Use of shuttle vectors to study the molecular processing of defined carcinogen-induced DNA damage: mutagenicity of single O⁴-ethylthymine adducts in HeLa cells

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ABSTRACT

We developed a simian virus 40 based shuttle vector system to study the molecular consequences of distinct carcinogen-induced DNA lesions in human cells. To establish the mutagenicity of O⁴-ethylthymine adducts, oligonucleotides carrying a single O⁴-ethylthymine adduct at a unique position were ligated into the vector molecules. Following replication in HeLa cells on average 23% of the progeny molecules carried a mutation in the region of modification. The vast majority of these mutations represented single T → C transitions at the position of the modified base, most probably as a consequence of mispairing of the O⁴-ethylthymine residues during replication. To a minor extent the O⁴-ethylthymine adduct may also induce T → A transversions or double point mutations. The *in vivo* mutation frequency of the adduct was found to be comparable to that of a C-A mismatch at the same position, but was lower than that expected from *in vitro* experiments with adducted DNA templates and purified DNA polymerases.

INTRODUCTION

Alkylating agents can initiate neoplastic transformation through the binding of their reactive forms to DNA (1). Adducts may be formed at the ring-nitrogen atoms and exocyclic oxygen atoms of all four DNA bases and at the oxygen atoms of the internucleotide-phosphate bonds. Hence, usually a multitude of structurally distinct DNA lesions result from a single reaction (2,3).

The individual alkylation products are thought to possess different mutagenic and carcinogenic properties. This may be partly attributed to differences in the way the adducts are handled by DNA repair mechanisms. In many mammalian cell types and also in bacteria, alkyl adducts formed at the N³- and N⁷-position of purines are efficiently repaired by the

3-methyladenine-DNA glycosylase, while O⁶-alkylguanine (O⁶-alkylG) adducts are removed by the O⁶-alkylguanine DNA alkyltransferase (3,4,5). The bacterial glycosylase and alkyltransferase also repair O²-alkylthymine-, O²-alkylcytosine- and O⁴-alkylthymine (O⁴-alkylT) adducts (6). In contrast, these adducts are not a substrate for the corresponding mammalian enzymes (5). Moreover, a (relative) high persistency of these adducts has been observed in slowly dividing tissues (5,7,8).

A number of *in vitro* experiments clearly illustrated the differences in miscoding potential for different alkylated bases with replication on synthetic DNA templates (3,9). From these experiments both O⁴-alkylT and O⁶-alkylG residues have been implicated as strong miscoding bases (10,11). The *in vivo* mutagenicity of O⁶-alkylG adducts has been determined for bacterial cells and Chinese Hamster Ovary cells (12,13). In both cases mutations were only obtained in repair deficient cells. The genetic alterations induced by the distinct O⁶-methylG and O⁶-ethylG adducts were almost exclusively G → A transitions. The *in vivo* mutagenicity of O⁴-alkylT adducts has only been described for repair deficient (*ada*⁻) *E.coli* cells (14). In these studies the distinct O⁴-methylT and O⁴-ethylT (O⁴-EtT) adducts directed exclusively T → C transitions. Yet, no data exist on the specific mutagenicity of these adducts in mammalian cells. In these cells the persistency of the distinct O⁴-alkylT adducts varies considerably with the size of the alkyl group (5,7). This may reflect differences in the molecular processing of the individual adducts and as a consequence, also their mutagenicity may vary with adduct size. Therefore, the O⁴-alkylT adducts should be studied individually in order to obtain unambiguous information about their carcinogenicity.

The *in vivo* mutagenicity of the above mentioned adducts has been determined using (*in vitro* adducted) shuttle vectors. Also, various other (mainly SV40 based) vector systems have been successfully used to study the mutational spectrum of carcinogenic compounds in mammalian cells (15–19). However, up till now no system was available to investigate the effects of the individual

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carcinogen-induced DNA lesions in human cells. Using a SV40-pAT153 derived shuttle vector (pSVsupF), we have now examined the mutagenic specificity of distinct O⁴-EtT adducts in HeLa cells. Modification of the plasmids was achieved by ligating oligonucleotides that contained a single O⁴-EtT adduct into the vector molecules. The resulting plasmids all carried just one adduct at a defined position. With this system we could clearly determine the frequency and nature of the O⁴-EtT induced mutations in HeLa cells.

MATERIALS AND METHODS

Enzymes

The restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, Klenow enzyme, spleen phosphodiesterase, alkaline phosphatase, nuclease P1 and Venom phosphodiesterase were obtained from Boehringer Mannheim. The micrococcal endonuclease, apyrase and RNase A were from Sigma and DNase I was from Cooper Biomedical. These enzymes were used as recommended by the manufacturers or as described below. The *E. coli* O⁶-methylguanine DNA methyltransferase was obtained from Applied Genetics and used according to Green *et al* (20).

Cells

Human HeLa cells were obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% fetal calf serum, 10 IU/ml penicillin and 10 µg/ml streptomycin.

E. coli DH5α cells were obtained from Gibco-BRL as Hanahan competent cells (maximum transformation efficiency 10⁹/µg DNA) and used as prescribed by the supplier. The CaCl₂ competent DH5 cells were made and used according to standard procedures (21). The transformation efficiency was 10⁶–10⁷/µg DNA.

Shuttle vector

The shuttle vector pSVsupF was constructed from the plasmids pSVCA-1 (kindly provided by Dr. J.L.M.C. Geelen, Amsterdam

Medical Centre), piAN7 (a gift from Dr. H.V. Huang, Washington University), pIC-20R (22) and pAT 153 (23). The structure is given in figure 1.

Synthesis of the oligonucleotides

The modified 16-mer 5'-d(AATTCA(O⁴-Et)TCGATATCTA)-3' (with the O⁴-EtT adduct in the ClaI recognition sequence) and the unmodified 16-mers having the wild type and mutant sequences 5'-d(AATTCATCGATATCTA)-3' and 5'-d(AATTCACCGATATCTA)-3' were prepared in the laboratory of J.H. van Boom using solid phase phosphoramidite chemistry with alternative deprotection conditions (24,25).

Prior to ligation into the shuttle vector, the oligonucleotides were purified using reversed-phase HPLC on a PEP RPC column (Pharmacia-LKB). Elution was in a 0.1 M triethylammonium acetate buffer (pH 7.0) with a linear gradient of (5–30%) acetonitrile.

Analysis of the nucleotide composition of oligonucleotides

The modified oligonucleotide preparation was analyzed for the presence of de-ethylated 16 mers with anion exchange HPLC on a Mono Q column eluting with 10 mM NaOH (pH 12) and a linear gradient of 0.5–0.7 M NaCl (figure 3).

The composition of the oligonucleotides was verified by enzymically hydrolyzing 10 µg oligonucleotide to nucleosides using a modification of the procedure described by Crozatier *et al* (26). In addition to DNase I, alkaline phosphatase and nuclease P1, 12 mU Venom phosphodiesterase were added per reaction. Nucleosides were separated on a Supelco LE-18C reversed phase HPLC column. Elution was in a buffer of 40 mM ammonium acetate, 1% methanol (pH 4.0) with a linear gradient of 0–50% acetonitrile (figure 3). The O⁴-EtT adduct was identified both by its UV absorbance spectrum and by comigration with the reference O⁴-ethylT nucleoside.

Fig. 1 Schematic representation of shuttle vector pSVsupF. It contains the total early region of replication from Simian virus 40 (SV40 ori) and the pAT153 origin of replication (ori) and β -lactamase gene (Amp^r) from *E. coli*. The *E. coli* supF gene, coding for a tyrosine suppressor tRNA, was inserted as an additional marker. The O⁴-EtT adducts were located at the unique ClaI site in the polylinker (position indicated with *).

Fig. 2 Nucleotide composition of the oligonucleotides as determined by ³²P-postlabeling analysis (see Material and Methods). (a) Autoradiogram of the unmodified oligonucleotide (carrying the wild type sequence), (b) autoradiogram of the O⁴-EtT modified oligonucleotide.

The nucleotide composition was also determined more sensitively by ^{32}P -postlabeling analysis in order to detect minor amounts of additional modifications. Oligonucleotides were digested to 2'-deoxyribonucleotides 3'-monophosphates and labeled with excess $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ according to Gupta (27). Nucleotides were resolved by multidirectional anion-exchange chromatography on polyethyleneimine cellulose thin layers (PEI-TLC; Machery-Nagel). The directions of chromatography (figure 2) and the solvent systems were: (D1) 0.25 M LiCl to remove

$^{32}\text{P}_i$; (D2) 1.5 M ammonium formate pH 3.5; (D3) 0.12 M NaH_2PO_4 pH 6.0. Spots containing labeled nucleotides were identified by autoradiography, excised from the thin layers and counted. The percentage of aberrant bases was calculated as being the radioactivity present in background spots divided by the total radioactivity counted.

Detection of 5-methylcytidine in oligonucleotides

^{32}P -Labeled 3', 5'-biphosphate nucleotides were converted to 5'- ^{32}P monophosphate nucleotides by digestion with nuclease P1 (28). Nucleotides were resolved by 2D chromatography on cellulose sheets (Machery-Nagel, Polygram Cel-300). Development in D1 was with isobutyric acid/ water/ ammonia, 66 : 20 : 1 (29). Development in D2 was with saturated ammonium sulphate/ isopropanol/ 1M NaAc, 80 : 2 : 18 (30). Radioactive spots were located and counted as described above. The 5-methylcytidine (5-meC) content was determined from the relative ratios of the amount of radioactivity measured in the spots representing 5-meC and C.

Introduction of the oligonucleotides into the shuttle vector

Oligonucleotides were inserted into the shuttle vector using a modification of the gapped duplex method as described by Essigmann and coworkers (20,31). pSVsupF (200–300 μg) was digested with EcoRI and BglII to remove the endogenous polylinker and mixed with an equimolar amount of the vector that had been digested with BamHI. The DNA was diluted to a concentration of 20 $\mu\text{g}/\text{ml}$ in 50 mM Tris-HCl, 2 mM EDTA, 0.5 M NaAc (pH 7.5) and 50% v/v deionized formamide. Denaturation and reannealing was achieved by heating for 5 min. at 80 °C and slow cooling from 60°C to 30°C, respectively. The DNA was precipitated with ethanol and the gapped duplexes were separated from ds linear molecules by electrophoresis on 1% agarose-gels (Biorad standard low m_r agarose). Gapped duplex DNA was recovered by electro elution using a buffer of 12.5 mM Tris-boric acid (pH 8.0), 1.5 mM EDTA and extracted with phenol. Phosphorylation of the oligonucleotides and ligation into the gapped duplex DNA was performed essentially as described by Green *et al* (20) and Naser *et al* (31). The covalently closed molecules were separated from the nicked or gapped duplexes by CsCl-EtBr gradient centrifugation in a Beckman VTi80 or VTi65.2 rotor.

Transfection and reisolation of the vector molecules

HeLa cells were seeded in 60 mm culture dishes. Transfection was performed 24 h later at approximately 50% confluency with 2 to 10 μg vector DNA using the modified calcium-phosphate precipitation technique as described by Chen and Okayama (32). The precipitate was left on the cells for 15 h and removed by washing twice with fresh medium. The cells were lysed 72 h after the start of transfection using, per dish, 0.5 ml lysis-buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM EDTA and 1% SDS. The plasmid DNA was isolated from the cell lysate using a modification of the Hirt protocol (33) and finally digested with DpnI to remove unreplicated material.

Selection and analysis of the mutants

A portion of the DNA isolated from HeLa cells was cleaved with ClaI and used to transform *E. coli* DH5 α cells along with an equal portion of undigested material. The mutation frequency was calculated from the ratio of the number of clones obtained in both transformations. Mutant DNA's (48–96 per construct) were

Fig. 3 HPLC of unmodified and $\text{O}^4\text{-EtT}$ adducted oligonucleotides. (a) Analysis of the $\text{O}^4\text{-EtT}$ modified oligonucleotide on an anion exchange Mono Q column. Elution was in a 10 mM NaOH solution (pH 12) with a linear gradient of 0.5–0.7 M NaCl. (b) The same analysis after addition of an equal amount of the corresponding unmodified oligonucleotide. The oligonucleotides were hydrolyzed to nucleosides as described in Materials and Methods. (c) Hydrolysate of the unmodified oligonucleotide. (d) Hydrolysate of the $\text{O}^4\text{-EtT}$ containing oligonucleotide. (e) Hydrolysate of the adducted oligonucleotide with the $\text{O}^4\text{-EtT}$ nucleoside added as internal reference.

reisolated from the DH5 α cells. Part of this DNA was digested with ClaI and analyzed by agarose gel electrophoresis to ensure the presence of a mutation at the ClaI site. Further analysis was performed by sequencing 12–40 of the mutant DNA's using the chain termination ('dideoxy') method on ds plasmid DNA (34).

RESULTS

Purity of the oligonucleotides

We wanted to be certain that oligonucleotides ligated into the vector (figure 1) did not contain groups that could induce additional mutations and therefore interfere with the mutation spectrum of the adduct under investigation (e.g. protecting groups that are not completely removed from the oligonucleotides upon deprotection). The percentage of aberrant nucleotides, as determined by 32 P-postlabeling analysis was 0.2–0.3% per 16-mer for both the modified and unmodified oligonucleotide preparations (figure 2).

We also considered the possible conversion of the O⁴-EtT moiety into either 5-methylcytidine (5-meC) or T, which has been reported to occur during deprotection of oligonucleotides carrying O⁴-alkylT residues (35,36). To prevent this, a 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU)/waterfree ethanol mixture was used as an alternative for the concentrated ammonia normally used for deprotection. An independent 32 P-postlabeling analysis was performed to detect 5-meC, taking ss ϕ X174 DNA as a reference. This DNA has been reported to contain one 5-meC out of 1155 normal C's (0.09%) (37). In our analysis, the amount of 5-meC in ss ϕ X174 DNA was found to be 0.3% of the total C content, which was 10-fold higher than the background level of 0.03%. The 5-meC/C ratios of the unmodified oligonucleotides (having the wild type sequence) and ethylated oligonucleotides were 0.05% and 0.11%, respectively (that is 0.15% and 0.33% per single nucleotide, since there are 3 C's present per oligonucleotide). This indicates that at most 0.18% of the O⁴-EtT nucleotide had been converted into 5-meC.

Finally, O⁴-alkylT residues are both acid and alkali labile moieties (35,36,38). Loss of the ethyl group could have occurred during deprotection and purification of the O⁴-T ethylated oligonucleotide with the alkaline conditions used. Anion exchange and reversed phase HPLC analysis showed that the O⁴-EtT residues had not been de-ethylated to a detectable extent (figure 3).

Induction of mutations during replication of the vector in HeLa cells

We performed a series of control experiments in order to ensure that the mutations obtained were indeed generated during replication of the vector molecules in HeLa cells.

A small portion of every vector preparation was directly used to transform *E. coli* DH5 α cells. The number of colonies obtained from this material generally exceeded 10^8 per μ g DNA (data not shown), while no transformants were ever obtained from an equal amount of DpnI digested material. This shows that the DpnI digestion is sufficient to exclude all unreplicated material from further analysis. In addition, vector DNA was isolated from 94 individual transformants obtained from the uncleaved material. Analysis of the ClaI digestion patterns on agarose gel revealed that none of the clones carried a mutation at the ClaI site. From this we concluded that unreplicated plasmids, that for some reason may have escaped the DpnI selection, will not significantly (less than 1%) contribute to the observed mutagenicity.

Fig. 4 Time course of the transient replication of the pSVsupF shuttle vector in HeLa cells. Ten μ g portions of the plasmid were transfected into these cells. The plasmid DNA was reisolated at different time points (see Materials and Methods). Half of the DNA was then digested with DpnI, while the other half was left undigested before transformation of *E. coli* DH5 cells. — Number of transformants (per μ g transfected DNA) obtained from undigested material. ---- Number of transformants obtained from DpnI digested material. After $\pm$ 48 hours the numbers of colonies obtained from both DNA samples were approximately the same (indicated with — — —)

Table I. Mutation frequencies at the ClaI site of various vector preparations transfected into HeLa cells. A. represents unmanipulated plasmids, B. constructs containing the O⁴-EtT adduct, C. plasmids containing either an oligonucleotide from which the adduct had been removed by treatment with the O⁶-methylguanine DNA methyltransferase (MT) or the corresponding unmodified oligonucleotide, D. constructs carrying a C-A mismatch at the position of modification.

Transfected construct	Mutation frequency (% ClaI resistant clones)	No. of <i>E. coli</i> colonies per μ g transfected DNA
A. unmanipulated	0	4200
"	0	1900
"	0	4300
"	0	1700
B. + O ⁴ -EtT	23	2100
"	19	2800
"	27	6900
C. + unmodified oligo	2	3400
O ⁴ -EtT removed by MT	4	6600
D. O ⁴ -EtT replaced by C	19	1900
"	30	1100
"	11	1100

Time course experiments were conducted in which 10 μ g portions of the plasmid were transfected into HeLa cells and reisolated at different time points. Half of the isolated DNA was digested with DpnI, while the other half was left untreated before transformation of CaCl₂ competent *E. coli* DH5 cells. Figure 4 shows the number of *E. coli* colonies obtained in two independent experiments. The number of transformants obtained from the undigested material decreased dramatically during the first 24 hours after transfection. The number of colonies obtained from DpnI digested material steadily increased with time and equalled the number of transformants from the undigested material at approximately 48 hours after transfection.

Fig. 5 Mutagenic specificity of O⁴-EtT induced mutations in HeLa cells. The site of modification of vector pSVsupF is shown along with the various restriction enzyme sites present in this region. The position of the adduct is indicated with *. The cleavage sites for the various restriction enzymes are indicated with arrows (solid for the adducted strand and stippled for the complementary strand). Mutations were selected and analyzed as described in Materials and Methods. (a) Distribution of mutations over the polylinker region of vectors carrying the adduct (+ O⁴-EtT) or containing a control oligonucleotide (- O⁴-EtT). The absolute mutation frequencies are depicted on the vertical axis. Open bars represent single T→C transitions. Other mutations are indicated by solid bars. (b) Sequence specificity of the mutations derived from constructs that contained the adduct (+ O⁴-EtT) or the control oligonucleotides (- O⁴-EtT). Open boxes represent single or multiple base pair deletions. The vector preparation from which a part had been treated with the O⁶-methylguanine DNA methyltransferase in order to remove the O⁴-EtT adducts, is indicated with **.

Mutations induced by O⁴-ethylthymine adducts

Plasmids containing the single O⁴-EtT adduct at the Cla I site were passed through the HeLa cells, reisolated and digested with Dpn I to remove unreplicated material. Selection of the mutants, calculation of the mutation frequency and analysis of the mutations was carried out as described below. In addition, we investigated the type of background mutations that had been introduced either by the way of transfection or by the insertion of the oligonucleotides. A similar analysis was conducted with DNA preparations that carried a control oligonucleotide (that is an unmodified oligonucleotide having the wild type sequence or an oligonucleotide from which the adduct had been removed by

treatment with the *E. coli* O⁶-methylguanine DNA methyltransferase prior to transfection). Also, in all experiments a parallel analysis with the unmanipulated vector was included. Finally, since the presence of the O⁴-ethyl group was likely to disturb the normal T-A basepairing, as a comparison constructs were also transfected that contained a C-A mispair. For these constructs only the mutation frequency was determined. Data are given in table I and figure 5.

The initial background mutation frequency of the unmanipulated vector ranged from 0.1% to 4%, as was estimated from the number of *E. coli* transformants obtained from Cla I digested DNA isolates. Upon further analysis it appeared that none of the vector clones carried a real mutation since they could

all be cleaved by Cla I. This reduces the spontaneous mutation frequency to less than 0.02–0.15%. Sequence analysis of a few of the clones was consistent with the restriction enzyme analysis. None of the sequenced DNAs had a mutation in the polylinker region. (Data not shown.)

Vectors containing a control oligonucleotide yielded a Cla I mutation frequency of 2% and 4% respectively. In both cases, 0.5–1.5% represented single point mutations or 1 bp deletions, while 1–1.5% consisted of multiple, clustered base pair substitutions and small deletions. Among the progeny of the de-ethylated vector preparation also 2% T → C transitions were found. Most likely, these mutations were induced by O⁴-EtT adducts that had not been removed upon treatment with the DNA methyltransferase.

Vector preparations that carried the O⁴-EtT adduct obtained a Cla I mutation frequency of 19–27%. The majority (17–24%) represented single T → C transitions; in 1–5% the T → C transition was combined with a second base pair substitution or a T → A transversion or 1 bp deletion was found at the position of the modified base. The remaining 1–3% consisted again of multiple base pair substitutions combined with small deletions. These mutations had a similar pattern to those observed for plasmids containing a control oligonucleotide. We therefore concluded that they were not induced by the adduct.

Plasmids that contained the C-A mismatch yielded a mutation frequency of 11–30%, which is within the same range as the mutation frequencies obtained for the adducted plasmids. (Table I)

DISCUSSION

O⁴-Alkylthymine adducts have been implicated as strong mutagenic and carcinogenic lesions (3,9). However, no specific information is available on the mutagenicity of these adducts in human cells. Therefore, we constructed a shuttle vector system to investigate the molecular processing of the individual O⁴-alkylT lesions. Here we report on the mutagenic specificity of O⁴-EtT lesions in HeLa cells.

The mutational spectra obtained from vector molecules containing both modified and control oligonucleotides, clearly show the mutagenic effects of O⁴-EtT adducts in these cells (figure 5). A high mutation frequency (on average 23%) was found for vectors carrying the adduct. The majority of the mutations (on average 19%) consisted of single T → C transitions at the position of the modified base. This is the same as was found with both *in vitro* replication experiments using O⁴-EtT adducted DNA templates (3,9) and with *in vivo* experiments where ϕ X174 molecules containing site specific O⁴-alkylT residues were allowed to replicate in *ada*⁻ *E.coli* cells (14). In these experiments exclusively dGs were incorporated opposite O⁴-EtT residues, which resulted in the observed T → C transitions. It is not yet clear whether the minor amounts of double point mutations, T → A transversions and 1 bp deletions all represent O⁴-EtT induced mutations. Some of these mutations may also represent background mutations, since 1 bp deletions and transversions were also found among progeny of vectors containing a control oligonucleotide. However, these mutations were not found with unmanipulated plasmids. Therefore, their occurrence seems to be coupled to the incorporation of the oligonucleotides. In addition, the absolute frequency of these mutations appears to be slightly higher for modified plasmids than for the controls.

A small percentage of the mutations consisted of multiple point mutations and small deletions. These mutations were found both for modified vector preparations as well as for vectors containing a control oligonucleotide (figure 5). Therefore, they are related to the incorporation of the oligonucleotides and we favour the explanation that they arise as a consequence of an aberrant ligation reaction of the gapped duplex DNA. With the construction of the modified plasmids two types of gapped duplexes are being formed carrying the 16 b gap in either of the two strands. Both types of gapped duplexes were present during ligation with T4 DNA ligase. In this reaction a small amount of the duplexes that carried the gap in the unadducted strand, might have been converted into covalently closed molecules containing a 16 b ss loop. This feature of the ligase has also been described by others (31,39). Consistently, all the multiple mutations are located within the region that is covered by the resulting 16 b loop.

Every plasmid that had a modified oligonucleotide incorporated also contained an O⁴-EtT adduct. HPLC analysis (figure 3) showed that after deprotection and purification all modified oligonucleotides still carried the O⁴-EtT adduct. Therefore it is unlikely that a significant loss of ethyl groups would have occurred during the construction of the modified vector molecules. Moreover, adducted plasmids could not be linearized upon digestion with ClaI, which indicated the presence of a modification at the ClaI site. (Data not shown). Hence, the obtained mutation frequency truly reflects the miscoding frequency of the O⁴-EtT adduct in HeLa cells.

The mutation frequency was lower than was expected on the basis of the *in vitro* replication experiments mentioned above (3,10). Instead, the O⁴-EtT induced mutation frequency appeared to be comparable to that of a C-A mismatch at the same position. Therefore, the O⁴-EtT-A bp seems to mimic a C-A mismatch in this system. This is consistent with studies of Brennand *et al* which indicated that O⁴-MeT behaves as cytidine during DNA replication (40). At this point we cannot explain the discrepancy between the *in vivo* and *in vitro* mutation frequencies and we do not know to what extent the observed mutation frequencies are the result of repair or replication. By using this system it is now possible to determine the mutagenic effects of other (O⁴-alkylT) adducts and to define alterations in repair and replication functions in a variety of cell lines.

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