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ABSTRACT

We have developed and validated a novel site-specific mutagenesis assay, termed SSMA-MS, which incorporates MALDI-ToF mass spectrometry (MALDI-MS) analysis as a means of determining the mutations induced by a single DNA adduct. The assay involves ligating an adducted deoxyoligonucleotide into *supF* containing pSP189 plasmid. The plasmid is transfected into human Ad293 kidney cells allowing replication and therefore repair or a mutagenic event to occur. *Escherichia coli* indicator bacteria are transformed with recovered plasmid and plasmids containing the insert are identified colorimetrically, as they behave as frameshift mutations. The plasmid is then amplified and digested using a restriction cocktail of Mbo1 and Mnl1 to yield 12 bp deoxyoligonucleotides, which are characterized by MALDI-MS. MALDI-MS takes advantage of the difference in molecular weight between bases to identify any induced mutations. This analysis method therefore provides qualitative and quantitative information regarding the type and frequency of mutations induced. This assay was developed and validated using an O⁶-methyl-2'-deoxyguanosine adduct, which induced the expected GC→AT substitutions, when replicated in human or bacterial cells. This approach can be applied to the study of any DNA adduct in any biologically relevant gene sequence (e.g. p53) in human cells and would be particularly amenable to high-throughput analysis.

INTRODUCTION

Many structural and biological features influence the mutagenic potential of a genotoxic chemical. The chemical structure of the DNA adduct including stereochemistry, can have a major affect on repair and mutagenesis (1). Furthermore,

the DNA sequence surrounding an adduct can have a profound influence, probably due in part, to effects on the 3-dimensional structure of the adduct or interference with base pairing and the resulting DNA distortions. For example, the bulky C8-deoxyguanosine adduct of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) has been shown to cause significant bending of the double helix (2). In contrast, methylation of the N7 position of deoxyguanosine has almost no effect on DNA conformation whilst alkylation of the O⁶ group of deoxyguanosine has severe structural consequences due to alterations in hydrogen bonding (3). Changes in sequence context, even at bases distant to the site of adduction can have effects on the biological outcomes (4,5). Consequently, the mutagenicity of a particular adduct may often be dependent on the gene it is present in. It is therefore important to be able to study the repair and mutagenicity of individual structurally defined adducts in numerous sequence contexts, preferably in genes relevant to human cancer.

Many methods have been developed to investigate mutagenesis using synthetic oligonucleotides containing single DNA adducts and these can be grouped according to whether replication occurs in bacterial or mammalian cells and whether the adduct is situated in double or single stranded DNA. Numerous assays have been based on the M13 viral genomes containing a single DNA adduct, which are then transfected into *Escherichia coli*. The resulting mutations can be detected with a variety of methods, such as DNA sequencing (6), hybridization (7) or REAP analysis (8–10). Although methods like this increase our understanding of the mutagenicity of DNA lesions in *E.coli* in single stranded DNA, they do not pertain to mammalian systems and in particular human cells.

In order to investigate the mutagenicity of DNA adducts in mammalian systems a variety of methods have been developed. One such assay, using simian kidney (COS-7) cells has been used to investigate the mutagenic properties of a wide range of lesions (11). This method uses a single stranded shuttle vector which has the DNA adduct of choice (in this case dG-N²-tamoxifen) inserted. It was shown that these lesions induced primarily GC→TA transversions in this system, as expected for a bulky type DNA adduct and

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is consistent with mutation assays which use human cells (12,13).

Other types of approaches using DNA duplexes (14) and shuttle vector plasmids (15,16) have allowed the investigation of how various DNA repair enzymes cope with DNA lesions, specifically those derived from 2-acetylaminofluorene. For example, a recent report characterized the involvement of DnaE in mutagenesis; due to its lack of proof-reading functions, low processivity and relaxed active site, it can bypass lesions which would generally block high fidelity polymerases (17). A novel plasmid based method for homologous recombination (18) has also been recently added to the growing array of mutagenesis assays.

The *supF* assay (19,20) is another shuttle vector based forward mutation assay which has been widely used to investigate mutagenesis from a variety of carcinogenic compounds (4,5,12,13,21–27), mainly due to the benefit of its applicability to human cells. This assay is, however, not site specific as it involves treating a double stranded plasmid with a reactive compound to generate an array of adducts at a variety of positions throughout the plasmid. The assay does detect 97% of possible base substitutions within the 85 bp *supF* gene (22) as well as deletions and insertions and because the plasmid is treated *in vitro*, aliquots of the treated DNA can be analysed for adduct quantification in parallel to the mutation assay. The *supF* gene is however, a non-essential sequence, so any mutations which are induced would not affect the cell survival or other pathways of carcinogenesis. The use of CpG methylated plasmid DNA means that the pattern of mutations in the *supF* gene can be transformed to a possible p53 mutation spectrum using an algorithm described by Lewis and Parry (28). Methods such as the standard *supF* assay are therefore extremely useful for looking at the range of mutations which can be induced by a particular chemical and predicting mutations in other relevant genes. They cannot, however, distinguish the mutational potential of the different DNA adducts formed. To do this a single adducted deoxyoligonucleotide would need to be inserted at a defined place in the plasmid. In addition, the *supF* assay relies on DNA sequencing of recovered plasmid, either using labeled PAGE electrophoresis or automated methodologies and these systems are both time consuming and expensive.

In order to address these limitations we describe in this manuscript a novel site-specific mutation assay which is an adaptation of the *supF* assay routinely used in our laboratory and benefits from the use of MALDI-ToF mass spectrometry (MALDI-MS) for the characterization of mutations. Synthetic deoxyoligonucleotides containing an O⁶-methyl-deoxyguanosine (O⁶-MedG) were inserted into the *supF* gene of the pSP189 plasmid (19,20). When the plasmid is transfected into and recovered from, human cells the presence of the synthetic insert acts as a frameshift mutation, thus inactivating the *supF* gene, resulting in the production of white mutant colonies (Figure 1). PCR followed by digestion with Mbo11/Mnl1 yields small (12 base) double stranded deoxyoligonucleotides which were then analysed by MALDI-MS (Figure 2). The increased throughput and reduced cost of using mass spectrometry for analysis will allow the rapid screening of the mutations induced by multiple DNA lesions produced by a single compound in different sequence

contexts. This technique can ultimately provide insight into the initiation processes of carcinogenesis.

MATERIALS AND METHODS

Materials

All chemicals were from Sigma (Poole, Dorset, UK) unless otherwise stated.

Shuttle vector plasmid, bacterial strain and cell lines

The plasmid pSP189 containing the *supF* gene (19,20) and *E.coli* strain MBM7070 were gifts from M. Seidman, National Institute of Aging, NIH, Baltimore, MD, USA. Human embryonic adenovirus-transformed kidney cells (Ad293) were cultured from cells previously provided by Dr A. Dipple, National Cancer Institute, Frederick, MD, USA. Ad293 cells were grown in DMEM, supplemented with 10% fetal calf serum at 37°C, in 5% CO₂ in air.

Deoxyoligonucleotides

Deoxyoligonucleotide inserts (INS1A and INS1B, where G is the proposed site of adduction for adduct containing deoxyoligonucleotides), mass spectrometry standards (STANDARD1 through to STANDARD8) and PCR primers ALMS3a and 4 were synthesized by Biomers.net GmbH (Ulm, Germany). The O⁶-MedG containing deoxyoligonucleotide was synthesized by Sigma-Genosys (Haverhill, Suffolk). **INS1A:** CTCCTCGCTCTTC; **INS1B:** GAAGA-GUGAGGAAG; **STANDARD1:** TCCTCACTCTTC; **STANDARD2:** AAGAGTGAGGAA; **STANDARD3:** TCCTCGCTCTTC; **STANDARD4:** AAGAGCGAGGAA; **STANDARD5:** TCCTCCCTCTTC; **STANDARD6:** AAGAGGGAGGAA; **STANDARD7:** TCCTCTCTCTTC; **STANDARD8:** AAGA-GAGAGGAA; **ALMS3A:** GAA CCT TCG AAG TCG ATG ACG GCA GAT TTA GAG TCT GCT CCC TTT GGC CG, **ALMS4:** CTC GAG CTG TGG TGG GGT TCC CGA GCT.

Two versions of the INS1A insert were synthesized; one with a deoxyguanosine and one with an O⁶-MedG deoxyoligonucleotide. INS1B contains a uracil which will be situated opposite the variable G site of INS1A during vector construction and transfection, to encourage translesion synthesis (29) rather than damage avoidance and replication of the complementary strand.

Prior to use, all deoxyoligonucleotides were desalted by precipitation using 5 µl 3 M sodium acetate and 100 µl ethanol, per optical density unit (ODU). The deoxyoligonucleotides were then briefly vortexed and stored at –20°C overnight. The desalted deoxyoligonucleotides were recovered by centrifuging for 5 min at 14 000 r.p.m. at 4°C and removing the supernatant. Pellets were re-suspended twice more in 70% ethanol (800 µl), centrifuged (5 min at 14 000 r.p.m. at 4°C) and the supernatant removed. Finally the pellets were washed with 100% ethanol (100 µl per ODU) and centrifuged, as before, then the supernatant was removed and traces of solvent were removed by vacuum centrifugation of the deoxyoligonucleotide pellet (Savant Industries Inc., Farmingdale, NY, USA). The desalted deoxyoligonucleotides were dissolved in 100 µl sterile water and the concentration

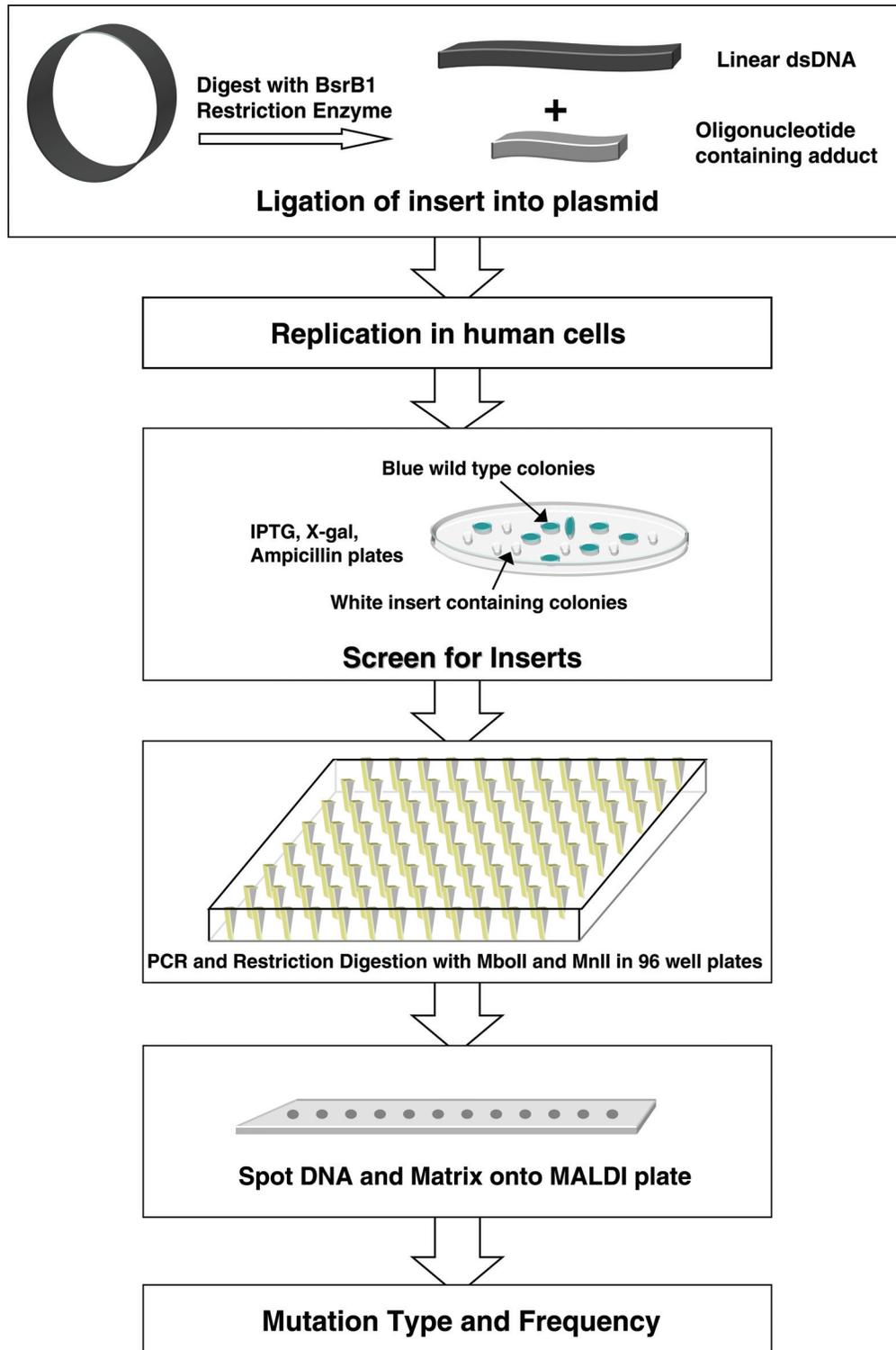


Figure 1. Outline of MALDI-MS site-selective mutation assay (SSMA-MS).

was calculated based on the optical density at 260 nm. The inserts (INS1A/B) were diluted to ~2–10 nmol/μl and the PCR primers (ALMS3A/ALMS4) to 50 pmol/μl with tissue culture grade water and stored at -20°C prior to use. Mass spectrometry standards were stored dry at -20°C until required.

Digestion of pSP189 plasmid

To enable insertion of the deoxyoligonucleotides, the pSP189 Plasmid (10 μg, 3 pmol) was digested with BsrB1 restriction enzyme [40 U; New England Biolabs (UK) Ltd, Hitchin, UK] in NEBuffer 2 [50 mM sodium chloride, 10 mM Tris-HCl,

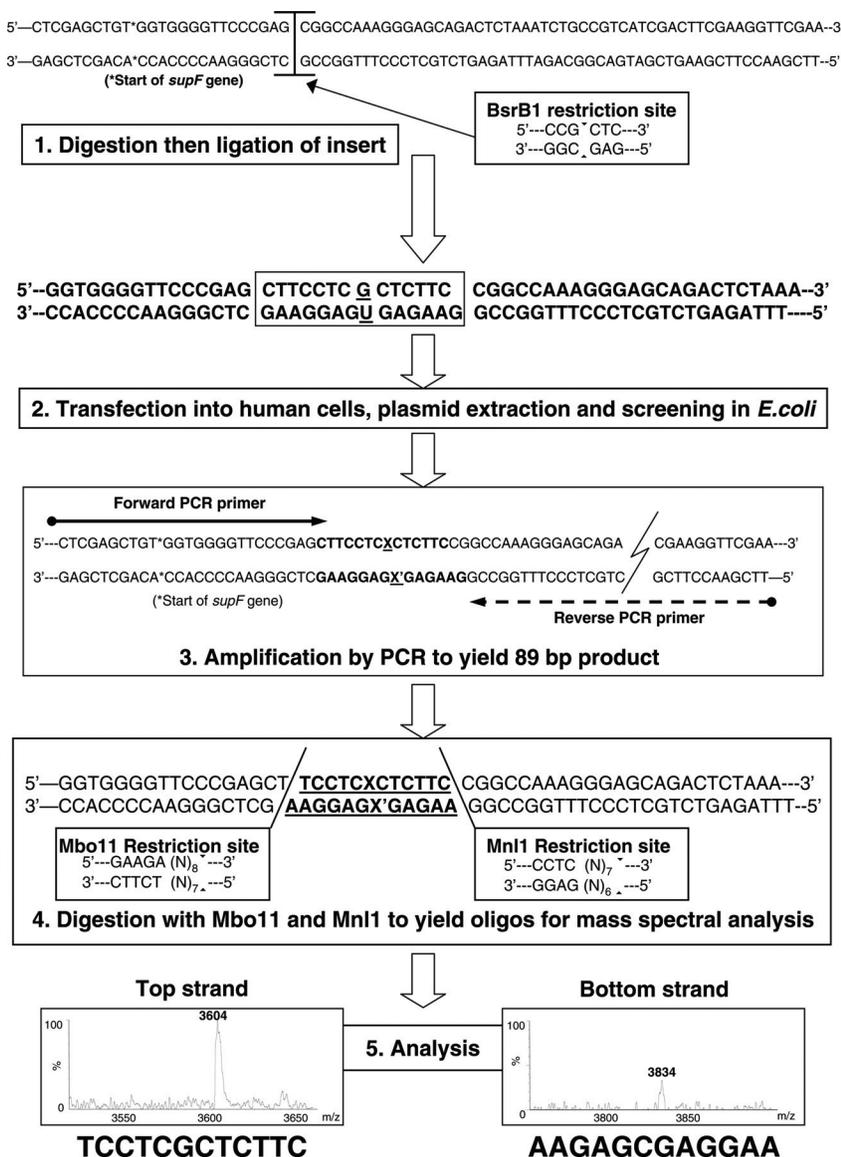


Figure 2. Flow diagram of methods for MALDI-MS site-selective mutation assay (SSMA-MS).

10 mM magnesium chloride, 1 mM DTT (pH 7.9 at 25°C)] supplemented with 100 µg/ml BSA, in a total volume of 50 µl, for 2 h at 37°C. BsrB1 cleaves at the GAG CGG restriction site producing blunt ended linear plasmid as shown in Figure 2. The restriction enzyme is not inactivated or removed post digestion to reduce re-annealing of the plasmid. The restriction site will be removed upon ligation of the insert.

Annealing, phosphorylation and ligation of deoxyoligonucleotide insert

Complimentary deoxyoligonucleotides (INS1A/B; 20 nmol of each) were annealed to form INS1 by heating to 98°C and slowly cooling to 30°C over 2 h. Double stranded deoxyoligonucleotides were then phosphorylated using polynucleotide kinase (USB, Cleveland, OH, USA; 5 µl, 150 U) in a total volume of 50 µl buffer [50 mM Tris-HCl

(pH 7.6), 10 mM MgCl₂, 10 mM 2-mercaptoethanol and 2 mM ATP] at 37°C for 2 h. Phosphorylated deoxyoligonucleotide inserts (2 nmol) were ligated into digested pSP189 plasmid (1 µg) using Boline Quickstick DNA ligase (Boline, London, UK) in 3.6 mM Tris-HCl, pH 7.4, 36 mM NaCl, 360 µM DTT, 36 µM EDTA and 18% glycerol in a total volume of 14 µl, at room temperature for 20 min.

Transfection

Ad293 human kidney cells were transfected with the modified pSP189 plasmid as previously described (12,22). In short, sub-confluent cells were transfected with plasmid containing the INS1 insert (10 µg per 9 cm culture plate) using Fugene-6 transfection reagent (15 µl, Roche, Lewes, East Sussex, UK). The plasmid was recovered after a period of 48 h using plasmid purification kits (Qiagen, Crawley, UK) and then digested with Dpn1 restriction enzyme

(2 U; New England Biolabs, Hitchin, UK) which removes any plasmid DNA that did not undergo replication in the human cells.

Transformation of electrocompetent MBM7070 *E.coli*

Electrocompetent MBM7070 *E.coli* were transformed with aliquots of recovered plasmid by electroporation, using Gene Pulser apparatus (2.5 KV, 200 Ω , 25 μ F) (BioRad, Hercules, CA, USA). Transformants were plated onto LB agar plates containing ampicillin (100 μ g/ml), which selects for those cells containing the plasmid, 5-bromo-4-chloro-3-indolyl- β -D-galactose (X-gal) (75 μ g/ml) and isopropyl- β -D-thiogalactoside (IPTG) (25 μ g/ml). White mutant colonies result from INS1 being successfully ligated into the *supF* gene of pSP189 plasmid. All white colonies were picked, re-streaked on fresh LB agar plates containing ampicillin (100 μ g/ml), X-gal (75 μ g/ml) and IPTG (25 μ g/ml) and stored at 4°C prior to use. Blue non-mutant (wild-type) colonies arose from the transformation of plasmid which did not contain the insert and could therefore be excluded from subsequent analysis.

PCR

For each white colony an 89 bp region of the plasmid was amplified directly by PCR as follows: PCR HotMasterMix (20 μ l, 2.5 \times , Eppendorf, Hamburg, Germany), tissue culture grade water (27 μ l), ALMS3A (1 μ l, 50 pmol), ALMS4 (1 μ l, 50 pmol) and 1 μ l MBM7070 *E.coli* (colony picked and diluted into 20 μ l tissue culture grade water) was introduced into a 200 μ l thin walled PCR tube and subjected to the following optimized PCR program using a Dyad thermal cycler (MJ Research, Inc., Waltham, MA, USA): 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 60 s, final extension at 72°C for 5 min, then cooled to 4°C. Samples were then stored at -4°C until required.

Digestion of PCR products to yield deoxyoligonucleotides for MALDI-MS analysis

The 89 bp PCR products (50 μ l) were digested with Mnl1 and Mbo11 restriction enzymes (New England Biolabs (UK) Ltd, Hitchin, UK) (40 U each) in NEBuffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl, 1 mM DTT, pH 7.9 at 25°C) supplemented with 100 μ g/ml BSA in a total volume of 80 μ l for 2 or 8 h at 37°C. The restriction enzymes were heat inactivated at 65°C for 20 min.

Sample preparation for MALDI-MS

Digested PCR products were desalted and concentrated using Amicon Microcon (Millipore, Watford, UK) 3000 Da cut off spin columns, by centrifugation for 30 min. The samples were further washed with deionised water (18.2 M Ω) and each concentrated sample was re-suspended in 10 μ l of MALDI matrix. The MALDI matrix was composed of ammonium citrate dibasic [0.2 M in 300 μ l of acetonitrile and deionised water (50:50)] and to this was added 0.2 M of nicotinic acid and 80 mM of anthranilic acid, along with 650 μ l of acetonitrile and 150 μ l of deionised water. To each sample, 12 μ l Dowex 80 W X200 beads (activated with ammonia) were added and left to exchange cationic adducts (30 min).

Mass spectrometry

All analyses were performed on a Waters QToF Ultima Global instrument (Waters, Manchester, UK) in MALDI mode. Calibration of the instrument was carried out using a number of oligonucleotide standards (STANDARD1-8) that covered the m/z range of interest (m/z 3000–7000 was typically chosen for analysis). Each digested deoxyoligonucleotide sample (1 μ l) was spotted on to a target in quadruplicate and the resultant spectra combined. The data was smoothed using a mean method and the monoisotopic (for standards) or average molecular weight (for samples) was determined from the molecular ion.

Sequencing

To independently confirm and validate the MS analyses, plasmid samples were also analyzed by standard DNA sequencing methodologies. In this case, plasmid DNA from white mutant colonies containing the insert underwent Templifi amplification and sequencing was carried out using the primer 5'-GGCGACACGGAAATGTTGAA-3' (Biomers.net GmbH, Ulm, Germany). Sequencing was performed using an Applied Biosystems Model 377 DNA sequencer using BigDye sequencing chemistries (Complement Genomics Ltd, Sunderland, UK).

Mutation classification

PCR products were digested as described and run on a gel (4% agarose, 30 min at 100–150 V or 20% PAGE, 250 V, O/N) to check for the presence of the digested insert. The deoxyoligonucleotide was then classified as a base substitution based on the mass of the deoxyoligonucleotide using MALDI-MS. Deletions were classified due to a reduced sized product on the gel and the lack of the appropriate m/z value in the MALDI-MS spectrum. All mutations were corroborated with the DNA sequence. Samples which did not yield full size PCR products were excluded from the analysis, as these will have deleted regions of the *supF* gene, which is probably due to incorrect ligation. Mutations in the BsrB1 restriction site will result in PCR products of approximately the correct size, but upon digestion with Mbo11 and Mnl1 no product will be produced.

RESULTS

Optimisation of molecular methodology

To develop and validate this assay the deoxyoligonucleotide sequence INS1A CTTCTCGTCTTTC was incorporated into the plasmid. This sequence was designed to contain a single guanine, enabling the future study of deoxyoligonucleotide adducts prepared by direct reaction of chemicals that preferentially bind to guanine residues in cases where phosphoramidite chemistry is not appropriate. We have previously investigated the mutagenicity of many such compounds using the standard *supF* assay, including tamoxifen, benzo(a)-pyrene diol epoxide, hydroquinone and *para*-benzoquinone, which preferentially form bulky adducts with deoxyguanosine (12,13,22,24–26). For the purpose of assay development, in order to avoid a lengthy synthesis and purification, the same deoxyoligonucleotide (INS1A) containing the

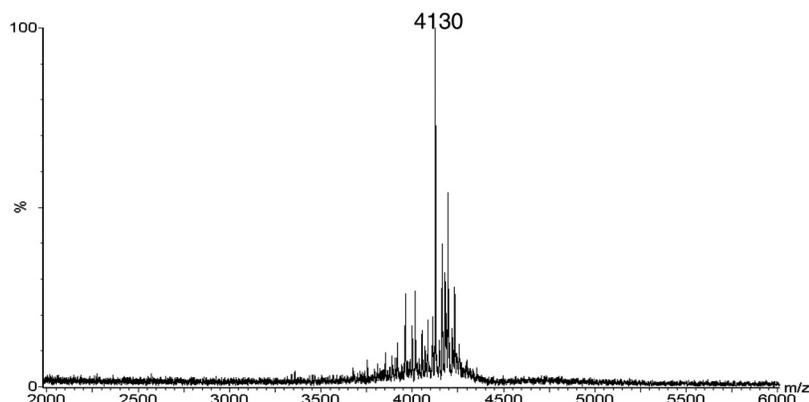
INS1A: CTT CCT C(O⁶-MeG)C TCT TC

Figure 3. Mass spectrum of the INS1A deoxyoligonucleotide with the O⁶-MedG adduct *in situ*.

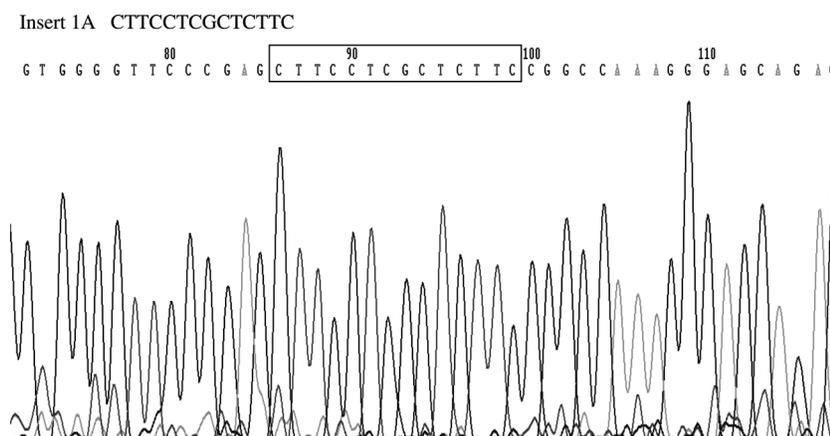


Figure 4. Sequencing electropherogram of pSP189 plasmid containing control INS1 deoxyoligonucleotide insert showing a GC→GC non mutation.

commercially available O⁶-MedG adduct was purchased. Prior to use the presence of the O⁶-MedG adduct was confirmed by MALDI-MS [protonated molecular ion of *m/z* 4130 for the adducted deoxyoligonucleotide compared to *m/z* 4115 for unmodified INS1A (Figure 3)].

Both standard inserts (INS1A containing unmodified dG) and inserts containing the O⁶-MedG adduct were annealed with their complementary deoxyoligonucleotide (INS1B) and ligated into the *supF* gene of the pSP189 plasmid at the BsrB1 restriction site. Purification of the fully ligated plasmid was not necessary at this stage, as the assay later selects for plasmids containing the insert: it can distinguish correctly ligated plasmid from all other products including the parent plasmid, when the replicated plasmid is screened *in E.coli*. The plasmids were then used to transform MBM7070 *E.coli*, which was plated on selective media; containing ampicillin to select for the presence of the plasmid and X-gal and IPTG to identify plasmids containing the deoxyoligonucleotide insert. The efficiency of ligation was in the range of 43–73%, i.e. between 43 and 73 white mutant colonies were seen per ~100 total (blue and white) colonies. Any mutations caused by incorrect ligation at this stage will result in a lack of observable digest product in later steps. White colonies containing the insert were picked and

re-streaked and plasmid DNA was amplified and sequenced to verify the presence of the deoxyoligonucleotide insert. Figure 4 illustrates an example of a sequencing electropherogram from a plasmid containing the ligated deoxyoligonucleotide insert, in this case as the dG control version of INS1. Figure 5 illustrates an electropherogram from a plasmid which contained the deoxyoligonucleotide INS1 insert with an O⁶-MedG adduct present, which has been converted to an adenine base during replication.

Various restriction enzymes were assessed for their ability to accurately and efficiently digest out the inserted deoxyoligonucleotides for MS sequence analysis. These included Bpm1 (30), which caused problems due to the fact that it is a type II restriction enzyme and appeared to remain bound to the digested fragments, adversely affecting recovery and subsequent MS detection. This issue was also recognized by Abdi *et al.* (31), who described a system using Hph1. Further developments led to the testing of Alu1, Msp1, Mbo11 and Mnl1 enzymes, with Mbo11 and Mnl1 together producing the cleanest and most reproducible digestion. The restriction site topography of the Mbo11 and Mnl1 cocktail results in deoxyoligonucleotides of the same size, whether or not the original insert was ligated in a forward or backward orientation at the blunt ended BsrB1 restriction

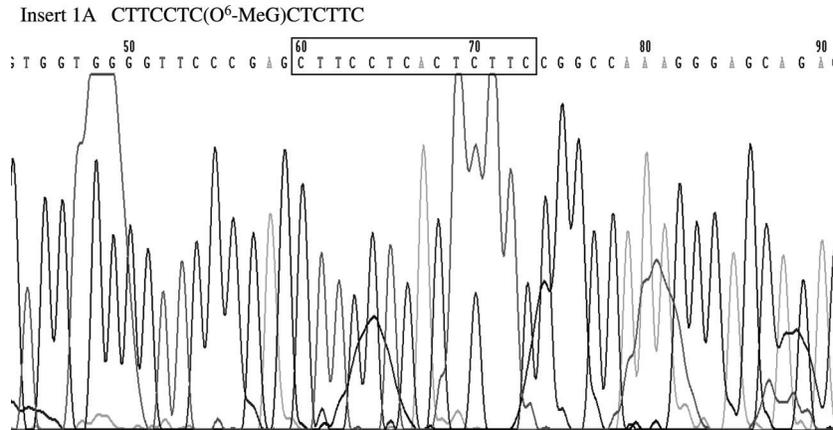


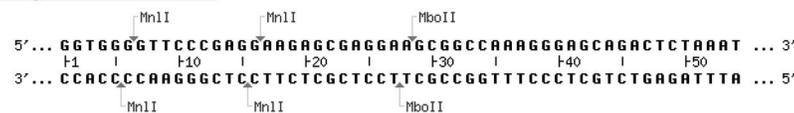
Figure 5. Sequencing electropherogram of pSP189 plasmid containing INS1 O⁶-MedG deoxyoligonucleotide insert showing a GC→AT transition.

Forward oligonucleotide



Results in 2 fragments: TCCTCGCTCTTC 12mer 3603 Da
 AAGAGCGAGGAA 12mer 3832 Da

Reversed Oligonucleotide



Results in the same 2 fragments: TCCTCGCTCTTC 12mer 3603 Da
 AAGAGCGAGGAA 12mer 3832 Da

Along with 2 more: GTTCCCGAGG 10mer 3124 Da
 CTCGGGAACC 9mer 3093 Da

Figure 6. Digestion of *supF* gene containing insert.

site. This simplifies the mass spectrometry of the purified deoxyoligonucleotide fragments. When the deoxyoligonucleotide is ligated in the forward orientation, there are two fragments from the digestion, whereas if the deoxyoligonucleotide insert is ligated in the reverse orientation, in addition to the same two digestion products, two smaller fragments, a 9 and 10mer, were also generated (Figure 6). The presence of these extra two deoxyoligonucleotides will enable determination of whether the DNA lesion was originally present on the leading or lagging strand and identification of any strand dependent differences in the mutagenic potential of the adduct under investigation.

The assay was further validated by ligating synthetic inserts (INS1) corresponding to all of the possible mutational outcomes (i.e. G, C, A or T) into the pSP189 plasmid and transforming MBM7070 *E.coli* with the plasmids. Resultant white colonies were picked and amplified prior to sequencing using the Templiphi method. It was found that the

deoxyoligonucleotides ligated correctly for all of the inserts and correctly matching sequences were detected for all possible outcomes. For example, when a T was in place of the dG, 100% of the plasmids sequenced contained a T in this position (data not shown).

Optimization of mass spectrometry using deoxyoligonucleotide standards

The assay was initially developed with a view to using electrospray ionization mass spectrometry (ESI-MS) analysis for mutation identification, since the clean up and concentration steps could be coupled to the mass spectrometer directly using LC-MS methodologies. However, this approach proved difficult and the inability to detect the expected molecular ions by mass spectrometry using ESI may have been due to high salt concentrations in the sample, therefore various methods were attempted to desalt the

Table 1. Monoisotopic masses of deoxyoligonucleotide standards

Oligo name	Sequence	Molecular weight		Error p.p.m.
		Observed	Calculated	
STANDARD1	TCCTCACTCTTC	3585.49	3585.58	25
STANDARD2	AAGAGTGAGGAA	3845.59	3845.67	19
STANDARD3	TCCTCGCTCTTC	3601.50	3601.57	20
STANDARD4	AAGAGCGAGGAA	3830.58	3830.67	23
STANDARD5	TCCTCCCTCTTC	3561.48	3561.57	25
STANDARD6	AAGAGGGAGGAA	3870.50	3870.67	44
STANDARD7	TCCTCTCTCTTC	3576.50	3576.57	19
STANDARD8	AAGAGAGAGGAA	3854.88	3854.68	-52

deoxyoligonucleotides. Different pre-HPLC desalting procedures, HPLC mobile phases, gradients and flow rates were all attempted but no significant improvement in ESI-MS analysis was observed. It was decided to pursue MALDI techniques in light of this to try and simplify sample preparation and analysis.

Deoxyoligonucleotide standards for all of the possible deoxyoligonucleotides were synthesized, i.e. INS1A containing either wild type G or possible mutant bases A, C or T. These were annealed with their appropriate complementary strands and analysed using MALDI-MS. The source conditions should denature the deoxyoligonucleotides, although all standards were also analyzed as pure single stranded deoxyoligonucleotides to confirm the results were comparable. Table 1 illustrates the observed masses and the equivalent calculated masses for all possible top and bottom strands. The difference in mass between observed and expected was acceptably low and in the range of 19–52 p.p.m. (i.e. 0.0019–0.0052%).

Detection of deoxyoligonucleotides recovered from *E.coli* using mass spectrometry

A random selection of bacterial colonies that were shown by conventional sequencing to contain the desired insert were picked and amplified as above and PCR products were digested with Mbo11 and Mnl1 restriction enzymes. The digested deoxyoligonucleotides were desalted using 3 kDa cut off spin columns (Microcon) and re-suspended in MALDI matrix, rather than distilled water. Each sample was applied to a MALDI target and left to dry thoroughly.

Of the 45 colonies that were prepared for MALDI mass analysis, all revealed the presence of a mutation of some type, as illustrated in Table 2. Forty-four (98%) of the mutations were in the form of GC→AT transitions. Of these, there was a 25:19 (56% : 42%) split in favour of the forward ligated insert compared to reversed insert. The remaining colony (2%) contained a large deletion, where part of the insert had been removed. Figure 7 illustrates examples of the possible mass spectra produced when deoxyoligonucleotides are processed through *E.coli*. Figure 7A shows a control insert, which originally contained a non-adducted deoxyguanosine. The peak with *m/z* 3604 corresponds to the unchanged top strand (TCCTCGCTCTTC), whilst the peak with *m/z* 3834 is due to the complimentary bottom strand (AAGAGCGAGGAA), which confirms the correct replication of the inserted deoxyoligonucleotide sequence. All other control deoxyoligonucleotides resulted in the appropriate unchanged masses after mass spectral analysis

Table 2. Table of mutations detected after processing in both human Ad293 cells and *E.coli*

Mutations induced in <i>E.coli</i> only (%)		
G-A transitions		
Total	44	98
Forward	25	56
Reversed	19	42
Deletions	1	2
Total mutations	45	100
Mutations induced in human Ad293 cells (%)		
G-A transitions		
Total	28	93
Forward	10	33
Reversed	18	60
Deletions	2	7
Total mutations	30	100

(i.e. none were mutated). Figure 7B illustrates an example of a mass spectrum from an insert that originally contained an O⁶-MedG, which was replicated in *E.coli*. The peak with *m/z* 3588 corresponds to the top strand (TCCTCACTCTTC), which now contains an adenine due to the induction of a G→A transition as a result of the adduct, whilst the peak with *m/z* 3849 is the bottom strand (AAGAGTGAGGAA) containing a T instead of the original C, thus confirming the induction of a GC→AT transition mutation, as would be predicted for an adduct of this type. A further eight colonies were sequenced which had lost regions of the *supF* gene, which resulted in a lack of PCR product and one colony which had a mutation in the BsrB1 restriction site, which would have not allowed insertion of the INS1 deoxyoligonucleotide.

Detection of deoxyoligonucleotides transfected into human Ad293 cells and recovered from *E.coli* using mass spectrometry

Control and O⁶-MedG containing deoxyoligonucleotide inserts were transfected into Ad293 adenovirus-transformed human kidney cells, where DNA repair, correct replication or misincorporation of a mutant base could take place. The processed plasmid was recovered after 48 h and used to transform MBM7070 *E.coli* indicator bacteria. White colonies were processed in the same way as described above prior to DNA sequencing or mass spectral analysis. All samples were analyzed in quadruplicate by MALDI-MS, with the resultant spectra being combined for subsequent mass and mutation classification. Table 2 illustrates the mutation status of the 30 white colonies analyzed. All inserts resulted in mutations. Twenty-eight (93%) of these were in the form of GC→AT transitions. Of these transition mutations, 10 (33%) were in the forward orientation and 18 (60%) had been ligated in reverse. Two (7%) of the analyzed plasmid samples contained deletions: where part of the deoxyoligonucleotide insert had been removed. Figure 8 illustrates typical examples of the mass spectra of deoxyoligonucleotides amplified and digested from plasmid which was replicated in human Ad293 cells then screened in MBM7070 *E.coli*. Figure 8A shows a deoxyoligonucleotide from a plasmid that originally had ligated a control, non-adducted dG containing insert. The *m/z* 3604 peak corresponds to the non-mutant top strand (TCCTCGCTCTTC), whilst the peak with *m/z* 3834 is the

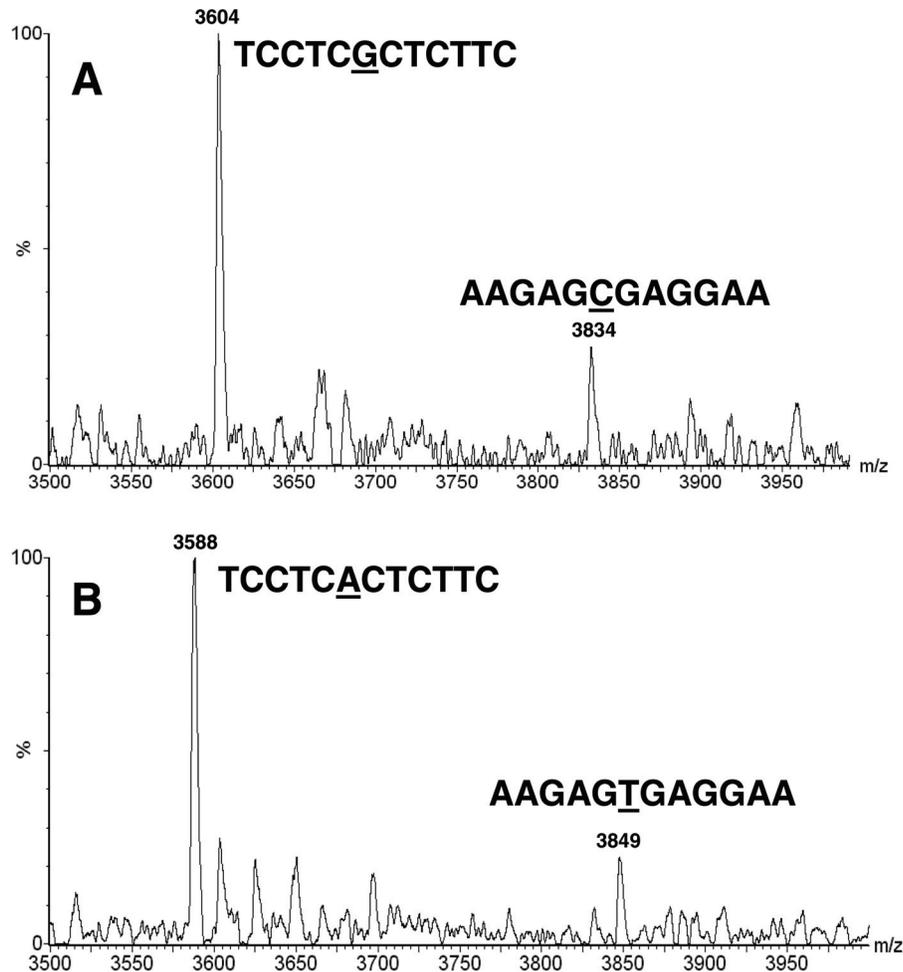


Figure 7. Examples of mass spectra for samples from *E.coli* only. (A) Control deoxyguanosine containing oligonucleotide. (B) O^6 -MedG containing oligonucleotide.

complimentary bottom strand (AAGAGCGAGGAA). This therefore, confirms the correct replication of the deoxyoligonucleotide insert in Ad293 cells, as previously observed for *E.coli* above. Figure 8B is a mass spectrum of an insert which originally contained an O^6 -MedG. The peak with m/z 3588 corresponds to the top strand (TCCTCACTCTTC), which contains an adenine due to the induction of a G→A transition, whilst the peak with m/z 3849 corresponds to the complimentary bottom strand (AAGAGTGAGGAA), containing a T rather than a C. This confirms that in this assay, when the O^6 -MedG adduct is replicated in human Ad293 cells it results in the incorporation of a deoxyadenosine and the induction of a GC→AT transition, rather than being repaired. A further 5 colonies lacked the PCR priming regions and 6 had mutations in the BsrB1 restriction site, both detected by DNA sequencing, which resulted in no PCR product or no digested deoxyoligonucleotide, respectively.

DISCUSSION

The site-specific mutagenesis assay (SSMA-MS) described in this paper offers considerable potential for future investigations of deoxyoligonucleotides containing important DNA lesions

in human cells. These preliminary experiments have shown that it is possible to insert a double stranded deoxyoligonucleotide into the *supF* gene of the pSP189 shuttle vector plasmid to knock out gene function. Recovered plasmids which are screened in *E.coli* for the presence of inserts can be picked and sequenced to determine whether a mutation has been induced, the adduct has been bypassed with incorporation of the correct base or repair has occurred. In this developmental work the plasmids have been allowed to replicate in bacteria only or in human Ad293 kidney cells prior to being screened in bacteria. PCR using the recovered plasmid as template, followed by digestion of the PCR product using a restriction cocktail of Mbo11 and Mnl1, results in the generation of small deoxyoligonucleotides which have been characterized by MALDI-MS. MALDI-MS provides an excellent platform for DNA analysis. It is more salt tolerant than ESI-MS, is very sensitive and allows rapid analysis of an entire sample, rather than requiring prior separation of components by HPLC. This results in a more streamlined assay that benefits from reduced costs, both in terms of time and consumables, compared to an ESI-MS method or DNA sequencing. These assets make the method particularly amenable to high-throughput analysis.

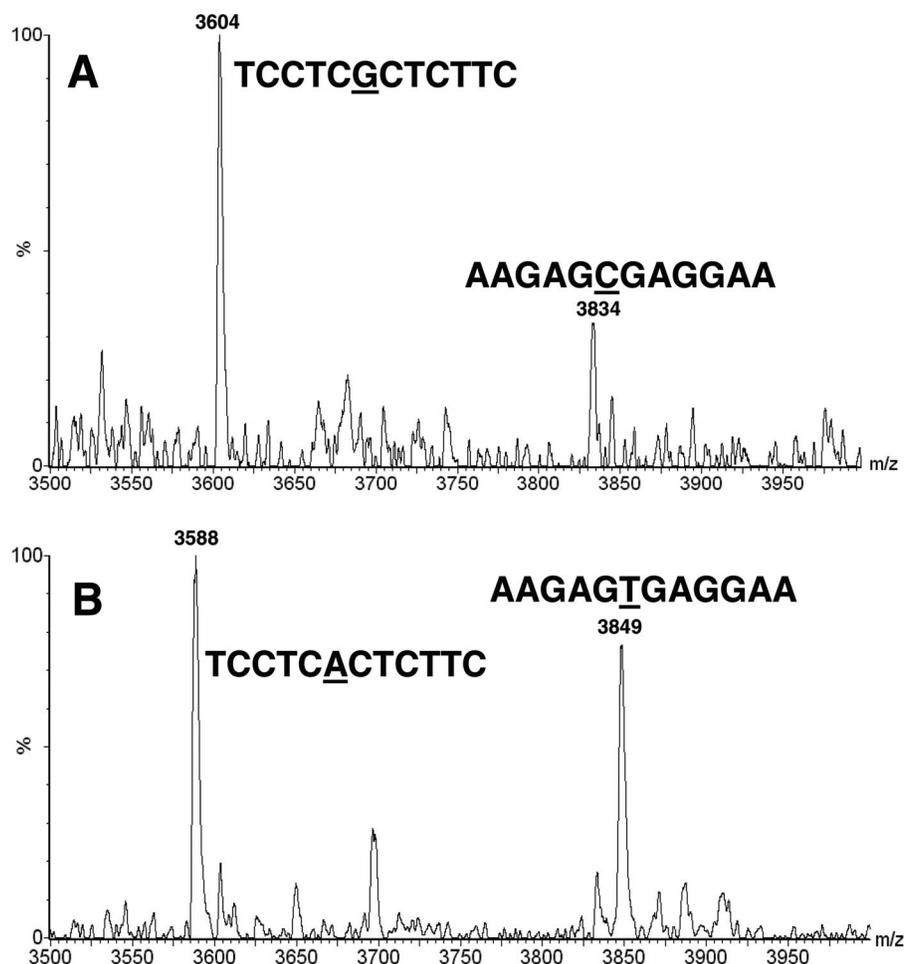


Figure 8. Examples of mass spectra for samples from human Ad293 cells, screened in *E.coli*. (A) Control deoxyguanosine containing oligonucleotide. (B) O⁶-MedG containing oligonucleotide.

Replication of the shuttle vector plasmid can be achieved in a variety of human cell lines, making it possible to study the effects of DNA adducts formed by a compound that is implicated in a certain type of cancer in the most relevant human cells. Furthermore, the adduct can be situated in a key gene for the cancer in question. Since the SSMA-MS method relies on chemical synthesis of the deoxyoligonucleotide inserts, any DNA lesion can be studied as long as it can be obtained in the form of phosphoramidites. Alternatively, if this is not possible, deoxyoligonucleotide adducts may be produced by direct reaction of the ultimate genotoxic species with a synthetic deoxyoligonucleotide, although the desired product will then need to be rigorously purified prior to insertion into the plasmid.

Using a commercially available DNA adduct, O⁶-MedG and control non-adducted deoxyoligonucleotide inserts, we have validated the method and obtained results which compare to those observed with other mutagenesis systems. O⁶-MedG is a pre-mutagenic lesion in bacterial (32) and mammalian cells (33), which predominantly results in GC→AT transitions both *in vitro* and *in vivo* (34,35). The presence of O⁶-MedG adducts has been correlated with increased organ specific tumorigenesis in rats induced by DNA alkylating agents (36,37) and O⁶-MedG has been

detected in both normal and tumour DNA isolated from colorectal tissue of men and women undergoing surgery for colorectal tumours (38). *In vitro* primer extension studies show that the frequency of misincorporation of dNTPs opposite or extension past, the O⁶-MedG lesion varies with the sequence context (39,40). Studies in bacterial cells using a single site-specific adduct have shown that O⁶-MedG is able to miscode DNA, resulting in the incorporation of the incorrect pyrimidine as its complementary base when replicated in *E.coli* (9,41) thus causing the characteristic GC→AT transitions observed in the present study (42). An excess of these mutations have also been detected in the *p53* tumour suppressor gene and *ras* oncogene in rodents after treatment with methylating agents (43–45) and are commonly seen in a variety of human tumour types (46,47).

The pSP189 plasmid used in this assay is double stranded, therefore a uracil base was incorporated opposite the O⁶-MedG lesion in the INS1 deoxyoligonucleotide insert. This should be removed by cellular uracil glycosylases after transfection, encouraging translesion synthesis by polymerases encountering the adduct rather than damage avoidance and preferential replication of the non-adducted strand. This increases the chances of a mutation occurring and allows a more accurate study of the types of mutations

induced by the O⁶-MedG adduct in human cells and bacterial cells, whilst also validating the system. This approach has been used to investigate O⁶-substituted guanines (29,48) and O⁴-substituted thymines (48) replicated in *E.coli* and similarly an increased mutagenicity was observed. A GC→AT transition base substitution was observed whether the deoxyoligonucleotide containing plasmid was replicated in human Ad293 cells or *E.coli*. In fact, no other base substitution was seen and the only other mutations detected in this system by adduct containing plasmid were manifested as a deletion of part of the deoxyoligonucleotide insert directly after the adduct position. This would suggest they arise as a consequence of the O⁶-MedG adducts, rather than the insertion of a shortened synthetic deoxyoligonucleotide in the initial stages of the assay prior to replication in human Ad293 cells or bacterial cells. In *E.coli*, O⁶-alkylguanine can be repaired by at least two alternative pathways: the base damage is directly removed by transfer of the alkyl group to an active cysteine residue on the inducible Ada or constitutive Ogt methyltransferases (49–51) or by the nucleotide excision repair (NER) pathway (52). Human cells have a single analogous O⁶-alkylguanine-DNA-alkyltransferase (MGMT) (53) and a complex NER pathway (54,55). When DNA adducts are repaired by NER a small region surrounding the lesion is excised prior to the gap being filled by polδ and polε (55). Any reduction in the efficiency or accuracy of this gap filling function can result in base deletions, whereas the use of direct repair pathways (Ada, Ogt or MGMT) should result in untraceable removal of the methyl group only. The attempted repair of the lesions by NER may therefore account for these observed deletions although, at present, the activity of this pathway in these cells is unknown. These results add further to the evidence that O⁶-alkylidG adducts are pro-mutagenic lesions and as such are important in the mutagenicity and carcinogenicity of alkylating agents which can form these lesions to varying degrees.

We are continually developing improved methods for the preparation of deoxyoligonucleotides prior to MS analysis, such as the use of appropriate size exclusion or solid phase columns, which will allow more efficient salt removal and greater purification of the deoxyoligonucleotides of importance. We are also presently developing an MS/MS method, which will allow us to further characterize deoxyoligonucleotide inserts, such that from the collision induced dissociation products, it will be possible to determine the DNA sequence and confirm the precise position of the mutation within the deoxyoligonucleotide.

In summary there are several benefits of this new SSMA-MS assay, both in terms of ease and speed in which deoxyoligonucleotides containing a DNA lesion can be inserted and analyzed. There is no need for laborious construction and purification of a shuttle vector using scaffold deoxyoligonucleotides, since MS analysis provides high specificity for vectors containing the adducted insert. The double stranded pSP189 shuttle vector plasmid used contains origins of replication for both bacterial and mammalian cells (19,20) so comparisons can be made between cells differing in source organ, species origin or repair proficiency. The increased throughput and speed of MALDI-MS analysis allows the comparison of either a set of DNA adducts in a fixed

sequence context or a single DNA adduct with variable sequence contexts, to be analyzed in a relatively short time frame.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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