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Potassium diazoacetate-induced *p53* mutations *in vitro* in relation to formation of O^6-carboxymethyl- and O^6-methyl-2′-deoxyguanosine DNA adducts: relevance for gastrointestinal cancer

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Nitrosated glycine derivatives react with DNA to form O^6-carboxymethyl-2′-deoxyguanosine (O^6-CMdG) and O^6-methyl-2′-deoxyguanosine (O^6-MedG) adducts concurrently. O^6-CMdG is not repaired by O^6-alkylguanine alkyltransferases and might be expected to lead to mutations via a similar mechanism to O^6-MedG. Potassium diazoacetate (KDA) is a stable form of nitrosated glycine and its ability to induce mutations in the *p53* gene in a functional yeast assay was studied. Treatment of a plasmid containing the human *p53* cDNA sequence with KDA afforded readily detectable levels of O^6-CMdG and O^6-MedG. The treated plasmid was used to transform yeast cells and coloured colonies harbouring a *p53* sequence with functional mutations were detected. Recovery of the mutated plasmids followed by DNA sequencing enabled the mutation spectrum of KDA to be characterised. The most common mutations induced by KDA were substitutions with >50% occurring at GC base pairs. In contrast to the methylating agent methylnitrosourea which gives predominantly (>80%) GC→AT transitions, KDA produced almost equal amounts of transitions (GC→AT) and transversions (GC→TA and AT→TA). This difference is probably due to a different mode of base mispairing for O^6-CMdG compared with O^6-MedG. The pattern of mutations induced by KDA was very similar to the patterns observed in mutated *p53* in human gastrointestinal tract tumours. These results are consistent with the hypothesis that nitrosation of glycine (or glycine derivatives) may contribute to characteristic human *p53* mutation profiles. This conclusion is borne out by recent observations that O^6-CMdG is present in human DNA both from blood and exfoliated colorectal cells and is consistent with recent epidemiological studies that have concluded that endogenous nitrosation arising from red meat consumption is related to an increased risk of colorectal cancer.

Abbreviations: O^6-CMG, O^6-carboxymethylguanine; O^6-CMdG, O^6-carboxymethyl-2′-deoxyguanosine; O^6-MeG, O^6-methylguanine; O^6-MedG, O^6-methyl-2′-deoxyguanosine; KDA, potassium diazoacetate; GI, gastrointestinal; MNU, *N*-methyl-*N*-nitrosourea.

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Introduction

We have shown that several nitrosated glycine derivatives react with DNA to form O^6-carboxymethyl-2′-deoxyguanosine (O^6-CMdG) and O^6-methyl-2′-deoxyguanosine (O^6-MedG) adducts concurrently (1). Since glycine is one of the most common and structurally simplest dietary amino acids, it would appear likely that nitrosation products of glycine would constitute a major source of alkylating agents in the human gastrointestinal (GI) tract (2,3). A scheme for the nitrosation of glycine, as well as pathways leading to the formation of both O^6-alkylguanine DNA adducts, is shown in Figure 1.

Recent *in vitro* studies lend further support to the hypothesis that reaction of glycine with nitrosating agents leads to formation of DNA-damaging species. Cupid *et al*. (4) demonstrated that glycine is nitrosated at neutral pH by nitric oxide in the presence of oxygen. Formation of diazoacetate was found to be linear with glycine and nitrosating agent concentration. Furthermore, incubation of the reaction mixtures with DNA gave rise to O^6-CMdG.

Further evidence of the likely human exposure to nitrosated glycine is based on the fact that O^6-CMdG is indeed detectable in gastric biopsies and human blood DNA samples using a sensitive immunoslot blot (ISB) assay (4,5). We had also previously noted that O^6-CMdG is not repaired by bacterial and mammalian O^6-alkylguanine-DNA alkyltransferases (6) suggesting that this adduct is likely to accumulate in the DNA of GI tract tissues and possibly be a promutagenic lesion. Interestingly, it has been shown that carboxymethylated bases are repaired by nucleotide excision repair (7) and appear to be an important contributor to lethal damage in human cells. It is therefore of some interest to determine whether O^6-CMdG is mutagenic and to see if the profile of induced mutations is characteristic.

One of the most frequently encountered genetic events in human malignancy is alteration of the *p53* gene and its encoded protein. Interpretation of mutational spectra induced by carcinogens could provide valuable information about the contribution of specific aetiologic factors in the development of human cancer. Specific mutational *p53* spectra have been reported for skin tumours in individuals exposed to UV, due to highly characteristic pairs of mutations due to pyrimidine photodimers (8), liver tumours from people with exposure to dietary aflatoxin B_1 [where N-7 guanine adducts give a

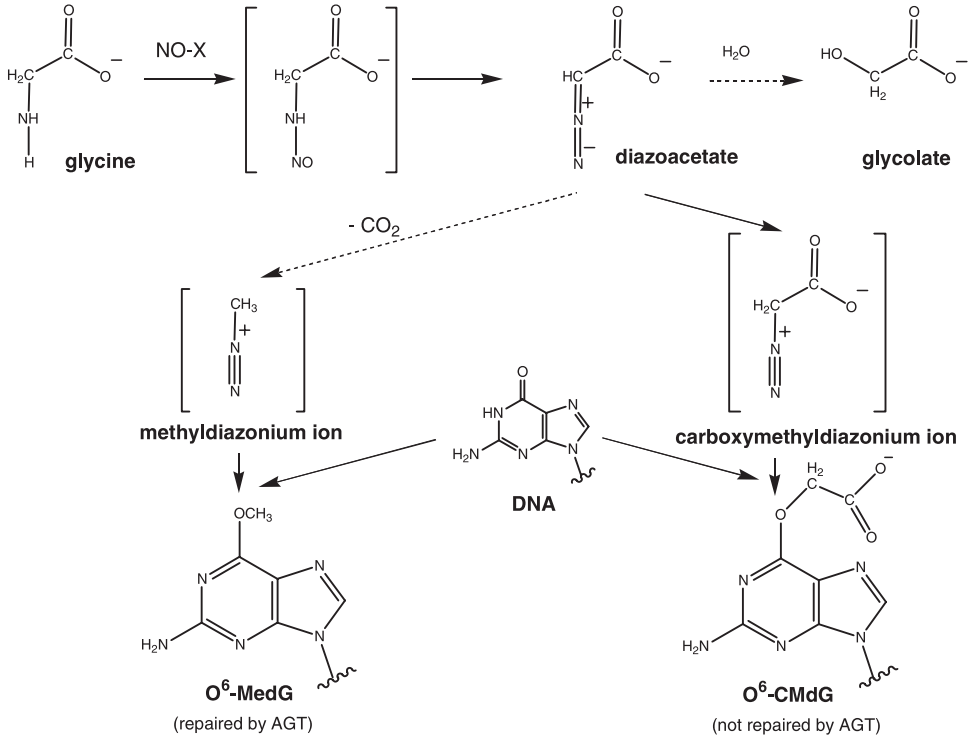

Fig. 1. Mechanism for the nitrosation of glycine and subsequent formation of O^6-MedG and O^6-CMdG DNA adducts. Nitrosation of glycine gives rise to alkylating agents which carboxymethylate and methylate DNA. Diazoacetate is formed as an intermediate that can generate both carboxymethyldiazonium and methyldiazonium ions, the latter reactive species being formed via decarboxylation of diazoacetate. Both diazonium ions are highly reactive electrophiles and potent alkylating agents.

mutational hotspot at codon 249 (9)] and for lung cancer in smokers [due to a complex mixture of DNA-damaging agents (10)].

We decided to use a functional assay for *p53* mutations in which mutations that block transcriptional competence are detected. The yeast expression vector pLS76 used in this study was first described and constructed by Ishioka *et al*. (11). A defect in transactivation ability enabled its use for mutagenesis studies (12–14). This assay was applied recently to the analysis of *p53* mutational spectra induced by the antineoplastic drug chloroethyl-cyclohexyl-nitrosourea (15,16). Another mutagenesis study investigated the effect of UV irradiation and demonstrated that the mutational spectrum in yeast was indistinguishable from *p53* mutations observed in human non-melanoma skin cancer (17).

The work presented here aims to establish a profile of mutations induced by nitrosated glycine in the *p53* gene using a functional yeast assay and to determine whether the profile is sufficiently characteristic to indicate a role for nitrosated glycine in human carcinogenesis.

Materials and methods

Caution

Reagents generating carboxymethyldiazonium ions are alkylating agents and should be handled with extreme caution (probable, potent carcinogens). Unused solutions of potassium diazoacetate should be decomposed by overnight treatment using 1 M aqueous acetic acid.

KDA treatment of plasmid pLS76

KDA was synthesised via alkaline hydrolysis of ethyl diazoacetate (Aldrich; 1). Plasmid pLS76 (~1 mg/ml) was treated *in vitro* in PBS (pH 7.3) using 0, 4, 6, 8 and 10 mM KDA, or in Tris–EDTA (ethylenediaminetetraacetic acid) buffer (10 mM Tris and 1 mM EDTA, pH 7.5) using 0, 8 and 10 mM KDA overnight at 37°C. Treatments with 8 mM KDA were performed multiple times in parallel in order to obtain enough mutants for *p53* analysis. Some aliquots were kept for O^6-CMdG and O^6-MedG adduct analysis. Isopropanol was used for DNA precipitation and recovered pellets were taken up in ultrapure water. Samples were then digested and immunopurified as described below for the determination of O^6-CMG and O^6-MeG by RP-HPLC.

Immunoaffinity-HPLC analysis of O^6-CMdG and O^6-MedG in DNA

DNA (24 µg) was digested to 2′-deoxynucleoside-3’-monophosphates using micrococcal nuclease (1.75 U) and calf spleen phosphodiesterase (30 mU), and 10 µl of 100 mM sodium succinate/50 mM calcium chloride buffer (pH 6) were added and the final volume adjusted with ultrapure water to 62.5 µl. The samples were mixed by vortexing, centrifuged and incubated at 37°C for 2 h. They were then dried down in a DNA speed vac. For the digestion to 2′-deoxynucleosides, 50 µl of nuclease P1 [at 2 µg/µl in 0.28 M sodium acetate (pH 5.0)/0.5 mM zinc chloride; ~20 U] were added and the mixture incubated overnight (17 h) at 37°C. Afterwards the samples were processed as described previously by Harrison *et al*. (1) with minor modifications as follows: Prior to HPLC analysis, the eluates from the O^6-MedG columns were heated at 50°C for 30 min to complete the hydrolysis of O^6-MedG to O^6-MeG using 100 µl of 0.1 M HCl. The solution was then neutralized using 0.1 M NaOH (100 µl) and dried down again. Depurination of O^6-CMdG to O^6-CMG takes place instantaneously in 1 M trifluoroacetic acid i.e. no further treatment of the eluates from the O^6-CMdG columns was needed.

Yeast based p53 functional assay and analysis of mutants

Aliquots of plasmid pLS76, a yeast expression vector that harbours wild-type human *p53* cDNA, were treated *in vitro* using KDA, purified by precipitation and resuspended in water. Treated plasmids (~200 ng) were transformed into yeast cells (*Saccharomyces cerevisiae*, strain yIG397) by a lithium acetate procedure (18). The transformants were cultured, plasmids recovered and *p53* sequences amplified using previously published procedures (17). The resulting sequences were analysed using ABI Prism Sequence Navigator software. KDA-induced mutations that were identified were then compared with the IARC TP53 mutation database (19). Only mutations between codons 90 and 290 in the IARC database were considered for analyses and

Table I. Survival and mutation induction in undamaged and KDA-treated pLS76 in relation to the buffer system used for treatments

Buffer	Treatment	Survival[a] (%)	p53 Mutant frequency[b]	MFR[c]
Tris–EDTA	control	100	1.1×10^{-4}	1
	8 mM KDA	32	2.2×10^{-3}	20
	10 mM KDA	3	3.0×10^{-3}	27
PBS	control	100	0.9×10^{-4}	1
	4 mM KDA	65	0.3×10^{-3}	3
	6 mM KDA	33	1.3×10^{-3}	14
	8 mM KDA	22	2.0×10^{-3}	22
	10 mM KDA	14	5.1×10^{-3}	57

[a]Survival = total number of colonies obtained for treated samples/number of colonies on control plates.
[b]Mutant frequency = number of red colonies/number of total colonies.
[c]MFR (mutant frequency ratio) = mutant frequency of KDA damaged/mutant frequency of undamaged DNA.

Table II. Buffer influence on the degree of O^6-MeG and O^6-CMdG formation following KDA treatment of plasmid pLS76

Treatment	O^6-MeG	O^6-CMdG
8 mM KDA in Tris–EDTA	402 pmol/mg DNA 124 adducts/10^6 bases	336 pmol/mg DNA 104 adducts/10^6 bases
8 mM KDA in PBS	100 pmol/mg DNA 31 adducts/10^6 bases	1306 pmol/mg DNA 403 adducts/10^6 bases

comparison to the *in vitro* induced spectra. In general, GC→AT transitions at CpG sites were not taken into account unless stated otherwise as these base pair substitutions are believed to originate from spontaneous deamination of 5-methylcytosine (20). This DNA modification does not occur naturally in yeast cells and the treated plasmid was unmethylated.

Results

Induction of p53 mutations by KDA treatment

The yeast expression vector that harboured a human wild-type *p53* cDNA and the LEU2 selectable marker was treated with KDA at different doses and in different buffer systems and transfected into yIG397 cells by lithium acetate procedure. A KDA dose dependent decrease in survival and increase in mutant frequency were observed (Table I). Molecular analysis was limited to mutant clones originating mainly from 8 mM KDA treatments. This particular KDA concentration resulted, independent of the treatment buffer, in a similar mutation frequency (~20-fold above background). Subsequent 8 mM KDA treatments of plasmid pLS76 in Tris–EDTA and phosphate-buffered saline (PBS) were carried out several times in parallel in order to obtain enough mutants for *p53* analysis and adduct analysis.

O^6-CMG and O^6-MeG adduct levels in plasmids treated with KDA

The levels of DNA O^6-CMdG and O^6-MedG were measured in plasmids treated with KDA in both Tris–EDTA and PBS buffers. Some marked differences in adduct levels were noted (Table II). The level of O^6-alkylguanine modification, following treatment with KDA (8 mM), based on the length of the *p53* coding sequence (393 amino acids i.e. 1179 bases in length) was calculated to be 0.12 O^6-CMG and 0.15 O^6-MeG adducts for KDA treatments in Tris–EDTA buffer and 0.48 O^6-CMG and 0.04 O^6-MeG adducts for the treatments carried out in PBS. Typical RP-HPLC traces obtained for KDA-treated plasmid pLS76 are shown in Figure 2A and B for O^6-CMG and O^6-MeG, respectively.

Analysis of KDA-induced p53 mutations at the DNA level

The most common mutations induced by KDA were base pair substitutions (85%, Table III). Of all the mutations induced by KDA treatment in Tris–EDTA buffer ~57% were at GC base pairs, whereas 28% were at AT base pairs. Of these substitutions, 55% were transitions (35/64), most of which were GC→AT base pairs (26/35). Transversions accounted for 45% of the base pair substitutions (29/64) and were comprised largely of GC→TA (12/29) and AT→TA (9/29). The remaining mutations (15%) were characterized as mainly base pair deletions (9/11). Similar observations were made for KDA treatments in PBS (Table III). Again the most common mutations induced were base pair substitutions (96%) and ~53% of all mutations induced by KDA treatments in PBS were found in GC base pairs. Some buffer-dependent variations in the percentage of mutations at AT base pairs was noted, namely 43% in PBS and 28% in Tris–EDTA buffer. Of the substitutions 49% were transitions (23/47), most of which were GC→AT (17/23). Transversions accounted for 51% of the base pair substitutions and encompassed mainly AT→TA (10/24). The remaining mutations (4%) were characterized as base pair deletions (2/2).

With regard to the variation in mutation frequency at various sites in the *p53* sequence using different incubation buffers we used a method for the comparison of mutational spectra at the same locus that has been developed by Cariello *et al*. (21,22). Application of this statistical tool to the comparison of KDA-induced spectra, i.e. Tris–EDTA buffer versus PBS gave a *P*-value of 0.64 following 10 000 iterations with the 95% confidence limits (CI) on the *P*-value ranging from 0.63 to 0.65. This *P*-value indicated that the two spectra were statistically indistinguishable.

Discussion

The yeast expression vector pLS76, harbouring wild-type *p53* cDNA, was treated *in vitro* using KDA in PBS or Tris–EDTA buffer and transfected into a yeast strain containing the *ADE2* gene regulated by a *p53*-responsive promoter. O^6-alkylguanine adduct levels were determined in 8 mM KDA-treated plasmid using immunopurification followed by HPLC fluorescence. Some variation in the absolute and relative levels of O^6-CMdG and O^6-MedG were observed using the two incubation buffers. However, since the application of a statistical test developed primarily for comparisons of *p53* mutation spectra demonstrated that the two KDA-induced spectra were indistinguishable, and therefore independent of the buffer system, both datasets were combined for future analyses and treated as one spectrum. The resulting classes of mutations are displayed in Figure 3 and compared with the ones induced in the same system by *N*-methyl-*N*-nitrosourea, a monofunctional methylating agent (23). The vast majority of mutations induced by MNU treatments were GC→AT transitions (82%). Transversions of GC→CG and AT→CG were not observed. Frameshifts accounted for ~5% of all mutations. Of all mutations ~84% were directed against GC base pairs, whereas those aimed at AT base pairs only accounted for 11%. AT→GC transitions accounted for the majority of mutations at AT

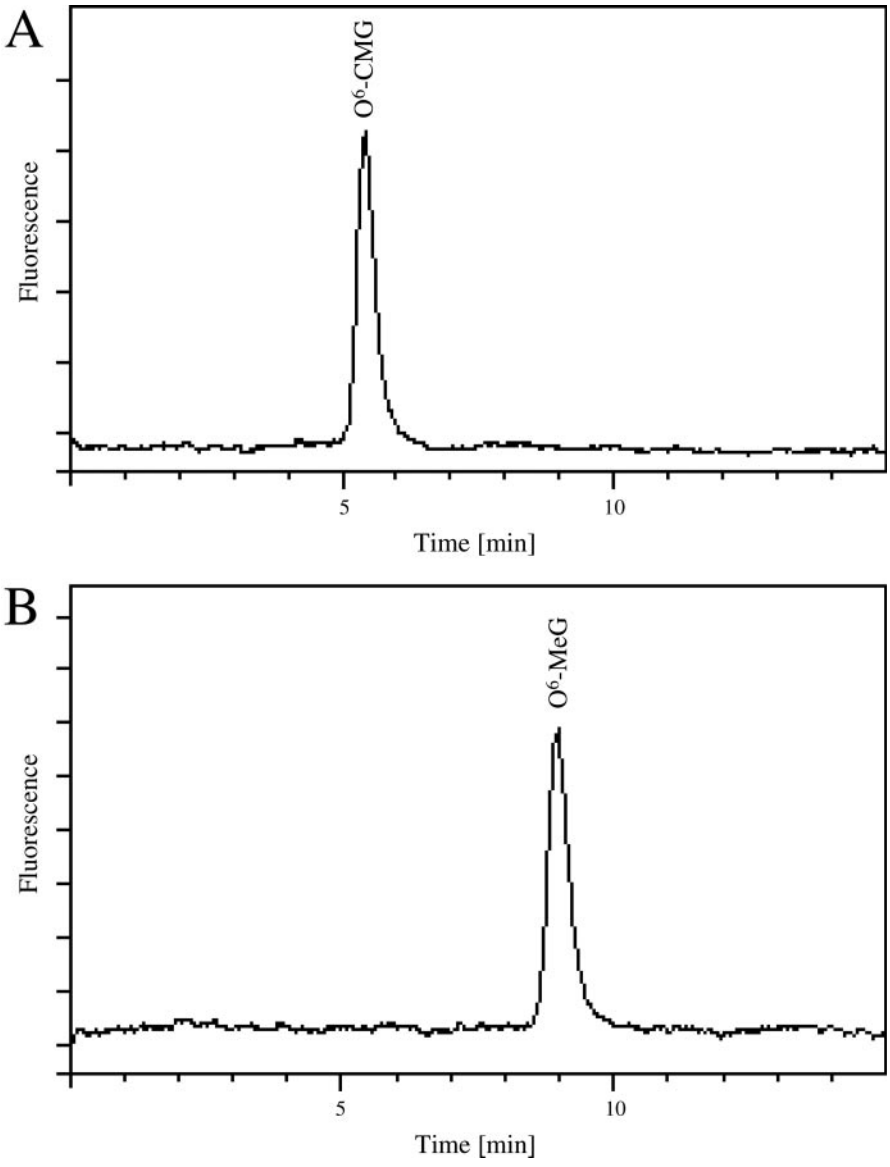

Fig. 2. Typical RP-HPLC chromatograms of O^6-CMG (**A**) and O^6-MeG (**B**) following enzymatic digestion and immunopurification of 8 mM KDA-treated plasmid pLS76. Overnight treatments using KDA were carried out in Tris–EDTA buffer.

Table III. Comparison of mutation spectra induced by KDA treatment in Tris–EDTA and PBS in the yeast functional *p53* mutation assay

	KDA in Tris–EDTA	KDA in PBS
GC targeted	43 (57)	26 (53)
GC→AT	26 (35)	17 (35)
GC→TA	12 (16)	5 (10)
GC→CG	5 (7)	4 (8)
AT targeted	21 (28)	21 (43)
AT→GC	9 (12)	6 (12)
AT→TA	9 (12)	10 (20)
AT→CG	3 (4)	5 (10)
Ins/Del/Comp[a]	11 (15)	2 (4)
TOTAL	**75**	**49**

The numbers found in each category of mutation are shown with the proportions in parentheses.
[a]The Ins/Del/Comp category includes insertions and deletions of sequences ranging from a single nucleotide base pair up to tens of base pairs plus complex mutations involving >1 bp.

base pairs (5/7), ~98% of all mutations at GC base pairs were GC→AT transitions, clearly dominating the MNU-induced spectrum.

In contrast to the spectrum induced by MNU only 56% of all KDA mutations were aimed at GC base pairs, whereas about a third were directed at AT base pairs. Of all base substitutions 48% (53/111) were accounted for by transversions and 52% (58/111) by transitions. Transitions were mainly comprised of GC→AT mutations (43/58), while AT→GC mutations accounted for 26% (15/58). Transversions were mainly GC→TA mutations (17/53) and AT→TA mutations (19/53). Frameshifts accounted for 10% of all KDA-induced mutations and were comprised largely of deletions (11/13). It is thought that GC→AT transitions induced by methylating agents are due to the miscoding properties of the O^6-MedG adduct. When the sites of GC→AT transitions induced by MNU in the *p53* gene were examined for sequence context a 4-fold bias was found in

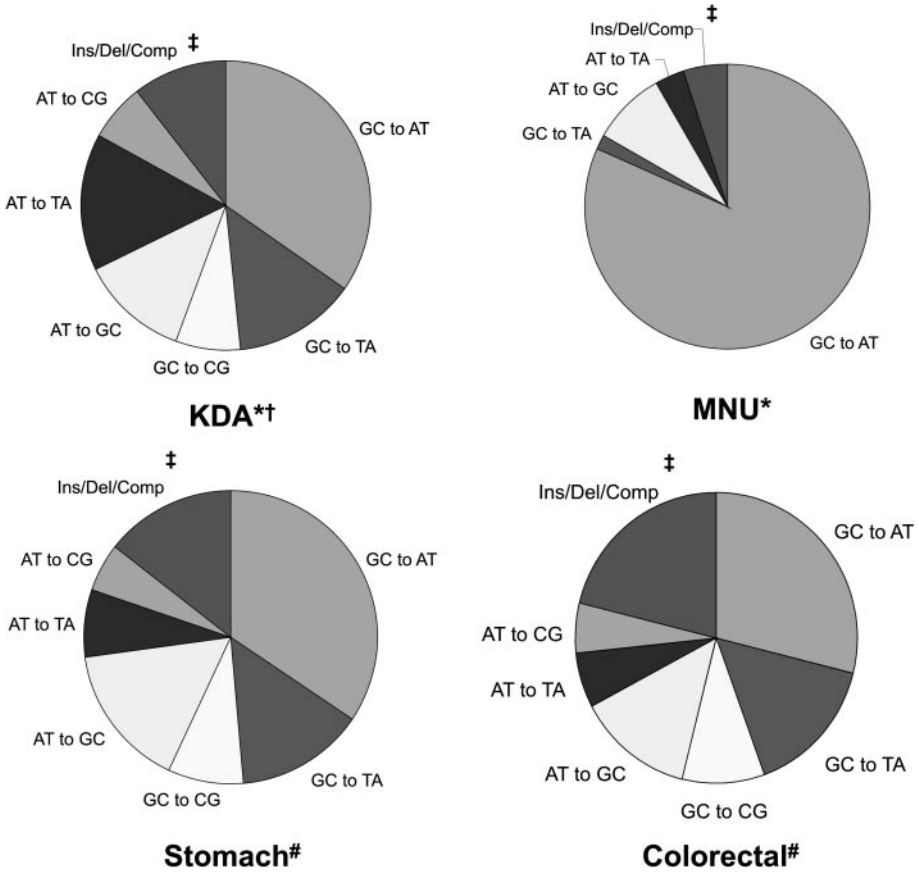

Fig. 3. Comparison of mutation spectra induced by KDA and MNU in the yeast functional *p53* mutation assay. Asterisk (*) denotes that the KDA and MNU spectra were obtained from the sequence analysis of the region of the *p53* cDNA spanning codons 90-290; dagger (†), KDA in Tris–EDTA and KDA in PBS induced spectra were statistically indistinguishable and thus combined here; hash symbol (#), the stomach and colorectal spectra were extracted from the most recent version (issue 10) of the IARC TP53 database. Mutations from outside the region spanning codons 90–290 were excluded, as were GC→AT transitions at CpG sites. Double plus (‡), the Ins/Del/Comp category includes insertions and deletions of sequences ranging from a single nucleotide base pair up to tens of base pairs plus complex mutations involving more than one base pair.

favour of mutations at guanine bases preceded (5′) by a purine (G. B. Scott and P. A. Burns, unpublished data). This is similar to earlier findings (24). No such bias was observed for the GC→AT transitions induced by KDA, suggesting not only that these mutations were more likely to result from O^6-CMdG adducts, but also that O^6-MedG adducts may be playing a relatively minor role in KDA mutagenesis.

Comparison of mutations induced by MNU and KDA to mutations seen in colon and gastric cancer (Figure 3) showed that the types of mutation observed in these human cancers matched more closely with those obtained for KDA. The nature and proportions of mutations were almost identical to those observed for KDA and strikingly different to those obtained for MNU. These findings support the hypothesis that the presence of O^6-CMdG might play an important role in the aetiology of GI tract cancer. Spectra obtained for other putative GI tract mutagens, such as benzo[α]pyrene diol epoxide (BPDE), 2-amino-3, 8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and hydroxyl radicals, showed a distinctly different pattern to that of human gastric and colon *p53* mutations (G. B. Scott and P. A. Burns, unpublished data).

A comparison of the types of mutation within the *p53* spectra of human colon and stomach tumours (excluding CpG sites) showed that the characteristics i.e. proportion of transitions, transversions and frameshifts were very similar (Figure 3). This resemblance could imply that the same agents may contribute to both types of cancer. The prevalence of GC→AT transitions in the MNU-induced *p53* spectrum is largely due to the miscoding properties of O^6-MedG, the latter forming a stable mispair with thymine (25). Differences between the two spectra might therefore suggest that mutations induced by KDA were mainly caused by the O^6-CMdG adduct. In all likelihood, KDA-induced O^6-MedG adducts were efficiently repaired by O^6-alkylguanine-DNA alkyltransferases (ATases) in the transformed yeast cells. Figure 4 shows the KDA-induced mutations in the yeast assay that also occur in human colorectal and stomach tumours. This comparison reveals that 48/111 (43%) of the KDA base substitutions observed in the yeast assay correspond with those seen in human colorectal tumours, while 39/111 (35%) are seen in stomach tumours. The KDA mutations account for 7.0 and 8.7% of the colorectal and stomach spectra, respectively. Correspondingly, there are a number of KDA mutations that do not appear in the human spectrum but it is important to note that the human spectrum is the result of not only mutation but also subsequent selection during the growth of the tumours. This selection pressure is minimal in the yeast assay. It is therefore not surprising that <10% of the human GI mutations can be theoretically accounted for by KDA mutagenesis as only 124 mutations were characterised

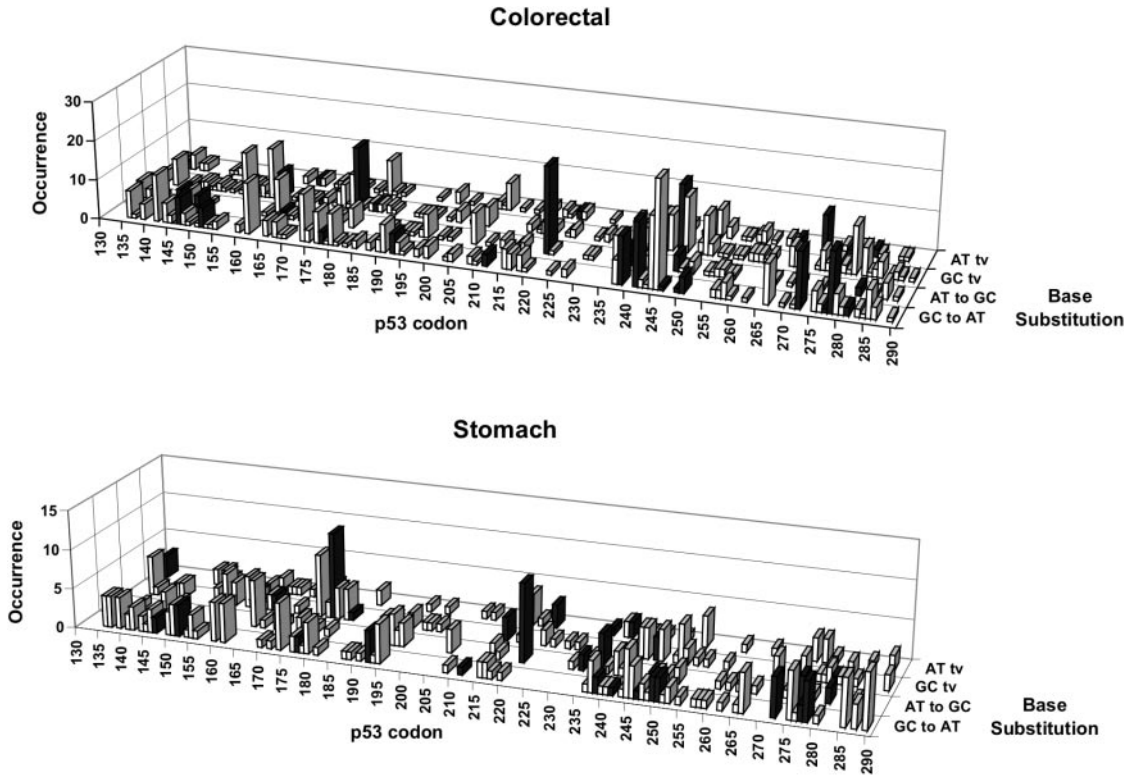

Fig. 4. The bars in this figure correspond to the sites and frequency of base substitution mutations in *p53* in human colorectal and stomach tumours (excluding those arising at CpG sites – see text). Those mutations that coincide with those induced in the yeast assay by treatment with KDA are filled in. These sites represent approximately half of the total induced by KDA in the yeast assay. tv = tranversions.

against >3000 GI mutations in the IARC *p53* database. It is also highly likely that other carcinogens and endogenous processes will play a significant role in GI tumorigenesis.

Some years ago we showed that O^6-CMdG was not repaired by ATases from a variety of sources (26). In principle, incubation of KDA-treated plasmids with excess ATase could have been used to remove O^6-MedG leaving only O^6-CMdG. However, it was decided to introduce O^6-CMdG unambiguously by chemical synthesis into a single site in the *p53* cDNA sequence in order to examine the mispairing potential of this base. The results are described in detail elsewhere but it was found that O^6-CMdG gives rise to both GC→AT and GC→TA mutations. Under identical assay conditions, O^6-MedG gave only GC→AT mutations at the same site (S. Ponnada, P.A. Burns and D.E.G. Shuker, manuscript under preparation). The reasons for these differences are not yet clear but may be due to a combination of the steric bulk of the carboxymethyl group and the presence of a negative charge leading to a different mode of mispairing than that seen for simple O^6-alkylguanines (27).

The mechanisms leading to the formation of *N*-nitroso compounds (NOC) and subsequent decomposition to DNA alkylating agents are complex. A model of the aetiology of gastric cancer has been proposed by Correa (28). Several mechanisms leading to the formation of NOC intermediates have been outlined: acid or bacterially catalysed nitrosation, and nitrosation from nitric oxide. The increased risk of developing gastric cancer has also been linked with high intake of smoked, salted and nitrated foods, high intake of carbohydrates and low intake of fruits, vegetables and milk. Another important link has been proposed between gastric cancer and *Helicobacter pylori* infection which is believed to play a role in ~60% of all cases (29). Bingham *et al*. (30) have recently established a link between high intake in red meat and increased endogenous intestinal production of NOC and nitrite. This association could not be made for white meat and fish.

Recent *in vitro* studies in our laboratory using conditions similar to those found in the GI tract *in vivo* showed that treatment of glycine with nitric oxide results in the formation of diazoacetate. Furthermore, incubation of the reaction mixture with 2′-deoxyguanosine and DNA formed O^6-CMdG and O^6-MedG DNA adducts. These findings suggested that diazoacetate was a key alkylating agent formed from the nitrosation of glycine under simulated physiological conditions (4).

Endogenous nitrosation of dietary amino acids and peptides has been proposed as a major route of exposure to genotoxic agents in the GI tract (31,32). Glycine is unique among the amino acids in having no substituent on the α-carbon. This appears to have a profound effect on the reactivity of the nitrosated amino acid in that any α-substituent, such as a methyl group in alanine, dramatically reduces its DNA-damaging potential (33). In any event, the O^6-CMdG adduct is structurally uniquely related to glycine (Figure 1) and its nitrosated derivatives. It would therefore seem likely that nitrosation products of glycine could constitute a major source of amino acid-derived alkylating agents in the gastric and intestinal contents. Thus the observations that O^6-CMdG has been detected in human gastric biopsies (5) and, more recently, in exfoliated colorectal

cells (32) lend further support to the hypothesis that nitrosation of glycine occurs in the human GI tract and may contribute to risk of cancers, particularly of the colon.

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