The standard single base insertion assays were performed with 6.25 ng/μl of pol ι and 200 μM of a single dNTP at 37 ^{o}C for 30 min.

.