cells, in which Y-family polymerases, such as polη, polκ, insert nucleotides opposite the lesion, and a B-family polymerase ζ extends the primer after insertion to the lesion (Shachar *et al*, 2009). The slow extension step by hpolη may be remedied by hpolζ in mammalian cells.

The mobile adducts destabilize the replicating base pair

Earlier biphasic kinetic data support that the Dpo4–DNA complex exists as at least two distinct populations (Brown *et al*, 2008). Pt-GG in different structural environments adopts different conformations with varied roll angles and α/β torsion angles (Sherman *et al*, 1985; Takahara *et al*, 1995; Ohndorf *et al*, 1999). The two guanine bases in the *cis*-$Pt(NH_3)_2$[d(pGpG)] (a single Pt-GG) crystal structure has a perpendicular conformation with a roll angle of ~90° and α/β angles differ from the ideal values in a narrow range of 5–30° (Sherman *et al*, 1985). When a Pt-GG adduct is incorporated in the middle of a duplex DNA, the roll angle is compressed to 30°, with the torsion angles α/β deviated from ideal values up to 50° in protein-free adducted DNA helix (Takahara *et al*, 1995). The distorted and angular Pt-GG causes the 12-base pair duplex bending to a 43° curve (Figure 3D; Takahara *et al*, 1995). In GG2 and GG3, the DNA helices are straight with bending angle <18° over a 12-bp helical region, which further compresses the Pt-GG adduct with roll angle as low as 22° (Figure 3B and C). The Pt-GG adduct is distorted with the α/β torsion angles up to 60° to fit into the active site and helical DNA, 10° more distortion than the ones observed in the Pt-GG adducts in protein-free DNA (Takahara *et al*, 1995). The α/β torsion angles in the Dpo4 complexes are energetically unfavourable based on the molecular simulation (Yao *et al*, 1994). Particularly, the additional depression on the roll angle is reinforced by the close contact of the finger domain on the replicating base pair in GG2 (Figure 2B and C). The depression, combined with alternate conformations, brings the adduct to nearly parallel conformations in GG2 (Figure 3B). The geometric strains from the distortion would bring the adduct to a high-energy state that takes alternate conformations in GG2. Overall, Pt-GG is highly mobile in the two insertion structures due to its solvent exposure (GG1) and geometric strains (GG2). A similar mobile Pt-GG adduct also exists in the cisplatinated nucleosome structure where the intrastrand platinum adducts are packed into helical DNA (Wu *et al*, 2008). At 3.4 Å resolution, elongated Pt-anomalous density indicates the inherent mobility of the platinum atom covalently bonded to the parallel purine bases, which contrasts with the spherical anomalous selenium peaks in the same structure (Wu *et al*, 2008).

In summary, destabilization of the adduct perturbs the DNA structure, so that catalytic efficiency and fidelity are reduced in Dpo4-mediated bypass of Pt-GG (Brown *et al*, 2008). The misaligned G*:dCTP in GG1 and alternate G*:dCTP base pair in GG2 make replicating base pairs unstable, which would increase the probability of mutations and decrease the populations of the productive complexes of Dpo4–DNA. The mobility is consistent with the following observations in solution: (1) the pronounced C base misincorporation in the primer extension assays (Figure 4); (2) 72- and 860-fold reduction in incorporation efficiency (k_p/K_d) at the first and second insertion steps, respectively, and six-fold reduction at the extension step, relative to control undamaged DNA (Brown *et al*, 2008); and (3) two orders of magnitude of fidelity reduction in Pt-GG adducted DNA replication compared with undamaged DNA replication by Dpo4 (Brown *et al*, 2008). Interestingly, the symmetric H-bonding in the misaligned G*:dCTP at the first insertion (GG1) and WC H-bonding in the second insertion (GG2) still provide specificity for selective dCTP incorporation against the Pt-GG lesion though enhanced misincorporation occurs in the disturbed base paring environment.

Conclusions

This work provides a new molecular model for TLS over DNA lesions. Our structures reveal that Pt-GG bypass is a unique, dynamic process in which the Pt-GG adduct undergoes conformational changes, as it is translocated through the 'close-off' active site of the Y-family polymerase. The angular double-base lesion adopts a depressed conformation to fit into the active site and the helical structure of DNA during the second insertion and extension stages. The incompatibility of angular adducts with helical DNA structure and the rigid active site, along with the stress caused by the depressed conformation, make the Pt-GG adduct in energetically unfavourable conformations. The disturbed and deformed DNA template leads to instability of the replicating base pair, resulting in low replication efficiency and reduced fidelity during Pt-GG bypass. Particularly, in the second insertion, the entropically costly conformational conversion may additionally impede nucleotide incorporation against the 5′G of Pt-GG. The *in vivo* observations support that the Y-family polymerase Dpo4 has an important function in tolerance of cisplatin, implicating possible contributions of Y-family polymerases to cisplatin resistance. The knowledge of how platinum cross-linked lesions are replicated by a Y-family DNA polymerase may help the development of cross-linking platinum agents for cancer therapy.

Materials and methods

Protein purification, DNA preparation, and crystallization

Dpo4 was expressed and purified as described earlier (Ling *et al*, 2001). DNA oligonucleotide preparation was similar to that in the earlier reported work (Brown *et al*, 2008). A hanging drop vapor diffusion method was used to grow crystals of the Dpo4–DNA–dNTP ternary complex by using our earlier conditions with some modifications (Bauer *et al*, 2007). The detailed procedures are reported in the Supplementary data.

Data collection and structure determination

Diffraction data for the GG1, GG2, and GG3 crystals were collected at beamline 24-ID-C (Argonne National Laboratory, IL) and processed using DENZO and SCALEPACK (Otwinowski and Minor, 1997). All crystal structures were solved with molecular replacement program PHASER (McCoy *et al*, 2005), with the type I structure as search model (PDB: 1JX4). Iterative cycles of simulated annealing, positional refinement, and B-factor refinement were performed using CNS (Brunger *et al*, 1998), along with manual rebuilding of the model using the graphics program COOT (Emsley and Cowtan, 2004). The GG1, GG2, and GG3 structures were refined to 2.9, 1.9, and 2.0 Å resolution, respectively.

In vitro assays

All the *in vitro* assays were carried out as described earlier (Brown *et al*, 2008; Wong *et al*, 2008), with reaction temperature at 23°C. The detailed procedures are reported in the Supplementary data.

Construction of the S. solfataricus dpo-4 mutant

The *dpo-4* knockout mutant of *S. solfataricus* was constructed as described earlier (Worthington *et al*, 2003; Schelert *et al*, 2004). The detailed procedures are reported in the Supplementary data.

Molecular biology and proteomic methods

DNA cloning, DNA sequencing, PCR, and plasmid transformation of *Escherichia coli* were performed as described (Rockabrand *et al*, 1998; Rolfsmeier *et al*, 1998; Haseltine *et al*, 1999). DNA concentrations were measured using a DyNA Quant 200 fluorometer (Hoefer). Protein concentrations were measured using the BCA Protein Assay Reagent Kit (Pierce). Chemiluminescent western blot analysis using anti-Dpo4 polyclonal sera was performed using the ECL system (Amersham Biosciences) as described (Rockabrand *et al*, 1998). Proteins were fractionated by two-dimensional SDS–PAGE as described (Hajduch *et al*, 2005). Protein samples were prepared using 50 ml of mid-exponential phase cultures collected by centrifugation at 3000*g* for 15 min at 26°C. Pelleted cells were extracted as described (Hajduch *et al*, 2005) and protein was precipitated for 1 h by exposure to five volumes of ice-cold 0.1 M ammonium acetate in 100% methanol at 22°C. Proteins were sequenced using tandem mass spectrometry (MS/MS) and peptides were identified by local BLAST against the *S. solfataricus* proteome as described (Worthington *et al*, 2003). Confirmed hits required a threshold peptide matching value (N) of at least five.

In vivo cisplatin assay

Cell lines were cultured at 80°C with aeration in a minimal salts medium (Allen, 1959), at pH 3.0 with tryptone 0.2% (wt/vol) as sole carbon and energy source. Growth was monitored at a wavelength of 540 nm using a Cary 50 spectrophotometer (Varian). Cisplatin was prepared as fresh aqueous solutions (2 mg/ml) before use and was added at early logrithmic phase.

Coordinates

The atomic coordinates and structure factors have been deposited in the Protein Data Bank, http://www.rcsb.org, with accession codes 3M9M, 3M9N, and 3M9O for the structures GG1, GG2, and GG3, respectively.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Albertella MR, Green CM, Lehmann AR, O'Connor MJ (2005) A role for polymerase eta in the cellular tolerance to cisplatin-induced damage. *Cancer Res* **65:** 9799–9806

Allen MB (1959) Studies with Cyanidium caldarium, an anomalously pigmented chlorophyte. *Arch Mikrobiol* **32:** 270–277

Alt A, Lammens K, Chiocchini C, Lammens A, Pieck JC, Kuch D, Hopfner KP, Carell T (2007) Bypass of DNA lesions generated during anticancer treatment with cisplatin by DNA polymerase eta. *Science* **318:** 967–970

Bassett E, Vaisman A, Havener JM, Masutani C, Hanaoka F, Chaney SG (2003) Efficiency of extension of mismatched primer termini across from cisplatin and oxaliplatin adducts by human DNA polymerases beta and eta *in vitro*. *Biochemistry* **42:** 14197–14206

Bassett E, Vaisman A, Tropea KA, McCall CM, Masutani C, Hanaoka F, Chaney SG (2002) Frameshifts and deletions during *in vitro* translesion synthesis past Pt-DNA adducts by DNA polymerases beta and eta. *DNA Repair (Amst)* **1:** 1003–1016

Bauer J, Xing G, Yagi H, Sayer JM, Jerina DM, Ling H (2007) A structural gap in Dpo4 supports mutagenic bypass of a major benzo[a]pyrene dG adduct in DNA through template misalignment. *Proc Natl Acad Sci USA* **104:** 14905–14910

Bergoglio V, Canitrot Y, Hogarth L, Minto L, Howell SB, Cazaux C, Hoffmann JS (2001) Enhanced expression and activity of DNA polymerase beta in human ovarian tumor cells: impact on sensitivity towards antitumor agents. *Oncogene* **20:** 6181–6187

Brown JA, Newmister SA, Fiala KA, Suo Z (2008) Mechanism of double-base lesion bypass catalyzed by a Y-family DNA polymerase. *Nucleic Acids Res* **36:** 3867–3878

Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL (1998) Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* **54:** 905–921

Canitrot Y, Cazaux C, Frechet M, Bouayadi K, Lesca C, Salles B, Hoffmann JS (1998) Overexpression of DNA polymerase beta in cell results in a mutator phenotype and a decreased sensitivity to anticancer drugs. *Proc Natl Acad Sci USA* **95:** 12586–12590

Cleland WW, Frey PA, Gerlt JA (1998) The low barrier hydrogen bond in enzymatic catalysis. *J Biol Chem* **273:** 25529–25532

Cohen SM, Lippard SJ (2001) Cisplatin: from DNA damage to cancer chemotherapy. *Prog Nucleic Acid Res Mol Biol* **67:** 93–130

Coll M, Sherman SE, Gibson D, Lippard SJ, Wang AH (1990) Molecular structure of the complex formed between the anticancer drug cisplatin and d(pGpG): C222(1) crystal form. *J Biomol Struct Dyn* **8:** 315–330

Davidson JP, Faber PJ, Fischer Jr RG, Mansy S, Peresie HJ, Rosenberg B, VanCamp L (1975) 'Platinum-pyrimidine blues' and related complexes: a new class of potent antitumor agents. *Cancer Chemother Rep* **59:** 287–300

Eastman A (1990) Activation of programmed cell death by anticancer agents: cisplatin as a model system. *Cancer Cells* **2:** 275–280

Emsley P, Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **60:** 2126–2132

Friedberg EC, Wagner R, Radman M (2002) Specialized DNA polymerases, cellular survival, and the genesis of mutations. *Science* **296:** 1627–1630

Garcia-Diaz M, Kunkel TA (2006) Mechanism of a genetic glissando: structural biology of indel mutations. *Trends Biochem Sci* **31:** 206–214

Gibbons GR, Kaufmann WK, Chaney SG (1991) Role of DNA replication in carrier-ligand-specific resistance to platinum compounds in L1210 cells. *Carcinogenesis* **12:** 2253–2257

Goodman MF (2002) Error-prone repair DNA polymerases in prokaryotes and eukaryotes. *Annu Rev Biochem* **71:** 17–50

Hajduch M, Ganapathy A, Stein JW, Thelen JJ (2005) A systematic proteomic study of seed filling in soybean. Establishment of high-resolution two-dimensional reference maps, expression profiles, and an interactive proteome database. *Plant Physiol* **137:** 1397–1419

Haseltine C, Montalvo-Rodriguez R, Carl A, Bini E, Blum P (1999) Extragenic pleiotropic mutations that repress glycosyl hydrolase expression in the hyperthermophilic archaeon Sulfolobus solfataricus. *Genetics* **152:** 1353–1361

Hoffmann JS, Pillaire MJ, Garcia-Estefania D, Lapalu S, Villani G (1996) *In vitro* bypass replication of the cisplatin-d(GpG) lesion by calf thymus DNA polymerase beta and human immunodeficiency virus type I reverse transcriptase is highly mutagenic. *J Biol Chem* **271:** 15386–15392

Hoffmann JS, Pillaire MJ, Maga G, Podust V, Hubscher U, Villani G (1995) DNA polymerase beta bypasses *in vitro* a single d(GpG)-cisplatin adduct placed on codon 13 of the HRAS gene. *Proc Natl Acad Sci USA* **92:** 5356–5360

Jamieson ER, Lippard SJ (1999) Structure, recognition, and processing of cisplatin-DNA adducts. *Chem Rev* **99:** 2467–2498

Kirouac KN, Ling H (2009) Structural basis of error-prone replication and stalling at a thymine base by human DNA polymerase iota. *EMBO J* **28:** 1644–1654

Kraut DA, Sigala PA, Pybus B, Liu CW, Ringe D, Petsko GA, Herschlag D (2006) Testing electrostatic complementarity in enzyme catalysis: hydrogen bonding in the ketosteroid isomerase oxyanion hole. *PLoS Biol* **4:** e99

Lehmann AR (2002) Replication of damaged DNA in mammalian cells: new solutions to an old problem. *Mutat Res* **509:** 23–34

Ling H, Boudsocq F, Plosky BS, Woodgate R, Yang W (2003) Replication of a cis-syn thymine dimer at atomic resolution. *Nature* **424:** 1083–1087

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

Ling H, Boudsocq F, Woodgate R, Yang W (2004a) Snapshots of replication through an abasic lesion; structural basis for base substitutions and frameshifts. *Mol Cell* **13:** 751–762

Ling H, Sayer JM, Plosky BS, Yagi H, Boudsocq F, Woodgate R, Jerina DM, Yang W (2004b) Crystal structure of a benzo[a]pyrene diol epoxide adduct in a ternary complex with a DNA polymerase. *Proc Natl Acad Sci USA* **101:** 2265–2269

Lippert B (1999) *Cisplatin: Chemistry and Biochemistry of a Leading Anticancer Drug*. Zürich: Helvetica Chimica

Lone S, Townson SA, Uljon SN, Johnson RE, Brahma A, Nair DT, Prakash S, Prakash L, Aggarwal AK (2007) Human DNA polymerase kappa encircles DNA: implications for mismatch extension and lesion bypass. *Mol Cell* **25:** 601–614

Mamenta EL, Poma EE, Kaufmann WK, Delmastro DA, Grady HL, Chaney SG (1994) Enhanced replicative bypass of platinum-DNA adducts in cisplatin-resistant human ovarian carcinoma cell lines. *Cancer Res* **54:** 3500–3505

McCoy AJ, Grosse-Kunstleve RW, Storoni LC, Read RJ (2005) Likelihood-enhanced fast translation functions. *Acta Crystallogr D Biol Crystallogr* **61:** 458–464

McCulloch SD, Kunkel TA (2008) The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases. *Cell Res* **18:** 148–161

Nair DT, Johnson RE, Prakash L, Prakash S, Aggarwal AK (2006) An incoming nucleotide imposes an anti to syn conformational change on the templating purine in the human DNA polymerase-iota active site. *Structure* **14:** 749–755

Ohndorf UM, Rould MA, He Q, Pabo CO, Lippard SJ (1999) Basis for recognition of cisplatin-modified DNA by high-mobility-group proteins. *Nature* **399:** 708–712

Otwinowski Z, Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* **276:** 307–326

Pinto AL, Lippard SJ (1985) Binding of the antitumor drug cis-diamminedichloroplatinum(II) (cisplatin) to DNA. *Biochim Biophys Acta* **780:** 167–180

Rabik CA, Dolan ME (2007) Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer Treat Rev* **33:** 9–23

Rechkoblit O, Malinina L, Cheng Y, Kuryavyi V, Broyde S, Geacintov NE, Patel DJ (2006) Stepwise translocation of Dpo4 polymerase during error-free bypass of an oxoG lesion. *PLoS Biol* **4:** e11

Richon VM, Schulte N, Eastman A (1987) Multiple mechanisms of resistance to cis-diamminedichloroplatinum(II) in murine leukemia L1210 cells. *Cancer Res* **47:** 2056–2061

Rockabrand D, Livers K, Austin T, Kaiser R, Jensen D, Burgess R, Blum P (1998) Roles of DnaK and RpoS in starvation-induced thermotolerance of Escherichia coli. *J Bacteriol* **180:** 846–854

Rolfsmeier M, Haseltine C, Bini E, Clark A, Blum P (1998) Molecular characterization of the alpha-glucosidase gene (malA) from the hyperthermophilic archaeon Sulfolobus solfataricus. *J Bacteriol* **180:** 1287–1295

Roush AA, Suarez M, Friedberg EC, Radman M, Siede W (1998) Deletion of the Saccharomyces cerevisiae gene RAD30 encoding an Escherichia coli DinB homolog confers UV radiation sensitivity and altered mutability. *Mol Gen Genet* **257:** 686–692

Schelert J, Dixit V, Hoang V, Simbahan J, Drozda M, Blum P (2004) Occurrence and characterization of mercury resistance in the hyperthermophilic archaeon Sulfolobus solfataricus by use of gene disruption. *J Bacteriol* **186:** 427–437

Shachar S, Ziv O, Avkin S, Adar S, Wittschieben J, Reissner T, Chaney S, Friedberg EC, Wang Z, Carell T, Geacintov N, Livneh Z (2009) Two-polymerase mechanisms dictate error-free and error-prone translesion DNA synthesis in mammals. *EMBO J* **28:** 383–393

Sherman SE, Gibson D, Wang AH, Lippard SJ (1985) X-ray structure of the major adduct of the anticancer drug cisplatin with DNA: cis-[Pt(NH3)2(d(pGpG))]. *Science* **230:** 412–417

Siddik ZH (2003) Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22:** 7265–7279

Suo Z, Lippard SJ, Johnson KA (1999) Single d(GpG)/cis-diammineplatinum(II) adduct-induced inhibition of DNA polymerization. *Biochemistry* **38:** 715–726

Takahara PM, Rosenzweig AC, Frederick CA, Lippard SJ (1995) Crystal structure of double-stranded DNA containing the major adduct of the anticancer drug cisplatin. *Nature* **377:** 649–652

Vaisman A, Chaney SG (2000) The efficiency and fidelity of translesion synthesis past cisplatin and oxaliplatin GpG adducts by human DNA polymerase beta. *J Biol Chem* **275:** 13017–13025

Vaisman A, Ling H, Woodgate R, Yang W (2005) Fidelity of Dpo4: effect of metal ions, nucleotide selection and pyrophosphorolysis. *EMBO J* **24:** 2957–2967

Vaisman A, Masutani C, Hanaoka F, Chaney SG (2000) Efficient translesion replication past oxaliplatin and cisplatin GpG adducts by human DNA polymerase eta. *Biochemistry* **39:** 4575–4580

Vaisman A, Varchenko M, Umar A, Kunkel TA, Risinger JI, Barrett JC, Hamilton TC, Chaney SG (1998) The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and oxaliplatin resistance: correlation with replicative bypass of platinum-DNA adducts. *Cancer Res* **58:** 3579–3585

Wong JH, Fiala KA, Suo Z, Ling H (2008) Snapshots of a Y-family DNA polymerase in replication: substrate-induced conformational transitions and implications for fidelity of Dpo4. *J Mol Biol* **379:** 317–330

Worthington P, Hoang V, Perez-Pomares F, Blum P (2003) Targeted disruption of the alpha-amylase gene in the hyperthermophilic archaeon Sulfolobus solfataricus. *J Bacteriol* **185:** 482–488

Wu B, Droge P, Davey CA (2008) Site selectivity of platinum anticancer therapeutics. *Nat Chem Biol* **4:** 110–112

Yang W, Woodgate R (2007) What a difference a decade makes: insights into translesion DNA synthesis. *Proc Natl Acad Sci USA* **104:** 15591–15598

Yao S, Plastaras J, Marzilli LG (1994) A molecular mechanics AMBER-type force field for modeling platinum complexes of guanine derivatives. *Inorg Chem* **33:** 6061–6077

Zorbas H, Keppler BK (2005) Cisplatin damage: are DNA repair proteins saviors or traitors to the cell? *Chembiochem* **6:** 1157–1166

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Strategies in case of positive *in vivo* results in genotoxicity testing☆

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ABSTRACT

At the 2009 International Workshop on Genotoxicity Testing in Basel, an expert group gathered to provide guidance on suitable follow-up tests to describe risk when basic *in vivo* genotoxicity tests have yielded positive results. The working group agreed that non-linear dose-response curves occur *in vivo* with at least some DNA-reactive agents. Quantitative risk assessment in such cases requires the use of (1) adequate data, *i.e.*, the use of all available data for the selection of reliable *in vivo* models to be used for quantitative risk assessment, (2) appropriate mathematical models and statistical analysis for characterizing the dose-response relationships and allowing the use of quantitative and dose-response information in the interpretation of results, (3) mode of action (MOA) information for the evaluation and analysis of risk, and (4) reliable assessments of the internal dose across species for deriving acceptable margins of exposure and risk levels. Hence, the elucidation of MOA and understanding of the mechanism underlying the dose-response curve are important components of risk assessment. The group agreed on the need for (i) the development of *in vivo* assays, especially multi-endpoint, multi-species assays, with emphasis on those applicable to humans, and (ii) consensus about the most appropriate mathematical models and statistical analyses for defining non-linear dose-responses and exposure levels associated with acceptable risk.

1. Introduction

The International Workshops on Genotoxicity Testing (IWGT), in addition to their historical focus on the refinement of genetic toxicology test protocols, have established working groups to recommend appropriate strategies for the use and interpretation of genetic toxicology tests and assessment of the risk of genotoxic exposures. Müller et al. [1] describe the objectives of this IWGT effort, identify areas of focus for the IWGT strategy working groups, and provide initial recommendations for hazard assessment. An IWGT working group has previously provided recommendations on follow-up testing in case of *in vitro* positive results in genotoxicity assays, defined criteria for developing a weight-of-evidence

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decision on marginal and non-reproducible results, and has provided a general decision tree for implementing a testing strategy [2]. This general strategy has been extended by a recent working group of the Health and Environmental Sciences Institute, a part of the International Life Sciences Institute (HESI/ILSI), with a focus on interpretation and follow-up testing of positive results *in vitro* [3].

At the August 17–19, 2009, meeting of the IWGT in Basel, Switzerland, a working group was charged with development of recommendations for appropriate follow-up actions when testing results clearly demonstrate genotoxic effects *in vivo*. The objectives of this working group were to develop consensus and to provide recommendations on the following specific topics:

(1) The use of *in vitro* and *in vivo* results in the interpretation and design of *in vivo* assays,
(2) The use of appropriate *in vivo* models for risk assessment,
(3) The evaluation and impact of mode of action (MOA) information, and
(4) Quantitative aspects of the interpretation and use of dose-response information.

This report summarizes the outcome of that working group meeting, including points on which consensus was achieved and those for which further consideration and discussion are needed.

2. Topic 1: Use of *in vitro* and *in vivo* results for risk assessment

In vivo tests are generally considered the most appropriate for quantitative risk estimations. However, they have certain limitations, which are associated both with the current lack of simple assays for relevant endpoints in certain tissues (and the consequent need to interpret surrogate information) and with the difficulty of obtaining mechanistic information in the context of often complex *in vivo* interactions. Thus it was agreed that *in vivo* assays should not be considered in isolation, but that information generated in *in vitro* assays is useful for interpreting the *in vivo* results, defining mode(s) of action, and offering guidance for any additional testing that may be necessary. *In vitro* studies can often be designed to address mechanistic questions that can aid extrapolation to humans. The working group affirmed that when *in vivo* testing is conducted as a follow-up to positive *in vitro* results, the endpoint(s) studied *in vivo* needs to be either the same as that affected *in vitro* or a surrogate demonstrated to be appropriate for predicting the affected endpoint [2] [3]. It was noted that most test systems in genetic toxicology are not specific to a single endpoint and that most DNA-damaging agents affect multiple endpoints, but many chemicals exhibit a predominance of certain types of damage (*e.g.*, point mutations *vs.* chromosomal breaks and rearrangements). Hence, the characteristics of the assays involved (both *in vitro* and *in vivo*) must be taken into account, with recognition of the spectrum of endpoints likely to be affected. Moreover, the selection of tissue(s) *in vivo* should consider pharmaco-/toxicokinetic and pharmaco-/toxicodynamic information about the test material, considering the relevant route(s) of administration. Furthermore, all pertinent toxicological information, including the identification of target organs in sub-acute and sub-chronic studies, should be considered in the design of follow-up *in vivo* genotoxicity studies.

Since absorption, distribution, metabolism, and elimination (ADME) of a compound are integral parts of *in vivo* assays, and ADME extrapolation across species is central to risk assessment, it was acknowledged that the results of the *in vivo* genotoxicity assays should generally have more weight than the *in vitro* assays in genotoxicity risk assessment. Moreover, *in vivo* test selection and design, including selection of tissues for analysis, should be based on appropriate information about anticipated human exposures and account for any known interspecies differences. As *in vivo* testing in genetic toxicology is usually conducted in rodents (mice and rats), well-known limitations exist when extrapolating results from such experiments to the human situation. This is in contrast to other areas of general toxicity testing (*e.g.*, for pharmaceuticals, food additives, and agricultural chemicals), in which extrapolation for humans is normally based on testing in both rodent and non-rodent species. Nonetheless, the working group agreed that if one or more *in vitro* tests are positive, and no measurable genotoxic effects are detected in appropriate *in vivo* endpoints in adequately exposed tissues in relevant animal species, the risk of *in vivo* genotoxic effects can be considered to be negligible. This requires that the follow-up testing *in vivo* is conducted at appropriate doses (*i.e.*, investigated tissues were exposed to sufficient levels of the test material) and that the experimental design is appropriate to show that the *in vitro* effects are not manifested *in vivo*.

The working group also agreed that when genotoxicity is identified in an animal model then an appropriate evaluation of risk in relation to anticipated human exposure should be conducted: *i.e.*, a quantitative risk assessment for the genetic effect should be conducted. The following sections briefly consider the appropriate *in vivo* models that can be used for risk assessment, and then discuss follow-up strategies that can be applied in order to characterize the genotoxic risk for humans.

3. Topic 2: Selection of appropriate *in vivo* models for risk assessment

There was limited discussion of the use of appropriate *in vivo* models for risk assessment. Selection of endpoints and species was not discussed extensively, but there was consensus that transgenic animal models in which neutral reporter genes are used to monitor mutation are acceptable surrogates for naturally occurring genes for assessment of *in vivo* mutagenic risk. The models include transgenic rodent assays with *lacI*, *lacZ* (phage and plasmid), *cII* and *gpt* delta target genes [4]. It appears that such non-transcribed transgenic constructs are useful genetic mutation markers as they are "neutral" and therefore mutations can accumulate during exposure, unlike transcribed genes that may be subject to selective pressure. Thus, the working group agreed that data from transgenic animals with recoverable neutral reporter genes were of comparable quality and predictability for carcinogenicity compared with other standard mutagenicity tests based on endogenous active genes, and that they fill an important need in current regulatory practices (*e.g.*, *in vivo* follow-up testing).

Promising assays include the new *Pig-a* assay [5–12], flow cytometric micronucleus assays [13–27], and *gpt* delta rat and mouse models [28–33]. In particular, the *Pig-a* assay, based on the loss of the glycosylphosphatidylinositol (GPI) membrane anchor of the cell membrane, shows great promise as a high throughput method which, when fully validated, should facilitate the acquisition of data necessary to define *in vivo* dose-response and kinetics relationships that are critical to risk assessment. This assay could easily be coupled with the analysis of micronuclei in peripheral blood. A major advantage of the *Pig-a* and micronucleus assays is that they are conducted using peripheral blood and are therefore relatively non-invasive and can be conducted in any species (including human) [34,35] as part of general toxicity.

The main disadvantage of the *Pig-a* and erythrocyte micronucleus assays is that at present they evaluate only damage in hematopoietic cells, and so are currently not amenable to many target tissues of mutagenesis and carcinogenesis (liver, GI-tract, lung, kidney). Therefore, other assays such as the comet assay or transgenic mutation assays (especially the *gpt* delta model, which

is able to detect both base substitutions and gene deletions) would be needed for the evaluation of *in vivo* genotoxicity in other target organs in which mutations play a significant etiological role in disease. There was consensus that there is a need to continue the development of *in vivo* assays, especially multi-endpoint, multi-species assays, with emphasis on those applicable to samples from human origin. In the spirit of the 3Rs (the Replacement, Reduction and Refinement of the use of experimental animals) in toxicology, integration of *in vivo* genotoxicity assays into 28-day repeat dose toxicity assays or short-term carcinogenicity assays may be an important future direction [4]. Integration into toxicology studies also facilitates comparison of genotoxic responses with other toxicity endpoints and with pharmacokinetic and metabolism information.

4. Topic 3: Quantitative aspects of the interpretation and use of dose-response information

As already reported in the literature [36–39], the working group considered the hypothesis that agents documented to induce genetic damage *via* interaction with non-DNA targets may exhibit a non-linear dose-response relationship with a "threshold" dose below which DNA damage is not expected to occur. For agents that act *via* such non-linear mechanisms, the No Observed Genotoxicity Effect Level (NOGEL) is generally considered an appropriate metric to which additional safety margins may be applied in determining an acceptable safe exposure limit [40]. In such cases, the risk assessment methods applied would be the same as those used for any other toxicological endpoint. For example, in the case of impurities in pharmaceutical agents, the calculation of a permissible daily exposure (PDE) starting with the no observed effect level (NOEL) (or lowest observed effect level (LOEL)) and using five different 'uncertainty factors' has been suggested [40]. The magnitude of the uncertainty factor depends on the degree of certainty for the respective extrapolations from the test systems to the human exposure situation. In the case of pharmaceuticals, an acceptable margin of exposure (MOE) also depends on the benefit of treatment to the exposed patient or population.

The main focus of the working group discussion was the use of quantitative dose-response information to assess the risk of genetic damage due to human exposure to DNA-reactive compounds. In the case of carcinogenicity, the current default assumption is that genotoxic carcinogens that interact with DNA will generally show linear non-threshold dose-responses. However, it has recently been demonstrated that some genotoxic carcinogens that interact with DNA show non-linear dose-response curves with apparent thresholds, *i.e.*, practical thresholds [41–51]. In this paper "threshold" is used to describe a dose below which the incidence of the measured genotoxic effects cannot be distinguished from the background and its associated confidence interval.

Among the non-linear dose-response examples a key case is the recent incident in which the pharmaceutical Viracept was contaminated with ethyl methane sulfonate (EMS), which led to intensive study of the genotoxicity exposure-response relationship for the well-studied genotoxic agent EMS. For this reason, this was selected by the working group as a case study for the quantitative evaluation of the dose-response curves. It was demonstrated in this case that assessment of exposure and response information could be used to define an exposure level, accepted by the European regulatory authority, below which intensive follow-up studies were not considered essential since there is no significant human safety concern. This information, reported at the meeting by Elmar Gocke and Lutz Müller, has now been published ([52,53]; all details in [51]) and is summarized briefly in Appendix A. In their analysis the authors reported that in the case of EMS:

(1) DNA adducts produced by EMS can be repaired error-free,
(2) The existence of several dose groups without any effect below the threshold and their comparison against a large cohort of vehicle controls allows the estimation of a threshold dose and its associated confidence interval,
(3) Assessment of exposure to free EMS in several species appears to be a reasonable basis for human exposure modelling,
(4) It appears that conventional cross-species exposure scaling methods (as used in other areas of toxicology), together with safety margin calculations to balance uncertainties about the exact threshold dose in other species (or other tissues or different age or disease conditions), can be used for risk management for this genotoxic carcinogen.

The direct nature of the genetic damage induced by EMS, which does not involve any major metabolic steps, makes cross-species scaling and risk assessment less complicated than in many other cases, in which metabolic activation or detoxification processes have to be taken into account.

After the Basel meeting, a cancer study using very large numbers of trout exposed to dibenzo[a,l]pyrene was published [54]. The size of this study allowed the determination of statistically significant increases of 1 cancer in 1000 animals. The sensitivity and hence statistical accuracy is more that two orders of magnitude higher than in a usual rodent cancer study with lifetime administration of the test substance.

It was shown that linear extrapolation of the dose-response in the low dose range overestimated the actual cancer risk, and appropriate modelling of the sublinear dose-response curve indicated that the virtual safe dose (VSD; 1 induced cancer in 1,000,000 individuals) was about 1000 fold higher than predicted by linear extrapolation. Notably, the dose-response of the induction of the bulky DNA adducts was close to linear, indicating that at low doses an error-free removal is apparently operative even for the bulky dibenzo[a,l]pyrene adducts. Alternatively, error-free bypass DNA synthesis across the lesion may occur, thereby suppressing the resultant mutations.

Based on the above data and other results recently reported in the literature (*e.g.*, see [41–45] [48] [50] [54–60]) the working group agreed that non-linear response curves and operational thresholds occur *in vivo* with at least some DNA-reactive agents. In other words, some agents will exhibit a "practical threshold": *i.e.*, a dose below which exposure does not add appreciably to existing background rates of DNA damage. Much more data are needed from studies with carefully determined dose-response curves to determine if generalizations across agents are possible. At present, each case requires appropriate data and careful statistical scrutiny. Possible mechanisms/modes of action underlying non-linear dose-response relationships should also be investigated. Consensus is needed on appropriate mathematical models and statistical analyses for characterizing these dose-response relationships and risk levels, and for deriving acceptable margins of exposure. While DNA primary damage can be used for exposure assessment (*i.e.*, as a biomarker of exposure), stable mutations (which are a biomarker of effect) should be given much more weight for risk assessment.

There was consensus that dose and exposure metrics must be justified for each situation of interest, that cross-species extrapolation should consider the same factors that are important for other toxicity endpoints, including relative metabolism, PK differences, surface area scaling, and internal dose, in addition to DNA repair and translesion DNA synthesis differences and relative apoptosis efficiency. Exposure metrics may include the traditional measures of plasma and tissue exposure (C_{max}/AUC).

The working group supported the approach suggested by Lutz and Lutz regarding the analysis of dose-response data for a continuous response variable with background to determine if a threshold

of response is present. This approach, recently published [61], involves definition of the background frequency and variability, followed by a statistical analysis of the data to check whether a fit by a "hockey stick" model is significantly better than a linear regression. If the hockey stick model applies, then the next step consists of a linear regression analysis for the data below the best estimate of the break point in the dose-response curve, estimating the slope of the upper limit of a confidence interval of the linear regression, and calculating the response at the threshold dose. In the context of EMS in Viracept, a 5% error level was proposed [62,63] and a 95% confidence interval was given for the estimate of the calculated threshold dose.

Conventions for unacceptable increases above the existing spontaneous levels need to be established within the scientific community, with consideration of the irreversible nature of mutation induction. The question of a theoretically-expected linear dose-related increase below the threshold dose could be addressed by linear regression of the data below the break point and estimation of an upper limit of the slope. The biological relevance of this slope can then be discussed against the normal variation of background measures in the controls [61]. Other approaches to analysis of thresholds (*e.g.*, [64]) should also be considered, and consensus is still needed about the most appropriate mathematical models and statistical analyses for defining threshold response and exposure levels associated with acceptable risk.

The working group also considered whether genotoxicity data can be used to derive acceptable MOEs, in a manner that is often applied to non-cancer risk factors (*e.g.*, [65]) and sometimes to risk from genotoxic carcinogens [66,67]. To this end, *in vivo* data can be modeled to estimate benchmark dose (BMD_x levels (*i.e.*, dose associated with a defined increase, *x*, of genotoxic damage above background) that could be compared with an estimated human exposure level, as proposed by the European Food Safety Authority (EFSA) for genotoxic carcinogens. As an example an MOE >10,000 relative to the carcinogenic BMD_{10} has been identified as a "low concern" for genotoxic carcinogens by EFSA (2005) [67]. In other words, the threshold of toxicological concern (TTC) levels can be defined for *in vivo* genotoxicants based on benchmark dose level and MOE determinations. Hence, one could determine a permitted daily exposure level with appropriate safety margins for genotoxic carcinogens [53]. For this MOE approach to be applied to *in vivo* genotoxicity data it will be necessary to define the relevant endpoint(s) to be considered and the biologically meaningful increase over background upon which the benchmark dose and safety margins would be chosen. For example, there would be a need for consensus on whether the NOGEL, a particular benchmark dose based on initial response, or other parameter was an appropriate reference exposure parameter for the genotoxic endpoint and also how that exposure metric related to the estimated cancer risk, or other endpoint of concern, for genotoxic agents. Moreover, it can be anticipated that the NOGEL will vary depending on the genetic effect induced and test method applied, and consensus on the selection of relevant endpoints and tests is needed.

The working group felt these approaches should be explored further, but was not able to define the necessary processes at the time of the meeting. The value of such approaches would be that mutagenicity dose-response curves can be determined with far greater precision than carcinogenicity dose-response curves, and so the acceptable margin to avoid genotoxic effects (which might lead to carcinogenicity or other adverse effects) could be determined with much better precision than the acceptable margin to avoid carcinogenicity.

In addition to application of quantitative dose-response information, secondary factors that may modify dose-response relationships were also considered [68–70]. Examples are cell proliferation state, modification or interspecies differences in repair and bypass DNA synthesis capacity or in levels of electrophilic "traps" such as thiols. It was recognized that these factors may be tissue specific and that such factors must be considered when applying quantitative methods to analyse dose-response information.

In summary, there was consensus that quantitative approaches to the assessment of the health risk of exposures to genotoxic agents are necessary when the potential for genotoxic damage that could lead to heritable changes is identified *in vivo*. IWGT will continue to develop recommendations for their implementation.

5. Topic 4: Evaluation and impact of mode of action (MOA) information

Elucidation of MOA of individual compounds is an important component of risk assessment. The better the information about MOA and dose-response relationships, the more certain is the interpretation of dose-response relationships and the determination of an acceptable exposure level in humans. When performing MOA analysis and extrapolating to humans, all available relevant data should be used—not only genotoxicity data.

One example presented was a drug candidate with positive *in vitro* findings that were due to species-specific metabolism that do not occur in humans (Appendix A). Results obtained with the chelating agent nitrilotriacetic acid (NTA) were presented as an example of a rodent nephrocarcinogen with an *in vivo* positive result due to an indirect mechanism of action (Appendix A). When carcinogenicity data are available, genotoxicity should be examined in the target organs for chemical carcinogenesis, using the same species and strains, when possible. Mechanisms underlying the shape of the dose-response curve should be investigated as thoroughly as is feasible both *in vitro* and *in vivo*.

Many chemicals are both mutagens and carcinogens. When conducting an MOA assessment for the induction of the tumors, it is important to consider whether the chemical is actually a mutagenic carcinogen. It should be noted that mutagens should not automatically be assumed to be mutagenic carcinogens. This determination depends on a comprehensive evaluation using a MOA framework and the assessment of key events [71,72].

A strategy for using *in vivo* mutation data to inform cancer MOA was presented. The strategy uses transgenic rodents to evaluate whether a carcinogen can induce mutation in the tumor target tissue. A modified Hill Criteria analysis [73,74] is used to determine whether the induced mutation response is consistent with a mutagenic MOA. This requires an assessment of temporality and dose-response concordance between the mutation dose-response and the tumor dose-response. A case study using riddelliine and dichloroacetic acid (DCA) was presented. Both of these chemicals are mutagens and liver carcinogens. Riddelliine induces mutations in the liver after only a few weeks exposure, while DCA induces mutations in the liver after 60 weeks exposure. A benchmark dose analysis of the mutation and cancer data dose-response curves indicates dose-response concordance for riddelliine but not for DCA. Taken together the temporality analysis and the dose-response concordance analysis for these two chemicals indicate that riddelliine is likely a mutagenic carcinogen, but DCA likely has a different mode of action. The details of this approach are published [75].

It was suggested that future *in vivo* mutation studies to inform MOA should be designed based on the cancer study. Species, dose route, and dose levels should be selected based on the cancer study and should include enough doses, particularly at the lower end of the dose-response curve, to provide an adequate assessment of the dose-response. The design should include chronic exposure and interim sacrifices to provide a dose-response curve at multiple time points. The timing of the interim sacrifices should be based on any

known preneoplastic lesions that occur prior to tumor development. It is possible that the treatment may need to be extended to up to a year, as was the case in the DCA example. Experiments can be designed to evaluate possible MOAs in addition to the induction of mutation.

The extent to which *in vivo* mutagenicity can be associated with adverse effects other than cancer, and the importance of risk assessment of genotoxicity, *per se*, was discussed. In addition to germline mutations that result in well-recognized human diseases, a number of human diseases are caused by *de novo* somatic mutations [76]. More recently, Borlak and co-workers have shown that both somatic and germline mutations result in cardiac septation defects [77–79]. Accordingly, it was affirmed that cancer is not the only adverse health outcome associated with genetic damage, and, therefore *in vivo* genotoxicity should be considered an adverse effect whatever the evidence of carcinogenicity. Data were also presented suggesting that negative carcinogenicity data may not always provide assurance of the lack of genotoxicity *in vivo* in other species or with different exposures. The Maillard reaction product 4-hydroxy-2,5-dimethylfuran-3(2H)-one, negative in a rat carcinogenicity and positive in mutagenicity studies *in vivo* in mouse somatic and germ cells, was discussed as an example [80].

These examples illustrate the need for expert evaluation of all available data to determine the appropriate follow-up investigation that may be necessary for *in vivo* and/or *in vitro* positive genotoxicity data, even when negative carcinogenicity data are available. It was agreed that further review and discussion is warranted before any specific recommendations can be provided on this topic.

6. Conclusions

In conclusion, appropriate models for risk assessment of *in vivo* genotoxicants have been discussed in an IWGT group, and the working group agreed on the following points:

(1) When *in vivo* testing is conducted as a follow-up to positive *in vitro* results, an appropriate experimental design should be used to determine if the *in vitro* effects are manifested *in vivo*, *i.e.*, in adequately exposed tissues in relevant animal species using the same endpoint as that affected *in vitro* or a surrogate demonstrated to be appropriate for predicting the affected endpoint.

(2) Transgenic animal models with recoverable neutral reporter genes are useful for assessing mutagenic activity in different tissues, and are of comparable quality and predictivity for assessment of *in vivo* mutagenic risk as compared to endogenous genes. They therefore fill an important need in current regulatory practice (*e.g.*, *in vivo* follow-up testing). The *Pig*-a assay, flow cytometric micronucleus assays, and *gpt* delta rat and mouse models are promising assays; *Pig-a* and micronucleus assays because they are conducted using peripheral blood and can be conducted in any species, and *gpt* delta model because it is able to detect both point mutations and gene deletions. The comet assay and transgenic mutation assays remain the principal assays allowing the evaluation of *in vivo* genotoxicity in any target organ. There is a need to continue the development of *in vivo* assays, especially multi-endpoint, multi-species assays, with emphasis on those applicable to samples from human origin. Integration of *in vivo* genotoxicity assays into general toxicity assays, such as 28-day repeat dose toxicity assays, is worth considering in the light of its advantages in efficiency, provision of comparative toxicology, pharmacokinetic and metabolic information, and the spirit of the "3Rs" in regulatory toxicology.

(3) Non-linear response curves may occur *in vivo* with non-DNA-reactive and at least some DNA-reactive agents. More data are needed to determine if generalizations across types of agents are possible. Each case requires appropriate data, justified doses and exposure metrics, and careful statistical scrutiny. Consensus is needed on appropriate mathematical models and statistical analyses for characterizing these dose-response relationships and risk levels, and for deriving acceptable margins of exposure. Among the possible approaches are (a) the use of mathematical models and statistical analyses (*e.g.*, "hockey stick" model) to define the background frequency and its variability, and to analyse the dose-response curves, (b) the estimation of the break point in the dose-response curve, *e.g.*, NOGEL with additional safety margins, or BMD to which may be applied an acceptable safe margin of exposure (MOE). In some instances, human exposure levels thought to pose negligible safety concerns can be defined for *in vivo* genotoxicants.

(4) Genotoxic effects *in vivo* generally have more weight than *in vitro* effects in genotoxicity risk assessment, and the absence of measurable *in vivo* effects in target tissues with adequate exposure and metabolic activity indicates that the risk of *in vivo* genotoxic effects can be considered to be negligible in relation to the anticipated human exposure. While DNA primary damage can be used for exposure assessment, stable mutations, *i.e.*, biomarkers of effect, should be given much more weight for risk assessment.

(5) All pertinent toxicological information should be considered in the design of follow-up *in vivo* genotoxicity studies. Elucidation of MOA of individual compounds is an important component of risk assessment, *i.e.*, mechanisms underlying the shape of the dose-response, MOA framework and the assessment of key events, temporality and dose-response concordance between the mutation dose-response, and the tumor dose-response when carcinogenicity data are available.

Conflict of interest

None.

Appendix A. Case examples presented and discussed

Roche Viracept® case

Roche's protease inhibitor nelfinavir mesylate (Viracept®) produced between March 2007 and June 2007 was found to contain elevated levels of EMS, a known mutagen (alkylating agent), leading to a global recall of the drug. EMS levels present in the contaminated drug were predicted not to exceed a dose of ~2.75 mg/day (~0.055 mg/kg/day for a 50 kg patient) based on the daily dose of 2500 mg Viracept/day. As existing toxicology data on EMS did not permit an adequate patient risk assessment, a comprehensive animal toxicology evaluation of EMS was conducted. The general toxicity of EMS was investigated in rats exposed for 28 days. Two studies that assessed DNA damage were performed in mice: chromosomal damage was assessed using a micronucleus assay and gene mutations were detected using the Muta™Mouse transgenic model. In addition, experiments designed to extrapolate animal exposure to humans were undertaken. A general toxicity study showed that the toxicity of EMS occurred only at doses ≥60 mg/kg/day, which is far above the doses received by patients. Studies for chromosomal damage and *lacZ* mutants in mice (in bone marrow and gastrointestinal tract) demonstrated a clear threshold effect with EMS, with no measurable effect at and below 25 mg/kg/day, under 4-week continuous dosing conditions. In the same experiment, a threshold in liver was determined to exist

Table 1
Threshold analysis (hockey-stick model).

Study	Organ	No observed effect level (mg/kg)	Threshold dose (mg/kg)	95% Confidence interval of threshold dose (mg/kg)
Micronucleus test	Bone marrow	80	89.81	56.67–118.25
Muta™mouse	Bone marrow	25	35.45	21.46–45.73
Muta™mouse	Liver	50	51.31	25.67–99.10
Muta™mouse	GI-tract	25	24.51	12.97–38.51

Table 2
Slope analysis for the low dose range.

Study	Organ	Slope at low dose region	95% Confidence interval of slope
Micronucleus test	Bone marrow	−0.10	−0.20 to −0.001
Muta™mouse	Bone marrow	−0.19	−1.19 to 0.81
Muta™mouse	Liver	−0.10	−0.69 to 0.48
Muta™mouse	GI-tract	0.48	−0.96 to 1.92

(The slope is given as number of micronucleated polychromatic erythrocytes out of 4000PCE/mg/kg, and mutant frequency per million cells/mg/kg, for *in vivo* micronucleus test and gene mutation assay in Muta™Mouse, respectively).

at 50 mg/kg/day. A detailed statistical analysis using the approach developed by [61] estimated the 95% statistical confidence intervals for the threshold dose and the slopes below the threshold for the investigated endpoints and organs [62,63]. The confidence in this analysis reflecting a threshold is strengthened by the fact that four dose levels for each organ measured yielded no discernable mutation difference from the control and that three independent control groups were used for the experiment (Tables 1 and 2).

Exposure analysis (C_{max}) in mice, rats and monkeys demonstrated that ~370-fold higher levels of EMS than that ingested by patients are needed to saturate known, highly conserved, error-free, mammalian DNA repair mechanisms for alkylation. Yet, as the half-life of EMS was higher in rats than in mice, and higher in non-human primates than in rats, the calculations of its AUC (area under the exposure-time curve) at the threshold dose of 25 mg/kg/day yielded an AUC-based safety factor of ~28 (*vs.* the C_{max}-based factor of ~370) [81]. Because all mutagenic DNA alkylations seem to be repairable at daily doses up to 25 mg/kg EMS, it can be argued that the C_{max} (which is largely half-life independent) is the main factor for risk assessment in this "EMS in Viracept" case.

In summary, the animal studies suggested that patients who took nelfinavir mesylate (Viracept) with elevated levels of EMS are not at increased risk for carcinogenicity, mutagenicity, or teratogenicity, since mutations are prerequisites for these events. As exposure biomarkers such as adducts on globin or DNA, do generally follow linear dose-response relationships in the case of EMS, these data clearly show that such biomarkers cannot be used for risk assessment or risk management processes in this case but that risk assessment should be based on "fixed" mutational events. Although non-linear behaviour of mutations *in vivo* has been demonstrated previously, these data give the first reliable experimental basis for comprehensive risk management in a low dose exposure scenario.

In vitro effects due to species-specific metabolism

An example of irrelevant *in vivo* positive findings due to an interspecies difference in metabolic capacity was presented. A drug candidate was positive in a comet assay performed on rat stomach and negative in rat liver. This compound is known to be hydroxylated in the stomach and then glucuroconjugated by UDP-glucuronosyltransferase (UDPGT) 1A8. This UDPGT 1A8 isoform is not expressed in the rat gastrointestinal tract [82] while it is highly expressed in human gastric mucosa [83]. *In vitro* assays on isolated mucosa were performed and demonstrated that (1) the glucuroconjugated metabolite was observed in human gastric mucosa but not in the rat, (2) the hydroxylated metabolite was present in gastric rat mucosa but not in the human, and (3) the hydroxylated metabolite was a direct genotoxic compound in the Ames assay and in the *in vitro* micronucleus assay. It was concluded that the parent compound is positive in the comet assay in rat gastric mucosa but most probably not in human, and that there was no genotoxic concern in human associated with this compound.

Rodent nephrocarcinogen with an indirect mechanism of action

Nitrilotriacetic acid (NTA) induced marked increases in DNA damage after a single oral treatment at high doses in the *in vivo* rodent comet assay on kidney cells at both short (3–6 h) and long-term (22–26 h) sampling times. NTA demonstrated no mutagenic activity in the Ames test but was positive in the *in vitro* micronucleus assay on L5178Y mouse lymphoma cells without and with metabolic activation by aroclor 1254-induced liver or kidney rat S9-mix. An assay on CTLL2/Bcl2 cells coupled to the apoptosis measurement with and without metabolic activation demonstrated a positive response and confirmed the absence of interference of apoptosis. The direct mutagenic activity of NTA was confirmed in the mouse lymphoma *tk*+/− gene mutation assay and in the chromosomal aberrations test on human lymphocytes. However, tested in combination with an excess of Ca^{2+}, NTA gave negative results on L5178Y mouse lymphoma cells, in the *in vitro* comet and in the micronucleus assays, while Ca^{2+} only partly abolished the formation of DNA strand breaks on rat primary kidney cells. The higher sensitivity of renal cells to Ca^{2+} variations could explain the positive response observed *in vivo*. The carcinogenicity of NTA could be a consequence of the intracellular variations of Ca^{2+}, leading to a local and indirect genotoxic mechanism. This suggests that in the case of NTA, a threshold dose may exist beyond which kidney tumor-generating events will be displayed [84].

References

[1] L. Müller, D. Blakey, K.L. Dearfield, S. Galloway, P. Guzzie, M. Hayashi, P. Kasper, D. Kirkland, J.T. MacGregor, J.M. Parry, L. Schechtman, A. Smith, N. Tanaka, D. Tweats, H. Yamasaki, Strategy for genotoxicity testing and stratification of genotoxicity test results—report on initial activities of the IWGT expert group, Mutat. Res. 540 (2003) 177–181.
[2] V. Thybaud, M. Aardema, J. Clements, K. Dearfield, S. Galloway, M. Hayashi, D. Jacobson-Kram, D. Kirkland, J.T. MacGregor, D. Marzin, W. Ohyama, M. Schuler, H. Suzuki, E. Zeiger, Strategy for genotoxicity testing: hazard identification and risk assessment in relation to *in vitro* testing, Mutat. Res. 627 (2007) 41–58.
[3] K.L. Dearfield, V. Thybaud, M.C. Cimino, L. Custer, A. Czich, J. Harvey, S. Hester, J. Kim, D. Kirkland, D. Levy, E. Lorge, M.M. Moore, G. Ouédraogo-Arras, M. Schuler, W. Suter, K. Sweder, K. Tarlo, J. Van Benthem, F. van Goethem, K. Witt, Follow-up actions from positive results of *in vitro* genetic toxicity testing, Environ. Mol. Mutagen. in press.
[4] V. Thybaud, S. Dean, T. Nohmi, J. de Boer, G. Douglas, B. Glickman, N. Gorelick, J. Heddle, R. Heflich, I. Lambert, H. Martus, J. Mirsalis, T. Suzuki, N. Yajima, *In vivo* transgenic mutation assays, Mutat. Res. 540 (2003) 141–151.

[5] S.M. Bryce, J.C. Bemis, S.D. Dertinger, *In vivo* mutation assay based on the endogenous *Pig-a* locus, Environ. Mol. Mutagen. 49 (2008) 256–264.
[6] D. Miura, V.N. Dobrovolsky, Y. Kasahara, Y. Katsuura, R.H. Heflich, Development of an *in vivo* gene mutation assay using the endogenous Pig-A gene: I. Flow cytometric detection of CD59-negative peripheral red blood cells and CD48-negative spleen T-cells from the rat, Environ. Mol. Mutagen. 49 (2008) 614–621.
[7] D. Miura, V.N. Dobrovolsky, R.A. Mittelstaedt, Y. Kasahara, Y. Katsuura, R.H. Heflich, Development of an *in vivo* gene mutation assay using the endogenous Pig-A gene: II. Selection of Pig-A mutant rat spleen T-cells with proaerolysin and sequencing Pig-A cDNA from the mutants, Environ. Mol. Mutagen. 49 (2008) 622–630.
[8] D. Miura, V.N. Dobrovolsky, T. Kimoto, Y. Kasahara, R.H. Heflich, Accumulation and persistence of Pig-A mutant peripheral red blood cells following treatment of rats with single and split doses of N-ethyl-N-nitrosourea, Mutat. Res. 677 (2009) 86–92.
[9] S. Phonethepswath, D. Franklin, D.K. Torous, S.M. Bryce, J.C. Bemis, S. Raja, S. Avlasevich, P. Weller, O. Hyrien, J. Palis, J.T. MacGregor, S.D. Dertinger, Pig-a mutation: Kinetics in rat erythrocytes following exposure to five prototypical mutagens, Toxicol. Sci. 114 (2010) 5–70.
[10] V.N. Dobrovolsky, S.Y. Boctor, N.C. Twaddle, D.R. Doerge, M.E. Bishop, M.G. Manjanatha, T. Kimoto, D. Miura, R.H. Heflich, S.A. Ferguson, Flow cytometric detection of Pig-A mutant red blood cells using an erythroid-specific antibody: Application of the method for evaluating the *in vivo* genotoxicity of methylphenidate in adolescent rats, Environ. Mol. Mutagen. 51 (2010) 138–145.
[11] V.N. Dobrovolsky, S.A. Ferguson, D.R. Doerge, S.M. Morris, R.H. Heflich, Pig-A gene mutation in mammalian red blood cells, Abstr. Annual Meeting, Society ofToxicology, The Toxicologist. (2010b) 689, Salt Lake City, UT March 9, 2010.
[12] S.D. Dertinger, S. Phonethepswath, D. Franklin, P. Weller, D.T. Torous, S.M. Bryce, S. Avlasevich, J.C. Bemis, O. Hyrien, J. Palis, J.T. MacGregor, Integration of mutation and chromosomal damage endpoint into 28-day repeat dose toxicology studies Toxicol Sci. 115 (2010) 401–411.
[13] J.T. MacGregor, C.M. Wehr, R.A. Hiatt, B. Peters, J.D. Tucker, R.G. Langlois, R.A. Jacob, R.H. Jenson, J.W. Yager, M.K. Shigenaga, B. Frei, B.P. Eynon, B.N. Ames, Spontaneous genetic damage in man: evaluation of interindividial variability, relationship among markers of damage, and influence of nutritional status, Mutat. Res. 377 (1997) 125–135.
[14] J.T. MacGregor, M.E. Bishop, J.P. McNamee, M. Hayashi, N. Asano, A. Wakata, M. Nakajima, J. Saito, A. Aidoo, M.M. Moore, S.D. Dertinger, Flow cytometric analysis of micronuclei in peripheral blood reticulocytes: II. An efficient method of monitoring chromosomal damage in the rat, Toxicol. Sci. 94 (2006) 92–107.
[15] L. Abramsson-Zetterberg, G. Zetterberg, M. Bergqvist, J. Grawé, Human cytogenetic biomonitoring using flow-cytometric analysis of micronuclei in transferring-positive immature peripheral blood reticulocytes, Environ. Mol. Mutagen. 36 (2000) 22–31.
[16] S. Hamada, S. Sutou, T. Morita, A. Wakata, S. Asanami, S. Hosoya, S. Ozawa, K. Kondo, M. Nakajima, H. Shimada, K. Osawa, Y. Kondo, N. Asano, S. Sato, H. Tamura, N. Yajima, R. Marshall, C. Moore, D. Blakey, L. Schechtman, J. Weaver, D. Torous, R. Proudlock, S. Ito, C. Namiki, M. Hayashi, Evaluation of the rodent micronucleus assay by a 28-day treatment protocol: Summary of the 13th collaborative study by the collaborative study group for the micronucleus test (CSGMT)/Environmental Mutagen Society of Japan (JEMS)–Mammalian Mutagenicity Study Group (MMS), Environ. Mol. Mutagen. 37 (2001) 93–110.
[17] S.D. Dertinger, D.K. Torous, N.E. Hall, F.G. Murante, S.E. Gleason, R.K. Miller, R. Carol, Tometsko, Enumeration of micronucleated CD71-positive human reticulocytes with a single-laser flow cytometer, Mutat. Res. 515 (2002) 3–14.
[18] S.D. Dertinger, Y. Chen, R.K. Miller, K.J. Brewer, T. Smudzin, D.K. Torous, N.E. Hall, K.A. Olvany, F.G. Murante, C.R. Tometsko, Micronucleated CD71-positive reticulocytes: a blood-based endpoint of cytogenetic damage in humans, Mutat. Res. 542 (2003) 77–87.
[19] S.D. Dertinger, R.K. Miller, K. Brewer, T. Smudzin, D.K. Torous, D.J. Roberts, S.L. Avlasevich, S.M. Bryce, S. Sugunan, Y. Chen, Automated human blood micronucleated reticulocyte measurements for rapid assessment of chromosomal damage, Mutat. Res. 626 (2007) 111–119.
[20] J. Grawé, J. Biko, R. Lorenz, C. Reiners, H. Stopper, S. Vershenya, V. Vukicevic, K. Hempel, Evaluation of the reticulocyte micronucleus assay in patients treated with radioiodine for thyroid cancer, Mutat. Res. 583 (2005) 12–25.
[21] T. Offer, E. Ho, M.G. Traber, R.S. Bruno, F.A. Kuypers, B.N. Ames, A simple assay for frequency of chromosome breaks and loss (micronuclei) by flow cytometry of human reticulocytes, FASEB J. 19 (2005) 485–487.
[22] V.L. Harrod, T.A. Howard, S.A. Zimmerman, S.D. Dertinger, R.E. Ware, Quantitative analysis of Howell-Jolly bodies in children with sickle cell disease, Exp. Hematol. 35 (2007) 179–183.
[23] S.B. Harper, S.D. Dertinger, M.E. Bishop, A.M. Lynch, M. Lorenzo, M. Saylor, J.T. MacGregor, Flow cytometric analysis of micronuclei in peripheral blood reticulocytes III. An efficient method of monitoring chromosomal damage in the beagle dog, Toxicol. Sci. 100 (2007) 406–414.
[24] K.L. Witt, C.K. Cunningham, K.B. Patterson, G.E. Kissling, S.D. Dertinger, E. Livingston, J.B. Bishop, Elevated frequencies of micronucleated erythrocytes in infants exposed to zidovudine in utero and postpartum to prevent mother-to-child transmission of HIV, Environ. Mol. Mutagen. 48 (2007) 322–329.
[25] D. Torous, M. McKeon, G. Schmuck, Y. Xu, S. Burgess, S. Avlasevich, S. Dertinger, D. Kirkland, Cyclophosphamide and etoposide canine studies demonstrate the cross-species potential of the peripheral blood micronucleated reticulocyte endpoint, Environ. Mol. Mutagen. 49 (2008) 569, Meeting abstract.
[26] C.E. Hotchkiss, M.E. Bishop, S.D. Dertinger, W. Slikker Jr., M.M. Moore, J.T. MacGregor, Flow cytometric analysis of micronuclei in peripheral blood reticulocytes IV: an index of chromosomal damage in the Rhesus monkey (*Macaca mulatta*), Toxicol. Sci. 102 (2008) 352–358.
[27] S.M. Morris, V.N. Dobrovolsky, J.G. Shaddock, R.A. Mittelstaedt, M.E. Bishop, M.G. Manjanatha, S.D. Shelton, D.R. Doerge, N.C. Twaddle, J.J. Chen, C.-J. Lin, M.G. Paule, W. Slikker Jr., C.E. Hotchkiss, D. Petibone, J.D. Tucker, D.R. Mattison, The genetic toxicology of methylphenidate hydrochloride in non-human primates, Mutat. Res. 673 (2009) 59–66.
[28] T. Nohmi, M. Katoh, H. Suzuki, M. Matsui, M. Yamada, M. Watanabe, M. Suzuki, N. Horiya, O. Ueda, T. Shibuya, H. Ikeda, T. Sofuni, A new transgenic mouse mutagenesis test system using Spi- and 6-thioguanine selections, Environ. Mol. Mutagen. 28 (1996) 465–470.
[29] T. Nohmi, T. Suzuki, K. Masumura, Recent advances in the protocols of transgenic mouse mutation assays, Mutat. Res. 455 (2000) 191–215.
[30] H. Hayashi, H. Kondo, M. Masumura, Y. Shindo, T. Nohmi, A novel transgenic rat for *in vivo* genotoxicity assays using 6-thioguanine and Spi- selection, Environ. Mol. Mutagen. 41 (2003) 253–259.
[31] T. Nohmi, K. Masumura, Molecular nature of intrachromosomal deletions and base substitutions induced by environmental mutagens, Environ. Mol. Mutagen. 45 (2005) 150–161.
[32] K. Masumura, T. Nohmi, Spontaneous mutagenesis in rodents: spontaneous gene mutations identified by neutral reporter genes in gpt delta transgenic mice and rats, J. Health Sci. 55 (2009) 40–49.
[33] N. Toyoda-Hokaiwado, T. Inoue, K. Masumura, H. Hayashi, Y. Kawamura, Y. Kurata, M. Takamune, M. Yamada, H. Sanada, T. Umemura, A. Nishikawa, T. Nohmi, Integration of *in vivo* genotoxicity and short-term carcinogenicity assays using F344 *gpt* delta transgenic rats: *in vivo* mutagenicity of 2,4-diaminotoluene and 2,6-diaminotoluene structural isomers, Toxicol. Sci. 114 (2010) 71–78.
[34] B. Peruzzi, D.J. Araten, R. Notaro, L. Luzzatto, The use of PIG-A as a sentinel gene for the study of the somatic mutation rate and of mutagenic agents *in vivo*, Mutat Res. 705 (2010) 3–10.
[35] S. Phonethepswath, S.M. Bryce, J.C. Bemis, S.D. Dertinger, Erythrocyte-based *Pig-a* gene mutation assay: demonstration of cross-species potential, Mutat. Res. 657 (2008) 122–126.
[36] L. Müller, P. Kasper, Human biological relevance and the use of threshold-arguments in regulatory genotoxicity assessment: experience with pharmaceuticals, Mutat. Res. 464 (2000) 19–34.
[37] M. Kirsch-Volders, A. Vanhauwaert, U. Eichenlaub-Ritter, I. Decordier, Indirect mechanisms of genotoxicity, Toxicol. Lett. 140–141 (2003) 63–74.
[38] I.S. Pratt, T. Barron, Regulatory recognition of indirect genotoxicity mechanisms in the European Union, Toxicol Lett. 140–141 (2003) 53–62.
[39] Center for Drug Evaluation and Research (CDER) Guidance (2006): "Recommended Approaches to Integration of Genetic Toxicology Study Results", U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER). Available at www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079257.pdf.
[40] EMEA (European Medicines Agency) 2006 Guideline on the limits of genotoxic impurities, EMEA/CHMP/QWP/CHMP guideline on Genotoxic Impurities, EMEA/CHMP/QWP/251344/2006. Available at www.ema.europa.eu/pdfs/human/swp/519902en.pdf.
[41] M.J. Nivard, K. Czene, D. Segerbäck, E.W. Vogel, Mutagenic activity of ethylene oxide and propylene oxide under XPG proficient and deficient conditions in relation to N-7-(2-hydroxyalkyl)guanine levels in Drosophila, Mutat. Res. 529 (2003) 95–107.
[42] T. Sofuni, T. Nohmi, T. Ohta, M. Hayashi, Genotoxicity: is a threshold concept applicable to evaluate the mutagenic activity of DNA-targeting substances? Environ. Mutagen. Res. 27 (2005) 61–73.
[43] N. Asano, D.K. Torous, C.R. Tometsko, S.D. Dertinger, T. Morita, M. Hayashi, Practical threshold for micronucleated reticulocyte induction observed for low doses of mitomycin C, Ara-C and colchicine, Mutagenesis 21 (2006) 15–20.
[44] S.H. Doak, G.J. Jenkins, G.E. Johnson, E. Quick, E.M. Parry, J.M. Parry, Mechanistic influences for mutation induction curves after exposure to DNA-reactive carcinogens, Cancer Res. 67 (2007), 3904-3011.
[45] M. Nagao, S. Ishikawa, H. Nakagama, M. Watanabe, Extrapolation of the animal carcinogenesis threshold to humans, Genes Environ. 30 (2008) 160–165.
[46] D.P. Lovell, Experimental design and statistical analysis of studies to demonstrate a threshold in genetic toxicology: a mini-review, Genes Environ. 30 (2008) 139–149.
[47] J.A. Swenberg, E. Fryar-Tita, Y.C. Jeong, G. Boysen, T. Starr, V.E. Walker, R.J. Albertini, Biomarkers in toxicology and risk assessment: informing critical dose-response relationships, Chem. Res. Toxicol. 21 (2008) 253–265.
[48] P. Carmichael, M. Kirsch-Volders, H. Vrijhof, Thresholds for genotoxins and their application in risk assessment, Mutat. Res. 678 (2009) 71 (Preface to special issue on thresholds).
[49] D.A. Marsden, D.J.L. Jones, R.G. Britton, T. Ognibene, E. Ubick, G. Johnson, P.B. Farmer, K. Brown, Dose response relationships for N7-(2-hydroxyethyl)guanine induced by low dose [14C]-ethylene oxide: evidence for a novel mechanism of endogenous adduct formation, Cancer Res. 69 (2009) 3052–3059.
[50] E. Zeiger, L. Recio, T.R. Fennell, J.K. Haseman, R.W. Snyder, M. Friedman, Investigation of the low-dose response in the *in vivo* induction of micronuclei and adducts by acrylamide, Toxicol. Sci. 107 (2009) 247–257.

[51] Special Issue: Assessment of human toxicological risk of Viracept patients accidentally exposed to ethyl methanesulfonate (EMS) based on preclinical investigations with EMS and ethylnitrosourea, Toxicol. Lett. 190 (2009) 239–340.
[52] L. Müller, E. Gocke, T. Lave, T. Pfister, Ethyl methanesulfonate toxicity in Viracept–a comprehensive human risk assessment based on threshold data for genotoxicity, Toxicol. Lett. 190 (2009) 317–329.
[53] L. Müller, E. Gocke, Considerations regarding a permitted daily exposure calculation for ethyl methanesulafonate, Toxicol. Lett. 190 (2009) 330–332.
[54] G.S. Bailey, A.P. Reddy, C.B. Pereira, U. Harttig, W. Baird, J.M. Spitsbergen, J.D. Hendricks, G.A. Orner, D.E. Williams, J.A. Swenberg, Nonlinear cancer response at ultralow dose: a 40800-animal ED(001) tumor and biomarker study, Chem. Res. Toxicol. 22 (2009) 1264–1276.
[55] H.M. Bolt, The concept of "practical thresholds" in the derivation of occupational exposure limits for carcinogens by the scientific committee on occupational exposure limits (SCOEL) of the European Union, Genes Environ. 30 (2008) 114–119.
[56] S.M. Cohen, Thresholds in genotoxicity and carcinogenicity: urinary bladder carcinogenesis, Genes Environ. 30 (2008) 132–138.
[57] K.T. Kitchen, Theoretical and experimental approaches to address possible thresholds of response in carcinogenicity, Genes Environ. 30 (2008) 150–159.
[58] T. Nohmi, N. Toyoda-Hokaiwado, M. Yamada, K. Masumura, M. Honma, S. Fukushima, International symposium on genotoxic and carcinogenic thresholds, Genes Environ. 30 (2008) 101–107.
[59] T. Nohmi, Possible mechanisms of practical thresholds for genotoxicity, Genes Environ. 30 (2008) 108–113.
[60] S. Fukushima, A. Kakehashi, H. Wei Min, Wanibuchi, Existence of a threshold for the genotoxic carcinogens: evidence from mechanism-based carcinogenicity studies, Genes Environ. 31 (2009) 33–36.
[61] W.K. Lutz, R.W. Lutz, Statistical model to estimate a threshold dose and its confidence limits for the analysis of sublinear dose–response relationships, exemplified for mutagenicity data, Mutat. Res. 678 (2009) 118–122.
[62] E. Gocke, M. Wall, *In vivo* genotoxicity of EMS: statistical assessment of the dose-response curves, Toxicol. Lett. 190 (2009) 298–302.
[63] E. Gocke, L. Müller, *In vivo* studies in the mouse to define a threshold for the genotoxicty of EMS and ENU, Mutat. Res. 678 (2009) 101–107.
[64] L.H. Pottenger, M.R. Schisler, F. Zhang, M.J. Bartels, D.D. Fontaine, L.G. McFadden, B. Gollapudi, Dose-response and operational thresholds/NOAELs for *in vitro* mutagenic effects from DNA-reactive mutagens, MMS and MNU, Mutat. Res. 678 (2009) 138–147.
[65] L.L. Aylward, J.E. Goodman, G. Charnley, L.R. Rhomberg, A margin-of-exposure approach to assessment of noncancer risks of dioxins based on human exposure and response data, Environ. Health Perspect. 116 (2008) 1344–1351.
[66] J. O'Brien, A.G. Renwick, A. Constable, E. Dybing, D.J.G. Müller, J. Schlatter, W. Slob, W. Tueting, J. Van Benthem, G.M. Williams, A. Wolfreys, Approaches to the risk assessment of genotoxic carcinogens in food: a critical appraisal, Food Chem. Toxicol. 44 (2006) 1613–1635.
[67] European Food Safety Authority (EFSA) Opinion of the Scientific Committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. The EFSA J. 282 (2005) 1–30. Available at http://www.efsa.eu.int/science/sc_committee/sc_opinions/1201_en.html.
[68] D. Renault, F. Tombolan, D. Brault, F. Périn, V. Thybaud, Comparative mutagenicity of 7H-dibenzo[c,g] carbazole and two derivatives in Muta™Mouse liver and skin, Mutat. Res. 417 (1998) 129–140.
[69] F. Tombolan, D. Renault, D. Brault, M. Guffroy, O. Perin-Roussel, F. Périn, V. Thybaud, Kinetics of induction of DNA adducts cell proliferation and gene mutations in the liver of Muta™Mice treated with 5,9-dimethyldibenzocarbazole, Carcinogenesis 20 (1999) 125–132.
[70] F. Tombolan, D. Renault, D. Brault, M. Guffroy, O. Perin-Roussel, F. Périn, V. Thybaud, Effect of mitogenic or regenerative cell proliferation on *lacZ* mutant frequency in the liver of Muta™Mice treated with 5,9-dimethyldibenzocarbazole, Carcinogenesis 20 (1999) 1357–1362.
[71] M.E. Meek, J.R. Bucher, S.M. Cohen, V. Dellarco, R.N. Hill, L.D. Lehman-McKeeman, D.G. Longfellow, T. Pastoor, J. Seed, D.E. Patton, A framework for human relevance analysis of information on carcinogenic modes of action, Crit. Rev. Toxicol. 33 (2003) 591–653.
[72] M.E. Meek, Recent developments in frameworks to consider human relevance of hypothesized modes of action for tumours in animals, Environ. Mol. Mutagen. 49 (2008) 110–116.
[73] A.B. Hill, The environment and disease: association or causation? Proc. R. Soc. Med. 58 (1965) 295–300.
[74] U.S. Environmetal Protection Agency (EPA). Guidelines for Carcinogen Risk Assessment. 2005. Available at http://www.epa.gov/iris/cancer032505.pdf.
[75] M.M. Moore, R.H. Heflich, L. Haber, B.C. Allen, A.M. Shipp, R.L. Kodell, Analysis of *in vivo* mutation data can inform cancer risk assessment, Reg. Toxicol. Pharm. 51 (2008) 151–161.
[76] R.P. Erikson, Somatic gene mutations and human disease other than cancer, Mutat. Res. 543 (2005) 125–136.
[77] S.M. Reamon-Buettner, J. Borlak, GATA zinc finger mutations as a rationale for septation defects of the human heart, J. Mol. Genet. 41 (2004) 684–690.
[78] S.M. Reamon-Buettner, K. Spanel-Borowski, J. Borlak, Bridging the gap between anatomy and molecular genetics for an improved understanding of congenital heart disease, Ann. Anat. 188 (2006) 213–220.
[79] S.M. Reamon-Buettner, Y. Ciribilli, I. Traverso, B. Kuhls, A. Inga, J. Borlak, A functional genetic study identifies HAND1 mutations in septation defects of the human heart, Hum. Mol. Genet. 18 (2009) 3567–3578.
[80] European Food Safety Authority (EFSA) Flavouring Group Evaluation [220:] alpha,beta-Unsaturated ketones and precursors from chemical subgroup 4.4 of FGE.19: 3(2H)-Furanones. The EFSA Journal 1061 (2009), 1–23. Available at: http://www.efsa.europa.eu/en/scdocs/doc/1061.pdf.
[81] T. Lave, A. Paehler, H.P. Grimm, E. Gocke, L. Müller, Modelling of patient EMS exposure: translating pharmacokinetics of EMS *in vitro* and in animals into patients, Toxicol. Lett. 190 (2009) 310–316.
[82] M.K. Shelby, N.J. Cherrington, N.R. Vansell, C.D. Klaassen, Tissue mRNA expression of the rat UDP-glucuronosyltransferase gene family, Drug Metab. Dispos. 31 (2003) 326–333.
[83] N.K. Basu, L. Kole, S. Kubota, I.S. Owens, Human UDP-glucuronosyltransferases show atypical metabolism of mycophenolic acid and inhibition by curcumin, Drug Metab. Dispos. 32 (2004) 768–773.
[84] F. Nesslany, S. Simar-Meintières, M. Watzinger, I. Talahari, D. Marzin, Characterization of the genotoxicity of nitrilotriacetic acid, Environ. Mol. Mutagen. 49 (2008) 439–452.

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Preface

Nucleotide pool damage and its biological consequences

It is our pleasure and honor to publish a special issue of Mutation Research "Nucleotide Pool Damage and Its Biological Consequences". This is extended presentation of a session of "Health Effects of Nucleotide Pool Damage" in the 10th International Conference on Environmental Mutagens (10th ICEM) in Florence in Italy in August 2009. The session was organized by us (Bignami and Nohmi), and six out of 11 authors who contribute to this special issue were invited speakers in the session. After ICEM, we invited another five persons to join the group to publish this special issue and all kindly accepted the invitation. We are very pleased that this special issue is published within a relatively short period of time.

Needless to say, nucleotide pools as well as DNA are important substrates for DNA polymerases. Accurate DNA synthesis requires well balanced dNTP pools and the imbalance leads to mutations and cell death. In addition, excess oxidation of nucleotide pools in aerobic metabolism or inflammation results in a variety of cellular abnormalities including genome instability. The representative example of oxidation of dNTPs is the formation of 8-oxo-dGTP in nucleus and mitochondria, which may induce mutations, cellular senescence, neurological diseases and cancer. To combat the detrimental effects of oxidized dNTPs, cells evolve nucleotide pool sanitizing enzymes such as MTH1. However, some of the oxidized dNTPs escape from the defense systems and eventually incorporated into DNA by polymerases. In this issue, 11 authors discuss biological and health consequences of nucleotide pool damage from various viewpoints. The collected papers may be beneficial to people in pharmaceutical industries as well because modified dNTPs are an important class of pharmaceuticals. It is our hope that this special issue will contribute to wider recognition of the importance of nucleotide pool damage in health sciences.

Finally we acknowledge Dr. David Kirkland, an editor of special issue of Mutation Research, who encouraged us to publish this special issue.

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Minireview

DNA polymerases involved in the incorporation of oxidized nucleotides into DNA: Their efficiency and template base preference

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ABSTRACT

Genetic information must be duplicated with precision and accurately passed on to daughter cells and later generations. In order to achieve this goal, DNA polymerases (Pols) have to faithfully execute DNA synthesis during chromosome replication and repair. However, the conditions under which Pols synthesize DNA are not always optimal; the template DNA can be damaged by various endogenous and exogenous genotoxic agents including reactive oxygen species (ROS), and ROS oxidize dNTPs in the nucleotide pool from which Pols elongate DNA strands. Both damaged DNA and oxidized dNTPs interfere with faithful DNA synthesis by Pols, inducing various cellular abnormalities, such as mutations, cancer, neurological diseases, and cellular senescence. In this review, we focus on the process by which Pols incorporate oxidized dNTPs into DNA and compare the properties of Pols: efficiency, i.e., k_{cat}/K_m, k_{pol}/K_d or V_{max}/K_m, and template base preference for the incorporation of 8-oxo-dGTP, an oxidized form of dGTP. In general, Pols involved in chromosome replication, the A- and B-family Pols, are resistant to the incorporation of 8-oxo-dGTP, whereas Pols involved in repair and/or translesion synthesis, the X- and Y-family Pols, incorporate nucleotides in a relatively efficient manner and tend to incorporate it opposite template dA rather than template dC, though there are several exceptions. We discuss the molecular mechanisms by which Pols exhibit different template base preferences for the incorporation of 8-oxo-dGTP and how Pols are involved in the induction of mutations via the incorporation of oxidized nucleotides under oxidative stress.

Contents

1. Introduction

Chromosome DNA is continuously exposed to various endogenous and exogenous genotoxic agents. Among these agents, oxidation is one of the most common threats to genetic stability [1,2]. Each human cell is estimated to metabolize approximately 10^{12} molecules of oxygen per day, and approximately 1% of oxygen

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metabolism results in the production of reactive oxygen species (ROS) via one electron reduction [3,4]. These reactive molecules include superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, with hydroxyl radicals thought to be the most predominant reactive species [5]. ROS are also generated in cells when they are exposed to radiation and chemical carcinogens. To counteract the oxidative stress induced by ROS, cells have evolved multiple defense mechanisms. Enzymes, such as catalase or superoxide dismutase, detoxify ROS and low-molecular-weight scavengers, such as glutathione, reduce the toxicity of ROS [6]. Nevertheless, some ROS molecules escape from the defense systems and inevitably damage the bio-molecules. Thus, ROS have been implicated in the etiology of human degenerative diseases, aging, and cancer [7,8].

Although ROS generate a variety of modified bases in DNA, 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG) is the best characterized oxidized base in chemistry and biology [9–11] and is used as a biomarker of DNA oxidation. Approximately 10^3 8-oxo-dG molecules are generated in normal human cells per day [2,12]. 8-oxo-dG pairs with cytosine in the *anti* conformation but assume the *syn* conformation when pairing with adenine [13–15]. In fact, both dATP and dCTP are inserted opposite template 8-oxo-dG during DNA synthesis [16]. The latter pairing can result in G:C to T:A transversion [17]. To prevent the mutagenesis caused by the lesion, human cells possess multiple repair mechanisms [18], including DNA glycosylases in the base excision repair pathway, such as 8-oxoguanine glycosylase (OGG1) and MutY glycosylase homologue (MUTYH), which excise 8-oxo-G when paired with cytosine and adenine opposite 8-oxo-dG, respectively [19]. The Cockayne syndrome proteins CSA and CSB in transcription-coupled nucleotide excision repair also involve the exclusion of 8-oxo-dG from DNA [20–22]. Despite the presence of repair mechanisms, 8-oxo-dG accumulates in senescent cells and the brain cortex of aged humans, which may cause various cellular abnormalities [23–26].

In addition to the direct oxidation of deoxyguanosine (dG) in DNA, 8-oxo-dG can be generated by the incorporation of oxidized dGTP (8-oxo-dGTP) into DNA by DNA polymerases (Pols). 8-oxo-dGTP can be incorporated into the template strand opposite deoxycytidine (dC) or deoxyadenosine (dA) and the latter may cause A:T to C:G transversions [27]. In fact, *Escherichia coli mutT* mutants, which lack the ability to hydrolyze 8-oxo-dGTP to its mono-phosphate form, exhibit more than 1000 times higher frequencies of spontaneous A:T to C:G transversions than controls [28]. Mice lacking MTH1, a mammalian homologue of MutT, display enhanced tumor formation in the lung, liver, and stomach [29]. In human cells, suppression of MTH1 expression induces cellular senescence [30]. In contrast, the over-expression of hMTH1 reduces total cellular 8-oxo-dG levels in human cells and transgenic mice [31,32]. Over-expression also suppresses genome instability in cells with defective mismatch repair (MMR) mechanisms and causes ameliorated neuropathological and behavioral symptoms resembling Huntington's disease in mice. Thus, the oxidized dNTP pool is recognized as a source of spontaneous mutagenesis, carcinogenesis, cellular senescence, and neurological disease.

In human cells, MTH1 hydrolyzes 8-oxo-dGTP and other oxidized dNTPs, such as 2-hydroxy-dATP (2-OH-dATP) and 8-oxo-dATP, to the mono-phosphate forms in the nucleotide pool [33]. In addition to MTH1, cells possess MTH2, which hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP [34], and Nudix type 5 (NUDT5) protein, which hydrolyzes 8-oxo-dGDP to the mono-phosphate [35]. MMR prevents the mutations caused by the incorporation of 8-oxo-dGTP [31]. 8-oxo-dG incorporated during replication can become a target of MMR machinery, which removes the incorporated 8-oxo-dG from DNA and initiates DNA re-synthesis.

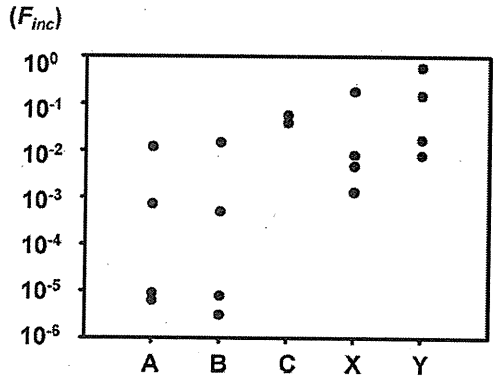

Fig. 1. Efficiency of the incorporation of 8-oxo-dGTP into DNA by family A, B, C, X, and Y Pols. F_{inc} indicates the ratio of the efficiency of incorporating 8-oxo-dGTP opposite a favorable template base versus that of incorporating normal dNTPs opposite the same template base. F_{inc} was calculated from the kinetic parameters in the references (see Table 1). Pols include hPolγ, *E. coli* Pol I Klenow fragment (exo⁻), and T7 Pol for family A; hPolα, bovine Polδ, *E. coli* Pol II, and φ29 Pol for family B; *E. coli* Pol III* and α subunit of Pol III for family C; hPolβ, hPolλ, and African swine fever virus Pol X for family X; and hPolη, Polι, and Polκ for family Y.

Measurements of intracellular levels of 8-oxo-dGTP in nucleotide pool are a challenging issue. Initial attempt to measure the level of 8-oxo-dGTP in *E. coli* was failed [36]. Recent measurements with improved sensitive methods indicate that the level of 8-oxo-dGTP in mitochondria is similar to that of normal dTTP, which is 1–10% of the level of normal dGTP [37]. It remains to be seen the levels of 8-oxo-dGTP and other oxidized dNTPs in nucleus in oxidative stressed and non-stressed human cells.

To exert adverse effects, oxidized dNTPs must be incorporated into DNA by Pols. In culture medium, 8-oxo-dG is readily incorporated into the genomic DNA of human cells upon phosphorylation [38]. As shown below, however, various Pols have distinct properties in terms of their efficiency in incorporating 8-oxo-dGTP into DNA. In this review, we use the term "efficiency" to mean k_{cat}/K_m or V_{max}/K_m in steady-state kinetic analyses and k_{pol}/K_d in pre-steady-state kinetic analyses. Some Pols incorporate 8-oxo-dGTP into DNA very efficiently, whereas others do so poorly. So far, we have surveyed the maximum difference of F_{inc} (i.e. the ratio of the efficiency for incorporating 8-oxo-dGTP versus that of incorporating normal dNTP) among various Pols to be 10^5 (Fig. 1, Table 1). For example, human Polη incorporates 8-oxo-dGTP opposite template dA with 20–60% efficiency compared to normal dTTP incorporation [39,40], whereas *E. coli* Pol II exo⁻ incorporates it opposite template dC with 0.0003% efficiency compared to normal dGTP incorporation [41]. In addition, the preference of the Pols for template bases is distinct (Table 2); Polη incorporates 8-oxo-dGTP opposite template dA almost exclusively [40], and *E. coli* Pol II exo⁻ incorporates it opposite template dC more favorably (the ratio for incorporation opposite template dC:dA is 22:1) [41].

In this review, we compare the relative efficiency and template base preferences of Pols for incorporating 8-oxo-dGTP. Because Pols are categorized into six families (A, B, C, D, X, and Y) based on their primary structures [42], we review the properties of Pols family by family. The A, B, or C families of Pols are involved in chromosome replication with high fidelity and high processivity, and the X and Y families are responsible for DNA repair synthesis and translesion synthesis (TLS). Pols family D is restricted to Archaea, and their properties for 8-oxo-dGTP incorporation have not been reported. Therefore, we omit the D-family Pols from this review. We also discuss mechanisms underlying the distinct properties of some Pols and the biological consequences of the incorporation of 8-oxo-dGTP.

Table 1
Relative efficiencies of DNA polymerases for incorporating 8-oxo-dGTP compared to normal dNTP.

Family	DNA polymerase	F_{inc}	Reference
A	KF (exo$^-$)	1.2×10^{-2}	[49]
	KF (exo$^-$)	7.2×10^{-4}	[47]
	T7 Pol	9.1×10^{-6}	[41]
	KF (exo$^-$)	6.4×10^{-6}	[41]
B	Polα (calf thymus)	1.5×10^{-2}	[49]
	φ29 Pol	5.0×10^{-4}	[56]
	Polα (calf thymus)	7.9×10^{-6}	[57]
	Pol II (exo$^-$)	3.1×10^{-6}	[41]
C	Pol III*	5.6×10^{-2}	Yamada et al., unpublished data
	α Subunit of Pol III	3.9×10^{-2}	[28]
X	hPolβ	1.8×10^{-1}	[68]
	hPolβ	8.0×10^{-3}	[69]
	hPolλ	4.7×10^{-3}	[69]
	ASFV Pol X	1.3×10^{-3}	[70]
Y	hPolη	5.9×10^{-1}	[40]
	hPolη	1.5×10^{-1}	[39]
	hPolι	1.7×10^{-2}	[40]
	hPolκ	7.9×10^{-3}	[39]

F_{inc} was calculated as the ratio of the efficiency of incorporating 8-oxo-dGTP opposite the favorite template base versus incorporating normal dNTPs opposite the same template base. The "efficiency" was defined as k_{cat}/K_m or V_{max}/K_m in steady-state kinetic analyses [28,39–41,47,49,56,57,68] or k_{pol}/K_d in pre-steady-state kinetic analyses [69,70].

2. Efficiency and template base preferences of Pols

2.1. A-family Pols

A-family Pols are defined as homologues of *E. coli* DNA Pol I, which was the first Pol to be described and is involved in the synthesis of Okazaki fragments during lagging strand synthesis and in DNA repair [43,44]. Representative members of this family are Polγ and Polθ in humans, and T7 Pol in phages. In general, Pols in this family inefficiently incorporate 8-oxo-dGTP into DNA compared to the incorporation of normal dNTPs (Fig. 1, Table 1). Polγ, which functions solely in mitochondrial DNA replication and has 3′ to 5′ exonuclease activity [45], incorporates 8-oxo-dGTP opposite template dA with a F_{inc} of approximately 10^{-4} [46]. This enzyme favorably incorporates 8-oxo-dGTP opposite template dA compared to template dC at a ratio of 13:1 (Table 2). The resulting 8-oxo-dG:dA pair is extended, rather than excised, by the exonuclease activity [46]. T7 Pol, which is involved in the replication of T7 phage, also poorly incorporates 8-oxo-dGTP with a F_{inc} of approximately 10^{-4} and preferably incorporates it opposite template dA compared to dC [41]. In contrast, the *E. coli* Pol I Klenow fragment deficient in exonuclease activity (KF exo$^-$) incorporates 8-oxo-dGTP opposite dA comparable to dC [41,47,48]. It incorporates 8-oxo-dGTP inefficiently ($F_{inc} = 10^{-5}$–10^{-6}). 8-oxo-dGTP may be more efficiently incorporated into template run sequences, such as CCCCCC, by KF(exo$^-$) and B-family Polα [49]. KF exo$^-$ also incorporates 5-hydroxydeoxycytidine tri-phosphate and 5-hydroxydeoxyuridine tri-phosphate into DNA more efficiently than 8-oxo-dGTP [47]. The degree of excision of 8-oxo-dGTP opposite template dA is enzyme-dependent: Polγ and KF excise it poorly while T4 Pol (B-family Pol) excises it efficiently [46,50]. The A-family Pols exhibit a broad range of template base preferences for the incorporation of 8-oxo-dGTP into DNA.

2.2. B-family Pols

B-family Pols are homologues of *E. coli* Pol II. This enzyme is encoded by *polB*, which is induced by DNA damage under the control of SOS regulation [51]. Pol II has both DNA Pol activity and 3′ to 5′ exonuclease activity in a single polypeptide [52]. Although Pol II is not involved in chromosome replication, the mammalian B-family members are responsible for the replication of the genome [42]. In humans, the B-family Pols, i.e., Polα, Polδ, and Polε, are involved in replication and DNA repair [53,54], and another B-family member, Polζ, plays a role in TLS with Y-family Pols [55]. Bacteriophage φ29 Pol is a protein-primed DNA-dependent replicase belonging to the B family. Similar to the A-family Pols, the B-family Pols poorly incorporate 8-oxo-dGTP into DNA. φ29 Pol incorporates 8-oxo-dGTP 2000-fold less efficiently than unmodified dGTP (Fig. 1, Table 1) [56]. The enzyme has a preference for pairing 8-oxo-dGTP with template dC (Table 2), and φ29 Pol extends the correct 8-oxo-dG:dC pair preferentially, with an efficiency similar to that of the

Table 2
Favorite template base for incorporation of 8-oxo-dGTP by various Pols.

Favorite template	DNA polymerase	Family	dA/dC[a]	Reference
dA » dC	hPolι	Y	dA only	[40]
	Dbh	Y	dA only	[59]
	Dpo4	Y	dA only	[59]
	hPolη	Y	660:1, 180:1	[39,40]
dA > dC	hPolλ	X	35:1	[69]
	T7 Pol	A	31:1	[41]
	hPolβ	X	24:1, 11:1	[68,69]
	Pol III *	C	20:1	Yamada et al., unpublished data
	hPolγ	A	13:1	[46]
	hPolκ	Y	11:1	[39]
	Pol IV	Y	ND	[48]
dA ≈ dC	KF (exo$^-$)	A	1.6:1, 0.44:1	[47,41]
	α Subunit of Pol III	C	1.3:1	[28]
	HIV-1 RT	RT	0.5:1	[41]
	ASFV Pol X	X	0.5:1	[70]
	φ29 Pol	B	0.33:1	[56]
	DNA Pol B1 (*Sso*)	B	ND	[59]
dA < dC	Bovine Polδ + PCNA	B	0.032:1	[84]
	Pol II (exo$^-$)	B	0.045:1	[41]
	hPolα	B	ND[b]	Katafuchi et al., unpublished data

[a] The ratio of dA/dC was determined as the ratio of the efficiency of incorporation 8-oxo-dGTP opposite template dA compared to that of incorporating it opposite template dC. The efficiency was defined as k_{cat}/K_m or V_{max}/K_m in steady-state kinetic analyses [28,39–41,47,48,56,59,68,84] or k_{pol}/K_d in pre-steady-state kinetic analyses [46,69,70].
[b] ND: the ratio was not determined. RT stands for reverse transcriptase.

normal dG:dC base pair. Calf thymus Polα incorporates 8-oxo-dGTP opposite dC with a F_{inc} of less than 10^{-5} [57]. The B-family Pols involved in chromosome replication appear to be highly resistant to mutations induced by the incorporation of 8-oxo-dGTP into DNA. An exception may be Polζ because knocking down the expression of *POLZ*, which encodes Polζ, in human cells significantly reduces mutations induced by 8-oxo-dGTP [58]. The Pol may be involved in an extension step from 8-oxo-dG:dA mismatch after the incorporation of 8-oxo-dGTP opposite template dA by other Pols, as suggested for TLS.

B-family Pols in other species also incorporate 8-oxo-dGTP inefficiently. Archaea *Sulfolobus solfataricus Sso* Pol B1 is a replicase that incorporates 8-oxo-dGTP ineffectively [59] and slightly more often opposite template dC than template dA (Table 2). *E. coli* Pol II exo$^-$ is the most inefficient enzyme examined thus far for the incorporation of 8-oxo-dGTP into DNA ($F_{inc} = 3.1 \times 10^{-6}$) and it has a preference for template dC [41].

2.3. C-family Pols

C-family Pols are homologues of the α subunit of *E. coli* Pol III holoenzyme, which is responsible for the replication of the *E. coli* genome [43]. *E. coli* Pol III holoenzyme is composed of the core (α, β and θ), γ complex, and β subunit. The core is a heterotrimer of the α Pol (catalytic subunit), ε subunit with 3′ to 5′ proofreading exonuclease, and θ subunit. The β subunit is a sliding clamp, which encircles duplex DNA and increases processivity, and the γ complex is the clamp loader. The C-family Pols are exclusively found in eubacteria, whereas all Archaea and eukaryotic replicative Pols belong to the B family. Although Pol III is responsible for replication in this organism, the α subunit of Pol III efficiently incorporates 8-oxo-dGTP opposite both dA and dC ($F_{inc} = 3.9 \times 10^{-2}$) [28]. This F_{inc} is substantially higher than the replicative Pols belonging to the A and B families (Table 1), which may be due to the structural resemblance of the α subunit to X-family Pols [60,61] (see more detail below). The probability that 8-oxo-dGTP is incorporated into DNA during *E. coli* replication may be higher compared to mammalian cells. *E. coli* Pol III holoenzyme lacking the β subunit is referred to as Pol III*. Unlike the α subunit, Pol III* tends to incorporate 8-oxo-dGTP opposite template dA more often than template dC (Yamada, et al., unpublished data). Complex formation with other subunits may alter the enzyme's template base preference. In addition to 8-oxo-dGTP, Pol III holoenzyme also incorporates 2-OH-dATP, an oxidized form of dATP, which induces a G:C to T:A transversion when it is incorporated opposite template dG [62].

2.4. X-family Pols

In humans, Polβ, Polλ, Polσ, Polμ, and terminal deoxynucleotidyl transferase (TdT) belong to the X family of Pols [63]. Because these Pols are involved in short DNA synthesis for repair rather than long DNA synthesis for chromosome replication, the family was termed X to differentiate from the A, B, and C families. Polβ plays important roles in gap-filling synthesis in base excision repair, and Polλ may have functions similar to Polβ [64–66]. Polλ also contributes to non-homologous end joining in the process of repairing double-strand DNA breaks [67]. Polβ and Polλ exhibit relatively high F_{inc} values when they incorporate 8-oxo-dGTP into gapped DNA (Fig. 1 and Table 1) [68,69]. In particular, Polβ incorporates 8-oxo-dGTP with an efficiency roughly 20% of that of normal dNTP incorporation. Both Pols prefer dA as a template base for incorporating 8-oxo-dGTP (Table 1). The priority of the enzymes may be the execution of DNA synthesis for repair rather than the exclusion of 8-oxo-dGTP from DNA to maintain genomic integrity. Among X-family Pols, Pol X from African swine fever virus (ASFV) prefers template dC for the incorporation of 8-oxo-dGTP [70].

2.5. Y-family Pols

The most remarkable feature of this family is the ability to bypass a variety of lesions in DNA that otherwise block chromosome replication by A-, B-, and C-family Pols [51,55,71]. Because the enzymes are involved in short track DNA synthesis rather than long DNA synthesis for chromosome replication, they are termed the Y family, following the X family. In general, these enzymes have large active sites to accommodate bulky lesions in DNA and lack 3′ to 5′ exonuclease activities [72]. The fidelity of DNA synthesis by Y-family Pols is much lower compared to that of replicative A-, B-, and C-family Pols [42]. In humans, Y-family Pols include REV1, Polη, Polκ, and Polι. Like Polβ in the X family, Polη, Polκ, and Polι efficiently incorporate 8-oxo-dGTP into DNA and favor dA as the template base. In particular, Polη exhibits the highest F_{inc} of 20–60% (Table 1), suggesting that it incorporates 8-oxo-dGTP opposite template dA almost as much as it incorporates dTTP into DNA [39,40]. Therefore, Y-family Pols seem to incorporate 8-oxo-dGTP into the cellular DNA efficiently although they have fewer chances to incorporate the oxidized nucleotide compared to the replicative Pols. Interestingly, suppressed expression of Polη and REV1 by siRNAs significantly reduces mutations in the *supF* plasmid in human cells in which 8-oxo-dGTP is introduced by osmotic shock [58]. In addition to 8-oxo-dGTP, Polη incorporates 2-OH-dATP opposite template T, G, and C with an efficiency of 2–6% of that of incorporating normal dNTPs [40]. In bacteria and Archaea, the Y-family Pols Pol IV (DinB) in *E. coli* and Dbh (*Sac* Pol Y1) and Dpo4 (*Sso* Pol Y1) in Archaea also incorporate 8-oxo-dGTP efficiently, favoring dA as the template base for incorporation [48,59]. Deficient *dinB* and/or *umu* expression (encoding Pol IV and Pol V, respectively), reduces the mutation frequency of A:T to C:G by 80–90% in *sod fur E. coli* mutants in which iron overload and superoxide stress occur [48]. The Y-family Pols in *E. coli* may be involved in the transversion mutations caused by 8-oxo-dGTP under SOS-induced conditions, and they may participate in sequential biochemical steps, such as the incorporation and extension of 8-oxo-dGTP during chromosome replication. *E. coli* Pol IV and Pol V appear to be involved in chromosome replication when the dNTP pool is depleted by treating the cells with hydroxyurea [73]. Whether the Pols incorporate 8-oxo-dGTP into DNA when they are involved in chromosome replication is of interest.

3. Structural insight into the template base preference by polymerases for incorporating 8-oxo-dGTP into DNA

The 8-oxo-dG molecule mainly exists in a 6,8-diketo form in solution at physiological pH, and its conformation is in equilibrium between the *anti* and *syn* formation, with the *syn* conformation being energetically favored [13–15,74]. Therefore, 8-oxo-dGTP is expected to pair with template dA more favorably than template dC. However, as described above, the template base preferred by Pols is significantly different, even in the same family (Table 2). This difference suggests that the sterical and/or electrostatic properties of the active site of Pols play important roles in the conformation of 8-oxo-dGTP in the enzyme where it pairs with template bases. Indeed, studies have indicated that particular amino acids in the active site greatly impact specificity for the incorporation of 8-oxo-dGTP into DNA by affecting the conformation of 8-oxo-dGTP in the active site.

In Polβ in humans, Asn 279 (N279) is the critical determinant of template base preference for incorporating 8-oxo-dGTP [68]. Wild-type Polβ exhibits a preference for template dC over template dA

at a ratio of 24:1 when it incorporates 8-oxo-dGTP. However, the mutant enzyme with alanine instead of N279 displays a reversed preference. The mutant (N279A) incorporates 8-oxo-dGTP opposite template dC and dA at a ratio of 14:1. The N279 appears to form a hydrogen bond with the O^8 of incoming 8-oxo-dGTP in the *syn* formation, which stabilizes the formation of 8-oxo-dGTP:dA (Fig. 2A). The active site of Polβ may alter the equilibrium of the conformation of 8-oxo-dGTP through interactions with N279.

φ29 Pol generally favors template dC for the insertion of 8-oxo-dGTP [56]; the ratio of pairing dA to 8-oxo-dGTP compared to pairing dC is 1:3. Structural modeling based on the crystal structure of the RB69 Pol active site suggests that this specificity is dominated by Lys 383 (K383), which sterically and/or electrostatically impinges the N^2 of 8-oxo-dGTP in the *syn* formation when paired with dA, thereby forcing it to form the *anti* conformation (Fig. 2B).

Human Y-family Pols, i.e., Polκ and Polη, have other mechanisms for selecting 8-oxo-dGTP [39]. In human Polκ, which exclusively incorporates 8-oxo-dGTP opposite template dA, the substitution of Tyr112 (Y112) with alanine eliminates the preference, mainly due to severely reduced efficiency for pairing with template dA (270-fold reduction) compared to that of pairing with template dC (15-fold reduction). Thus, the ratio of dA to dC for pairing with 8-oxo-dGTP is reduced from 11:1 to almost 1:1 by the amino acid substitution. Y112 is known as the 'steric gate', which distinguishes dNTPs from rNTPs by sensing the absence of the ribose 2′-hydroxy group. In addition, the residue is involved in an incorporation step of dCTP opposite a benzo[a]pyrene 7,8-dihydrodiol 9,10-epoxide-N^2-dG adduct in the template DNA and an extension step from mismatched termini [75]. Therefore, Y112 in human Polκ has multiple functions and may interact with both the sugar moiety and the base of the incoming dNTP, as well as stabilize the pairing of template dA with 8-oxo-dGTP in the *syn* conformation in the active site.

In contrast, phenylalanine 18 (F18) of human Polη, which corresponds to Y112 in human Polκ, does not affect the template base preferred by this Y-family Pol [39]. An amino acid substitution of F18 with alanine does not alter the enzyme's specificity for incorporating 8-oxo-dGTP into DNA; instead, arginine 61 (R61) affects the preference. Human Polη incorporates 8-oxo-dGTP opposite template dA almost exclusively. However, substitutions of R61 with alanine (R61A) or lysine (R61K) drastically alter the template base preference. The ratio of incorporation of 8-oxo-dGTP opposite template dA versus dC is 660:1 for the wild-type Pol, 65:1 for R61A, and 7:1 for R61K. Similar alterations in the template base prefer-

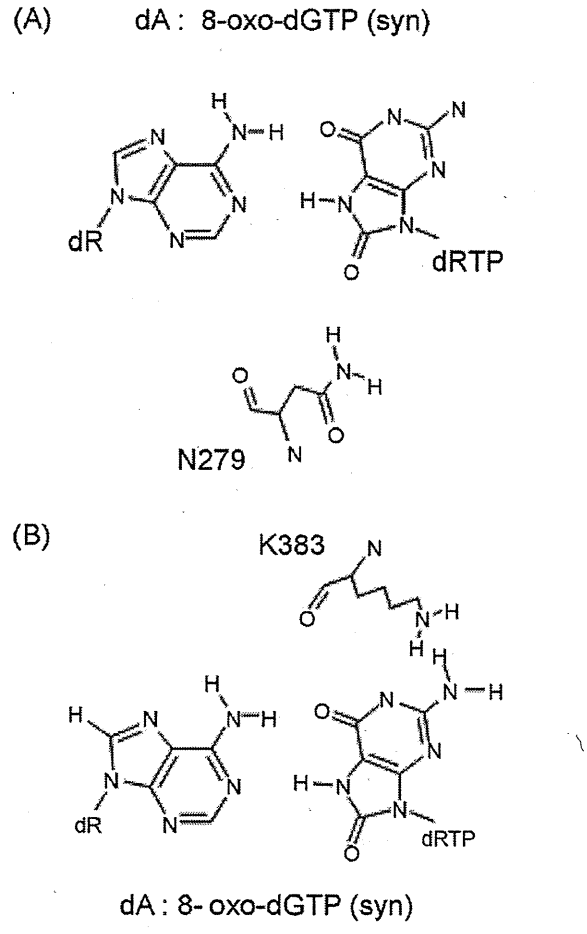

Fig. 2. Schematic representation of amino acids affecting the pairing of dA:8-oxo-dGTP(*syn*). (A) In hPolβ, a hydrogen bond can be present between NH_2 of N279 and the O^8 of 8-oxo-dGTP in the *syn* conformation. (B) In φ29 Pol, sterical and/or electrostatic collisions could occur between K383 and the N^2 of 8-oxo-dGTP in the *syn* conformation. The collision may force 8-oxo-dGTP to form the *anti* conformation in the active site.

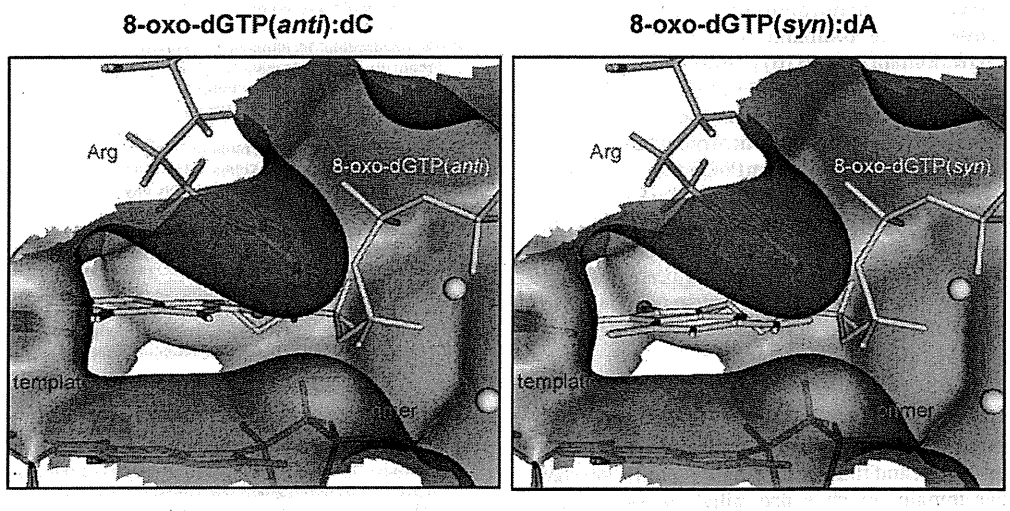

Fig. 3. The modeling structures of 8-oxo-dGTP(*anti*):dC and 8-oxo-dGTP(*syn*):dA in the active site of hPolη. R61 (ocher) may sterically clash with the O^8 of the incoming 8-oxo-dGTP in the *anti* conformation, leading to the exclusion of a pairing with template dC.

ence occur in the case of 8-oxo-dATP but not 2-OH-dATP. Molecular modeling studies suggest that R61 causes steric and/or electrostatic hindrance with the O^8 of 8-oxo-dGTP and 8-oxo-dATP in the *anti* conformation (Fig. 3). This context may be the basis for the preference of human Polη for pairing 8-oxo-dGTP with template dA. When R61 is replaced by lysine, the ε-amino group and O^8 of 8-oxo-dGTP in the *anti* conformation may interact electrostatically. Thus, the lysine residue (K61), but not the arginine residue (R61), may stabilize 8-oxo-dGTP in the *anti* conformation, which enhances the preference for correctly pairing 8-oxo-dGTP with template dC. The counterpart of R61 in yeast Polη is R73, which is involved in bypass reactions across 1,2-(GpG) cisplatin adducts in DNA [76].

4. Biological implications of incorporation of oxidized dNTPs into DNA by Pols

The incorporation of 8-oxo-dGTP into DNA opposite template dA may cause mutations because the incorporated 8-oxo-dG can pair with dCTP in the next round of DNA replication, thereby inducing A:T to C:G transversions. Reasonably, A- and B-family Pols, most of which are involved in chromosome replication, incorporate 8-oxo-dGTP into DNA inefficiently and most disfavor the pairing of 8-oxo-dGTP with template dA (Fig. 1 and Table 1). The eukaryotic genome may be dually protected from the mutagenic threats of 8-oxo-dGTP incorporation into DNA by the presence of MTH1 and other sanitizing enzymes that hydrolyze oxidized nucleotides, and by the poor ability of the Pols to incorporate the oxidized nucleotides into DNA.

In contrast, the C-family Pols, such as *E. coli* Pol III (both the assembled holoenzyme, Pol III*, and the catalytic α-subunit alone), have high activity for incorporating 8-oxo-dGTP into DNA and pairs it with template dA rather than dC (Tables 1 and 2). The enzyme incorporates the oxidized dNTP opposite template dA at approximately 4% of the efficiency of incorporating the normal dTTP opposite template dA [28]. The high efficiency of incorporating 8-oxo-dGTP opposite dA may account for the extremely high mutation frequency of the *mutT E. coli* mutants in which A:T to C:G transversion mutations are increased more than 1000 times over the wild-type strain. In this respect, the bacterial genome is less protected from the mutagenic effect of 8-oxo-dGTP compared to eukaryotes, which may be due to the structural similarity of the α-subunit of Pol III to Polβ in family X rather than replicative Pols in family A or B [60,61,77]. The structure of the palm domain of the α-subunit of *Thermus aquaticus* Pol III is similar to that of the palm domain of rat Polβ [60]. The amino acid sequence of the α-subunit of *T. aquaticus* is roughly 40% identical to that of the *E. coli* homologue. A large fragment of the α-subunit of *E. coli* Pol III is partly similar to the catalytic domain of Polβ [61]. However, whether the efficient incorporation of 8-oxo-dGTP by *E. coli* Pol III is achieved by mechanisms similar to those in Polβ remains unknown.

The X- and Y-family Pols also efficiently incorporate 8-oxo-dGTP and prefer template dA. In particular, human Polβ and Polη have the pair 8-oxo-dGTP with dA comparable to dTTP with dA [39,40,68]. The enzymes may incorporate 8-oxo-dGTP into DNA at a high frequency and cause A:T to C:G transversions. The erroneous incorporation of 8-oxo-dGTP opposite template dA may be analogous to the error-prone TLS by the Y-family Pols because two pathways enhance the induction of mutations during DNA synthesis.

5. Future perspective

Most of Pols seem to share a common architecture with three domains, the fingers, palm, and thumb, although Y-family Pols have an extra little finger domain, which is also called the polymerase-associated domain (PAD) or wrist domain [72,78–80]. The fingers interact with incoming dNTPs and the single-stranded template DNA, the palm holds two catalytic metals, and the thumb binds duplex DNA. Despite the structural similarities, Pols have remarkably divergent properties for the incorporation of 8-oxo-dGTP into DNA. In addition, the mechanism underlying the erroneous incorporation of 8-oxo-dGTP opposite template dA is distinct among φ29 Pol, human Polβ, human Polκ, and human Polη, suggesting the convergent evolution of Pols to incorporate the mutagenic oxidized nucleotide into DNA. The biological or evolutionary merits of the efficient incorporation of 8-oxo-dGTP opposite template dA are of great interest. In addition, it is worth investigating how accessory factors such as PCNA/RFA affect the efficiency and template preference of Pols [81,82]. Erroneous TLS or error-prone Pols have been proposed to be needed to adequately adapt to environmental changes during evolution [83]. Similarly, the incorporation of 8-oxo-dGTP, and perhaps other oxidized dNTPs, into DNA may have be advantageous in competition with other organisms during evolution, though it is harmful for multicellular organisms, such as humans, maintaining their genomic integrity and longevity.

Conflict of interest

No conflicting interest exists.

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References

[1] T. Lindahl, Instability and decay of the primary structure of DNA, Nature 362 (1993) 709–715.
[2] M. Dizdaroglu, Chemical determination of free radical-induced damage to DNA, Free Radic. Biol. Med. 10 (1991) 225–242.
[3] A.L. Jackson, L.A. Loeb, The contribution of endogenous sources of DNA damage to the multiple mutations in cancer, Mutat. Res. 477 (2001) 7–21.
[4] R.A. Floyd, Measurement of oxidative stress in vivo, in: K.J.A. Daview, F. Ursini (Eds.), The Oxygen Paradox, Cleup University Press, Cleup, 1995, pp. 89–103.
[5] M. Dizdaroglu, Mechanisms of oxidative damage: lesions and their measurement, in: M. Dizdaroglu, A. Karakaya (Eds.), Advances in DNA Damage and Repair, Kluwer Academic Publishers/Plenum Press, New York, 1999, pp. 67–87.
[6] Y.Z. Fang, S. Yang, G. Wu, Free radicals, antioxidants, and nutrition, Nutrition 18 (2002) 872–879.
[7] B.N. Ames, Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases, Science 221 (1983) 1256–1264.
[8] D.I. Feig, T.M. Reid, L.A. Loeb, Reactive oxygen species in tumorigenesis, Cancer Res. 54 (1994) 1890s–1894s.
[9] H. Kasai, Chemistry-based studies on oxidative DNA damage: formation, repair, and mutagenesis, Free Radic. Biol. Med. 33 (2002) 450–456.
[10] M.L. Michaels, J.H. Miller, The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine), J. Bacteriol. 174 (1992) 6321–6325.
[11] H. Kasai, S. Nishimura, Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents, Nucleic Acids Res. 12 (1984) 2137–2145.
[12] T.A. Kunkel, The high cost of living, in: American Association for Cancer Research Special Conference: Endogenous Sources of Mutations, Fort Myers, Florida, USA, 11–15 November 1998, Trends Genet. 15 (1999) 93–94.
[13] M. Kouchakdjian, V. Bodepudi, S. Shibutani, M. Eisenberg, F. Johnson, A.P. Grollman, D.J. Patel, NMR structural studies of the ionizing radiation adduct 7-hydro-8-oxodeoxyguanosine (8-oxo-7H-dG) opposite deoxyadenosine in a DNA duplex. 8-Oxo-7H-dG(syn).dA(anti) alignment at lesion site, Biochemistry 30 (1991) 1403–1412.

[14] Y. Oda, S. Uesugi, M. Ikehara, S. Nishimura, Y. Kawase, H. Ishikawa, H. Inoue, E. Ohtsuka, NMR studies of a DNA containing 8-hydroxydeoxyguanosine, Nucleic Acids Res. 19 (1991) 1407–1412.

[15] L.A. Lipscomb, M.E. Peek, M.L. Morningstar, S.M. Verghis, E.M. Miller, A. Rich, J.M. Essigmann, L.D. Williams, X-ray structure of a DNA decamer containing 7,8-dihydro-8-oxoguanine, Proc. Natl. Acad. Sci. USA 92 (1995) 719–723.

[16] S. Shibutani, M. Takeshita, A.P. Grollman, Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG, Nature 349 (1991) 431–434.

[17] K.C. Cheng, D.S. Cahill, H. Kasai, S. Nishimura, L.A. Loeb, 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G–T and A–C substitutions, J. Biol. Chem. 267 (1992) 166–172.

[18] T. Nohmi, S.R. Kim, M. Yamada, Modulation of oxidative mutagenesis and carcinogenesis by polymorphic forms of human DNA repair enzymes, Mutat. Res. 591 (2005) 60–73.

[19] H. Ide, M. Kotera, Human DNA glycosylases involved in the repair of oxidatively damaged DNA, Biol. Pharm. Bull. 27 (2004) 480–485.

[20] H. de Waard, J. de Wit, J.O. Andressoo, C.T. van Oostrom, B. Riis, A. Weimann, H.E. Poulsen, H. van Steeg, J.H. Hoeijmakers, G.T. van der Horst, Different effects of CSA and CSB deficiency on sensitivity to oxidative DNA damage, Mol. Cell Biol. 24 (2004) 7941–7948.

[21] M. D'Errico, E. Parlanti, M. Teson, P. Degan, T. Lemma, A. Calcagnile, I. Iavarone, P. Jaruga, M. Ropolo, A.M. Pedrini, D. Orioli, G. Frosina, G. Zambruno, M. Dizdaroglu, M. Stefanini, E. Dogliotti, The role of CSA in the response to oxidative DNA damage in human cells, Oncogene 26 (2007) 4336–4343.

[22] J. Tuo, P. Jaruga, H. Rodriguez, V.A. Bohr, M. Dizdaroglu, Primary fibroblasts of Cockayne syndrome patients are defective in cellular repair of 8-hydroxyguanine and 8-hydroxyadenine resulting from oxidative stress, FASEB J. 17 (2003) 668–674.

[23] Y. Homma, M. Tsunoda, H. Kasai, Evidence for the accumulation of oxidative stress during cellular ageing of human diploid fibroblasts, Biochem. Biophys. Res. Commun. 203 (1994) 1063–1068.

[24] T. Kaneko, S. Tahara, T. Taguchi, H. Kondo, Accumulation of oxidative DNA damage, 8-oxo-2′-deoxyguanosine, and change of repair systems during in vitro cellular aging of cultured human skin fibroblasts, Mutat. Res. 487 (2001) 19–30.

[25] I. Schmerold, H. Niedermuller, Levels of 8-hydroxy-2′-deoxyguanosine in cellular DNA from 12 tissues of young and old Sprague–Dawley rats, Exp. Gerontol. 36 (2001) 1375–1386.

[26] T. Lu, Y. Pan, S.Y. Kao, C. Li, I. Kohane, J. Chan, B.A. Yankner, Gene regulation and DNA damage in the ageing human brain, Nature 429 (2004) 883–891.

[27] M. Sekiguchi, T. Tsuzuki, Oxidative nucleotide damage: consequences and prevention, Oncogene 21 (2002) 8895–8904.

[28] H. Maki, M. Sekiguchi, MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis, Nature 355 (1992) 273–275.

[29] T. Tsuzuki, A. Egashira, H. Igarashi, T. Iwakuma, Y. Nakatsuru, Y. Tominaga, H. Kawate, K. Nakao, K. Nakamura, F. Ide, S. Kura, Y. Nakabeppu, M. Katsuki, T. Ishikawa, M. Sekiguchi, Spontaneous tumorigenesis in mice defective in the *MTH1* gene encoding 8-oxo-dGTPase, Proc. Natl. Acad. Sci. USA 98 (2001) 11456–11461.

[30] P. Rai, T.T. Onder, J.J. Young, J.L. McFaline, B. Pang, P.C. Dedon, R.A. Weinberg, Continuous elimination of oxidized nucleotides is necessary to prevent rapid onset of cellular senescence, Proc. Natl. Acad. Sci. USA 106 (2009) 169–174.

[31] C. Colussi, E. Parlanti, P. Degan, G. Aquilina, D. Barnes, P. Macpherson, P. Karran, M. Crescenzi, E. Dogliotti, M. Bignami, The mammalian mismatch repair pathway removes DNA 8-oxodGMP incorporated from the oxidized dNTP pool, Curr. Biol. 12 (2002) 912–918.

[32] M.T. Russo, M.F. Blasi, F. Chiera, P. Fortini, P. Degan, P. Macpherson, M. Furuichi, Y. Nakabeppu, P. Karran, G. Aquilina, M. Bignami, The oxidized deoxynucleoside triphosphate pool is a significant contributor to genetic instability in mismatch repair-deficient cells, Mol. Cell Biol. 24 (2004) 465–474.

[33] K. Fujikawa, H. Kamiya, H. Yakushiji, Y. Fujii, Y. Nakabeppu, H. Kasai, The oxidized forms of dATP are substrates for the human MutT homologue, the hMTH1 protein, J. Biol. Chem. 274 (1999) 18201–18205.

[34] J.P. Cai, T. Ishibashi, Y. Takagi, H. Hayakawa, M. Sekiguchi, Mouse MTH2 protein which prevents mutations caused by 8-oxoguanine nucleotides, Biochem. Biophys. Res. Commun. 305 (2003) 1073–1077.

[35] T. Ishibashi, H. Hayakawa, M. Sekiguchi, A novel mechanism for preventing mutations caused by oxidation of guanine nucleotides, EMBO Rep. 4 (2003) 479–483.

[36] M.L. Tassotto, C.K. Mathews, Assessing the metabolic function of the MutT 8-oxodeoxyguanosine triphosphatase in *Escherichia coli* by nucleotide pool analysis, J. Biol. Chem. 277 (2002) 15807–15812.

[37] Z.F. Pursell, J.T. McDonald, C.K. Mathews, T.A. Kunkel, Trace amounts of 8-oxo-dGTP in mitochondrial dNTP pools reduce DNA polymerase gamma replication fidelity, Nucleic Acids Res. 36 (2008) 2174–2181.

[38] S.S. Hah, J.M. Mundt, H.M. Kim, R.A. Sumbad, K.W. Turteltaub, P.T. Henderson, Measurement of 7,8-dihydro-8-oxo-2′-deoxyguanosine metabolism in MCF-7 cells at low concentrations using accelerator mass spectrometry, Proc. Natl. Acad. Sci. USA 104 (2007) 11203–11208.

[39] A. Katafuchi, A. Sassa, N. Niimi, P. Gruz, H. Fujimoto, C. Masutani, F. Hanaoka, T. Ohta, T. Nohmi, Critical amino acids in human DNA polymerases η and κ involved in erroneous incorporation of oxidized nucleotides, Nucleic Acids Res. 38 (2010) 859–867.

[40] M. Shimizu, P. Gruz, H. Kamiya, C. Masutani, Y. Xu, Y. Usui, H. Sugiyama, H. Harashima, F. Hanaoka, T. Nohmi, Efficient and erroneous incorporation of oxidized DNA precursors by human DNA polymerase η, Biochemistry 46 (2007) 5515–5522.

[41] H.J. Einolf, N. Schnetz-Boutaud, F.P. Guengerich, Steady-state and pre-steady-state kinetic analysis of 8-oxo-7,8-dihydroguanosine triphosphate incorporation and extension by replicative and repair DNA polymerases, Biochemistry 37 (1998) 13300–13312.

[42] K. Bebenek, T.A. Kunkel, Functions of DNA polymerases, Adv. Protein Chem. 69 (2004) 137–165.

[43] A. Kornberg, T.A. Baker, DNA Replication, W.H. Freeman and Co., New York, 1992, pp. 165–182.

[44] C.M. Joyce, W.S. Kelley, N.D. Grindley, Nucleotide sequence of the *Escherichia coli polA* gene and primary structure of DNA polymerase I, J. Biol. Chem. 257 (1982) 1958–1964.

[45] P.A. Ropp, W.C. Copeland, Cloning and characterization of the human mitochondrial DNA polymerase, DNA polymerase gamma, Genomics 36 (1996) 449–458.

[46] J.W. Hanes, D.M. Thal, K.A. Johnson, Incorporation and replication of 8-oxo-deoxyguanosine by the human mitochondrial DNA polymerase, J. Biol. Chem. 281 (2006) 36241–36248.

[47] A.A. Purmal, Y.W. Kow, S.S. Wallace, 5-Hydroxypyrimidine deoxynucleoside triphosphates are more efficiently incorporated into DNA by exonuclease-free Klenow fragment than 8-oxopurine deoxynucleoside triphosphates, Nucleic Acids Res. 22 (1994) 3930–3935.

[48] M. Yamada, T. Nunoshiba, M. Shimizu, P. Gruz, H. Kamiya, H. Harashima, T. Nohmi, Involvement of Y-family DNA polymerases in mutagenesis caused by oxidized nucleotides in *Escherichia coli*, J. Bacteriol. 188 (2006) 4992–4995.

[49] P. Macpherson, F. Barone, G. Maga, F. Mazzei, P. Karran, M. Bignami, 8-oxoguanine incorporation into DNA repeats in vitro and mismatch recognition by MutSα, Nucleic Acids Res. 33 (2005) 5094–5105.

[50] Y.I. Pavlov, D.T. Minnick, S. Izuta, T.A. Kunkel, DNA replication fidelity with 8-oxodeoxyguanosine triphosphate, Biochemistry 33 (1994) 4695–4701.

[51] T. Nohmi, Environmental stress and lesion-bypass DNA polymerases, Annu. Rev. Microbiol. 60 (2006) 231–253.

[52] F. Wang, W. Yang, Structural insight into translesion synthesis by DNA Pol II, Cell 139 (2009) 1279–1289.

[53] P.M. Burgers, Eukaryotic DNA polymerases in DNA replication and DNA repair, Chromosoma 107 (1998) 218–227.

[54] T. Kesti, K. Flick, S. Keranen, J.E. Syvaoja, C. Wittenberg, DNA polymerase epsilon catalytic domains are dispensable for DNA replication, DNA repair, and cell viability, Mol. Cell 3 (1999) 679–685.

[55] S. Prakash, R.E. Johnson, L. Prakash, Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function, Annu. Rev. Biochem. 74 (2005) 317–353.

[56] M. de Vega, M. Salas, A highly conserved tyrosine residue of family B DNA polymerases contributes to dictate translesion synthesis past 8-oxo-7,8-dihydro-2′-deoxyguanosine, Nucleic Acids Res. 35 (2007) 5096–5107.

[57] A.S. Kamath-Loeb, A. Hizi, H. Kasai, L.A. Loeb, Incorporation of the guanosine triphosphate analogs 8-oxo-dGTP and 8-NH2-dGTP by reverse transcriptases and mammalian DNA polymerases, J. Biol. Chem. 272 (1997) 5892–5898.

[58] K. Satou, M. Hori, K. Kawai, H. Kasai, H. Harashima, H. Kamiya, Involvement of specialized DNA polymerases in mutagenesis by 8-hydroxy-dGTP in human cells, DNA Repair (Amst) 8 (2009) 637–642.

[59] M. Shimizu, P. Gruz, H. Kamiya, S.R. Kim, F.M. Pisani, C. Masutani, Y. Kanke, H. Harashima, F. Hanaoka, T. Nohmi, Erroneous incorporation of oxidized DNA precursors by Y-family DNA polymerases, EMBO Rep. 4 (2003) 269–273.

[60] S. Bailey, R.A. Wing, T.A. Steitz, The structure of *T. aquaticus* DNA polymerase III is distinct from eukaryotic replicative DNA polymerases, Cell 126 (2006) 893–904.

[61] M.H. Lamers, R.E. Georgescu, S.G. Lee, M. O'Donnell, J. Kuriyan, Crystal structure of the catalytic alpha subunit of *E. coli* replicative DNA polymerase III, Cell 126 (2006) 881–892.

[62] H. Kamiya, H. Kasai, 2-Hydroxy-dATP is incorporated opposite G by *Escherichia coli* DNA polymerase III resulting in high mutagenicity, Nucleic Acids Res. 28 (2000) 1640–1646.

[63] P.M. Burgers, E.V. Koonin, E. Bruford, L. Blanco, K.C. Burtis, M.F. Christman, W.C. Copeland, E.C. Friedberg, F. Hanaoka, D.C. Hinkle, C.W. Lawrence, M. Nakanishi, H. Ohmori, L. Prakash, S. Prakash, C.A. Reynaud, A. Sugino, T. Todo, Z. Wang, J.C. Weill, R. Woodgate, Eukaryotic DNA polymerases: proposal for a revised nomenclature, J. Biol. Chem. 276 (2001) 43487–43490.

[64] S. Aoufouchi, E. Flatter, A. Dahan, A. Faili, B. Bertocci, S. Storck, F. Delbos, L. Cocea, N. Gupta, J.C. Weill, C.A. Reynaud, Two novel human and mouse DNA polymerases of the polX family, Nucleic Acids Res. 28 (2000) 3684–3693.

[65] M. Garcia-Diaz, O. Dominguez, L.A. Lopez-Fernandez, L.T. de Lera, M.L. Saniger, J.F. Ruiz, M. Parraga, M.J. Garcia-Ortiz, T. Kirchhoff, J. del Mazo, A. Bernad, L. Blanco, DNA polymerase λ (Pol λ), a novel eukaryotic DNA polymerase with a potential role in meiosis, J. Mol. Biol. 301 (2000) 851–867.

[66] K. Nagasawa, K. Kitamura, A. Yasui, Y. Nimura, K. Ikeda, M. Hirai, A. Matsukage, M. Nakanishi, Identification and characterization of human DNA polymerase β 2, a DNA polymerase β-related enzyme, J. Biol. Chem. 275 (2000) 31233–31238.

[67] M. Garcia-Diaz, K. Bebenek, G. Gao, L.C. Pedersen, R.E. London, T.A. Kunkel, Structure-function studies of DNA polymerase lambda, DNA Repair (Amst) 4 (2005) 1358–1367.

[68] H. Miller, R. Prasad, S.H. Wilson, F. Johnson, A.P. Grollman, 8-oxodGTP incorporation by DNA polymerase β is modified by active-site residue Asn279, Biochemistry 39 (2000) 1029–1033.

[69] J.A. Brown, W.W. Duym, J.D. Fowler, Z. Suo, Single-turnover kinetic analysis of the mutagenic potential of 8-oxo-7,8-dihydro-2′-deoxyguanosine during gap-

filling synthesis catalyzed by human DNA polymerases λ and β, J. Mol. Biol. 367 (2007) 1258–1269.
[70] S. Kumar, B.J. Lamarche, M.D. Tsai, Use of damaged DNA and dNTP substrates by the error-prone DNA polymerase X from African swine fever virus, Biochemistry 46 (2007) 3814–3825.
[71] 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.
[72] 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.
[73] V.G. Godoy, D.F. Jarosz, F.L. Walker, L.A. Simmons, G.C. Walker, Y-family DNA polymerases respond to DNA damage-independent inhibition of replication fork progression, EMBO J. 25 (2006) 868–879.
[74] S.J. Culp, B.P. Cho, F.F. Kadlubar, F.E. Evans, Structural and conformational analyses of 8-hydroxy-2′-deoxyguanosine, Chem. Res. Toxicol. 2 (1989) 416–422.
[75] N. Niimi, A. Sassa, A. Katafuchi, P. Gruz, H. Fujimoto, R.R. Bonala, F. Johnson, T. Ohta, T. Nohmi, The steric gate amino acid tyrosine 112 is required for efficient mismatched-primer extension by human DNA polymerase κ, Biochemistry 48 (2009) 4239–4246.
[76] A. Alt, K. Lammens, C. Chiocchini, A. Lammens, J.C. Pieck, D. Kuch, K.P. Hopfner, T. Carell, Bypass of DNA lesions generated during anticancer treatment with cisplatin by DNA polymerase η, Science 318 (2007) 967–970.
[77] R.A. Wing, S. Bailey, T.A. Steitz, Insights into the replisome from the structure of a ternary complex of the DNA polymerase III alpha-subunit, J. Mol. Biol. 382 (2008) 859–869.
[78] C.M. Joyce, T.A. Steitz, Polymerase structures and function: variations on a theme? J. Bacteriol. 177 (1995) 6321–6329.
[79] L.F. Silvian, E.A. Toth, P. Pham, M.F. Goodman, T. Ellenberger, Crystal structure of a DinB family error-prone DNA polymerase from *Sulfolobus solfataricus*, Nat. Struct. Biol. 8 (2001) 984–989.
[80] 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.
[81] G. Maga, G. Villani, E. Crespan, U. Wimmer, E. Ferrari, B. Bertocci, U. Hubscher, 8-oxo-guanine bypass by human DNA polymerases in the presence of auxiliary proteins, Nature 447 (2007) 606–608.
[82] G. Maga, E. Crespan, U. Wimmer, B. van Loon, A. Amoroso, C. Mondello, C. Belgiovine, E. Ferrari, G. Locatelli, G. Villani, U. Hubscher, Replication protein A and proliferating cell nuclear antigen coordinate DNA polymerase selection in 8-oxo-guanine repair, Proc. Natl. Acad. Sci. USA 105 (2008) 20689–20694.
[83] B. Yeiser, E.D. Pepper, M.F. Goodman, S.E. Finkel, SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness, Proc. Natl. Acad. Sci. USA 99 (2002) 8737–8741.
[84] H.J. Einolf, F.P. Guengerich, Fidelity of nucleotide insertion at 8-oxo-7,8-dihydroguanine by mammalian DNA polymerase delta. Steady-state and pre-steady-state kinetic analysis, J. Biol. Chem. 276 (2001) 3764–3771.