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Formation and persistence of N7-methylguanine DNA adducts in the target pyloric tissue following chronic exposure to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine

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Outbred 7-week old male Wistar rats were exposed for 21 days to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) via the drinking water and N7-methyl deoxyguanosine 3′-monophosphate (N7-MedGp)levels in DNA from the pyloric mucosa (target tissue) and white blood cells (wbc: non-target tissue) were determined by ^{32}P-postlabelling. Exposure to MNNG resulted in the non-linear, dose-related formation of N7-medGp in both tissues. Adduct levels in the pyloric mucosa were determined to be 1058, 5.4 and 1.1 μmole N7-medGp mole^{-1} deoxyguanosine 3′-monophosphate (dGp) after exposure to 4.1, 0.62 and 0.006 mg MNNG kg^{-1} day^{-1} respectively whereas adduct levels in the wbc DNA were lower at 5.2, 0.52 and 0.68 μmoles N7-medGp mole^{-1} dGp after exposure to 4.1, 0.62 and 0.062 mg MNNG kg^{-1} day^{-1} respectively. In addition, the persistence of N7-medGp was investigated. Loss of adduct occurred rapidly, with a decrease of 87 and 97% respectively in target tissue and wbc DNA by 48 h after cessation of 4.1 mg MNNG kg^{-1} day^{-1} exposure; 14 days post-MNNG treatment, however, N7-medGp was still detectable (0.46 μmole N7-medGp mole^{-1} dGp) in pyloric mucosal DNA. The quantitation of N7-medGp after exposure to low doses of carcinogen, i.e. 0.006 mg MNNG kg^{-1} day^{-1}, approaching environmentally relevant levels has not been previously reported, and indicates that the ^{32}P-postlabelling assay developed here possesses sufficient sensitivity to quantitate N7-medGp in human DNA arising from environmental exposure to methylating agents.

Keywords: N7-methylguanine, target tissue, MNNG, ^{32}P-postlabelling.

Abbreviations: dG, deoxyguanosine; dGp, deoxyguanosine 3′-monophosphate; pdG deoxyguanosine 5′-monophosphate; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; N7-MeG, N7-methylguanine; N7-MedGp, N7-methyldeoxyguanosine 3′-monophosphate; N7-MepdG, N7-methyldeoxyguanosine 5′-monophosphate; NDMA, *N*-nitrosodimethylamine; O^6-MeG, O^6-methylguanine; wbc, white blood cells.

Introduction

N-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) is an animal carcinogen, which results in a high incidence of tumours of the forestomach and pyloric regions of the stomach when administered to rats as single/multiple oral doses (Schoental 1966, Zaidi *et al.* 1993a) and in the drinking water (Sugimura and Fujimura 1967, Sugimura *et al.* 1970, Zaidi *et al.* 1993a) respectively. The chronic exposure (drinking water) regime has been considered as a model for human gastric carcinogenesis (Mirvish 1983, Boeing 1991) and has provided direct evidence that high levels of DNA methylation are associated with tumour induction in the target

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(i.e. the pylorus) tissue (Kobori *et al.* 1988, Zaidi *et al.* 1993b). By exposing rats to MNNG in the drinking water as described by Zaidi *et al.* (1993b), the following questions were addressed. Firstly, was a newly developed ^{32}P-postlabelling assay (Haque *et al.* 1997) sensitive enough to quantitate N7-methylguanine (N7-MeG) in the pyloric mucosa of rats exposed to doses of MNNG lower than that previously investigated and at doses that may be relevant to humans? Secondly, for what period of time, after cessation of carcinogen exposure, did N7-MeG persist in the pyloric mucosa? Thirdly, could white blood cell (wbc) N7-MeG levels be used as an indicator of pyloric mucosal levels and do they accurately reflect DNA methylation in the target tissue?

Materials and methods

Treatment of animals

Male outbred Wistar rats (7-weeks old) were obtained from the Paterson colony. Animals were housed two per cage, maintained on a 12 h light/dark cycle and were allowed water and food (standard rodent chow) *ad libitum*. Animal experiments were performed in accordance with the Animals (Scientific Procedures) Act of the UK Parliament, 1986. The rats were exposed to MNNG at concentrations ranging from 0.04 to 40 μg ml^{-1} in drinking water for 3 weeks (four rats per MNNG dose). At this time, animals were sacrificed but further groups of animals exposed to 40 μg ml^{-1} MNNG were maintained on normal drinking water for either 2 or 14 days, whereupon they too were sacrificed. Untreated control animals were maintained on normal drinking water. MNNG solutions, kept in glass bottles covered with aluminum foil, were provided for the animals and replaced with freshly prepared solutions every third day.

The total amount of MNNG consumed by each group of rats over the 3 week treatment period was calculated from the daily water intake, taking into account the stability of MNNG in solution at room temperature (16% loss after 72 h [Zaidi, unpublished results]). The average daily consumption of MNNG ranged from 0.006 to 4.1 mg MNNG kg^{-1} day^{-1}: a total of approximately 0.02–14.2 mg MNNG per animal was consumed.

Collection of biological samples

Pyloric mucosa and blood from each animal were collected. Animals were first terminally anaesthetized by Enflurane inhalation. While the heart was still beating, blood was taken from the hepatic portal vein and transferred to a sterile universal containing tri-sodium citrate (final concentration 10 mM), and immediately stored at –20 °C. The stomach (with part of the oesophagus and duodenum attached) was then opened along the greater curvature, rinsed in cold phosphate buffered saline and the pyloric mucosa was collected by scraping the pyloric region using a clean, metal spatula. The mucosa was then snap-frozen in liquid nitrogen.

DNA isolation

Pyloric mucosa samples were thawed, homogenized with 0.5 ml of 50 mM Tris–HCl (pH8) containing 100 mM NaCl, 100 mM EDTA, 1% (w/v) SDS and 2% (v/v) β-mercaptoethanol, and then treated with proteinase K for 15 h at 4 °C (Haque *et al.* 1997); RNase A was added for a further incubation at 37 °C for 1 h. The DNA was extracted with phenol, chloroform and recovered by ethanol precipitation (Haque *et al.* 1997). DNA from the wbc was purified using a Nucleon II DNA extraction kit (Scotlab), using essentially the manufacturer's recommended procedure. Briefly, rat blood was lysed with 100 mM Tris–HCl (pH 8) containing 320 mM sucrose, 5 mM $MgCl_2$, 1% v:v Triton X-100 and the wbc nuclei were collected by centrifugation, resuspended in 400 mM Tris–HCl (pH 8) containing 60 mM EDTA, 150 mM NaCl, 1% v:v SDS, shaken and then extracted using phenol. The aqueous phase was extracted with chloroform, the Nucleon Silica suspension added and the sample was centrifuged. DNA in the aqueous phase was recovered with ethanol, dried, and dissolved in double distilled water for subsequent analysis.

Isolation of N7-MedGp

Equal amounts of DNA from each of the four animals per treatment group were pooled. Duplicate DNAs were digested to nucleoside-3′-monophosphates with micrococcal nuclease and calf spleen phosphodiesterase at neutral pH by incubation at 4 °C for 18 h (Haque *et al.* 1997). N7-MedGp was

then isolated by two-stage HPLC by elution with 0.5 M triethylamine acetate (pH 7) using a SynChropak AX300 column (5 µm, 250 mm ×4.6 mm) and then by elution with 0.1 M triethylamine acetate (pH 7) containing 1% v/v acetonitrile using a Chromex Hypersil ODS 5 column (5 µm, 250 mm × 4.6 mm) (Haque *et al.* 1997). HPLC column fractions corresponding to N7-MedGp were collected, pooled and dried *in vacuo* prior to ^{32}P-postlabelling. The amount of deoxyguanosine 3′-monophosphate (dGp) released by enzymic digestion was quantitated by the area under the appropriate HPLC peak (A_{254nm}) in the initial anion exchange separation.

Quantitation of N7-MedGp by ^{32}P-postlabelling

Briefly, N7-MedGp was postlabelled in the presence of an internal standard (dGp) at pH 8.6 using 20 µCi of [γ^{32}P]-ATP, and two units of T_4-polynucleotide kinase. After incubation at 37 °C for 60 min, ^{32}P-postlabelled 3′-, 5′-bisphosphates were converted to ^{32}P-postlabelled -5′-monophosphates by the addition of nuclease-P1 (0.6 units) and the samples were incubated for a further 30 min at 37 °C. ^{32}P-postlabelled, nuclease-P1 digested samples were then applied to methanol-washed PEI–cellulose TLC plates and then chromatographed with 1 M ammonium acetate (pH 8)/propan-2-ol (90:10, v/v) as the first dimension (D1) and with saturated sodium citrate/saturated ammonium sulphate/propan-2-ol (50:5:1, v/v/v) as the second dimension (D2). Adduct levels were then quantified from the ratio of [^{32}P]-N7-methyl deoxyguanosine 5′-monophosphate (N7-MepdG)/[^{32}P]-deoxyguanosine 5′-monophosphate (pdG) by using a Molecular Dynamics 425S Phosphorimager and ImageQuant™ software (Povey and Cooper 1995). The recovery of N7-MedGp was 40%, resulting in a detection limit of 1.3 fmole which is equivalent to 0.16 µmol mole^{-1} dG when analysing 10 µg DNA (Haque *et al.* 1997).

Results

Figure 1 shows representative phosphorimages of ^{32}P-postlabelled pyloric mucosa DNA samples from animals treated with 0.06 and 4.1 mg MNNG kg^{-1} day^{-1} and drinking water alone. N7-MedGp was easily detected in DNA from both the pyloric tissue and wbc of treated animals at levels ranging from 1.1 to 1058 and 0.68 to 5.2 µmole N7-MedGp mole^{-1} dGp respectively. N7-MedGp was also present in DNA from the control rats but these background levels were detected only after prolonged exposure (>5 h) of the TLC plates to storage-phosphor screens. Under these conditions the intensity of the pdG (internal standard) signal was no longer proportional to the amount of radioactivity present so that a direct comparison between the pdG and N7-MepdG signal was impossible. Hence background levels were estimated by the determining the ratio of signal generated by a ubiquitous contaminant (spot X; see figure 1) to that of pdG under quantitative conditions. This ratio was then used to correct the pdG signal when plates were exposed for greater than 5 h. Using the corrected pdG signal, adduct levels in control DNA were estimated to be 0.087 µmole N7-MedGp mole^{-1} dGp.

N7-MedGp levels in the pyloric mucosa were found to be related to the amount of MNNG consumed (figure 2). The relationship between dose and adduct levels was complex, however, as an ~6-fold increase in dose (0.62 vs 4.1 mg MNNG kg^{-1} day^{-1}), resulted in a more than 200-fold increase in adduct levels. Conversely a 100-fold decrease in dose (from 0.62 to 0.006 mg MNNG kg^{-1} day^{-1}) resulted in only a five-fold decrease in adduct levels. Levels of N7-MedGp in DNA from wbc were 10–200-fold lower than those found in the target tissue (figures 2 and 3). The levels of N7-MedGp in wbc DNA were similar at MNNG doses of 0.062 and 0.62 mg kg^{-1} day^{-1}: and in contrast to the target tissue, a further seven-fold increase in MNNG dose (to 4.1 mg kg^{-1} day^{-1}) resulted in only a 10-fold increase in adduct levels.

Loss of N7-MedGp adduct was rapid with only ~3% and 13% of the adduct still present in the pyloric and wbc DNA 2 days after the cessation of treatment (figure 3). Two weeks after the treatment, the levels of N7-MedGp in the pyloric mucosa

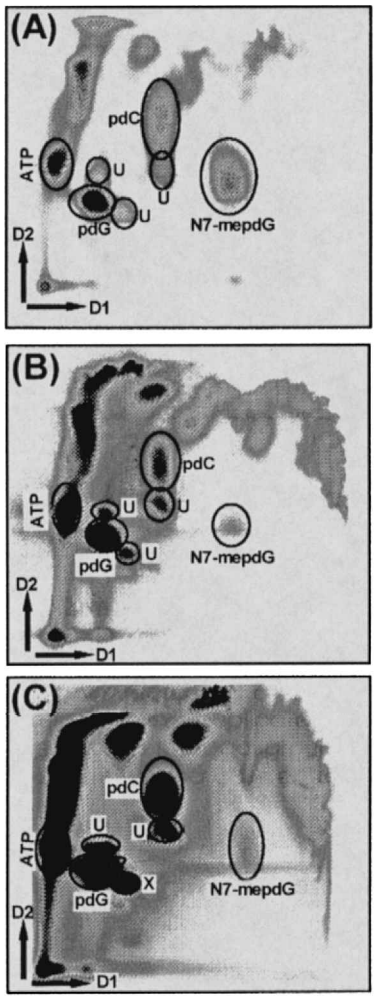

Figure 1. Two-dimensional-TLC phosphorimages of ^{32}P-postlabelled pyloric mucosa DNA. DNA from the pyloric mucosa of rats treated with 4.1 mg MNNG kg^{-1} day^{-1} (panel A), 0.06 mg MNNG kg^{-1} day^{-1} (panel B) or untreated (panel C) were enzymically digested (5, 20 and 20 µg DNA respectively) and separated by two-step HPLC. The appropriate HPLC column fractions were collected and ^{32}P-postlabelled (with 2 pmole dGp), nuclease-P1 digested and resolved by two-dimensional-TLC (solvent system C). TLC plates A and B were exposed to storage-phosphor screens for up to 5 h whilst TLC plate C was exposed for 48 h. U = unknown contaminant. X = unknown contaminant used to correct pdG signal after over-exposure (see text for details).

had decreased by a further 98% (to approximately 0.04% of the initial amount) to 0.46 µmole N7-MedGp mole^{-1} dGp, which was approximately five-fold above the level in untreated rats.

Discussion

An animal model system has been employed in order to assess the sensitivity and suitability of the N7-MedGp ^{32}P-postlabelling assay as a means of estimating human exposure to methylating agents. This assay was able to detect N7-MedGp

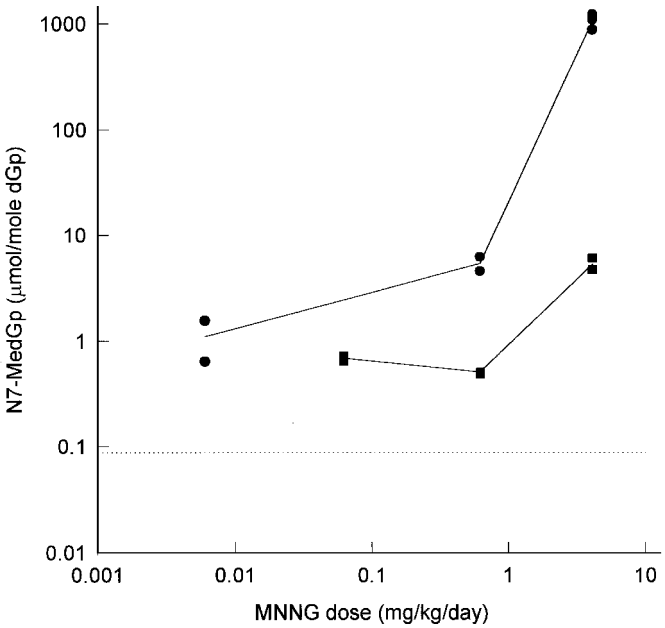

Figure 2. The levels of N7-MedGp measured in the DNA of rats exposed to MNNG in drinking water. N7-MedGp levels in pyloric mucosa (●) and wbc (■) DNA were determined by ^{32}P-postlabelling. The curve is plotted through the mean ± range of duplicate analyses of DNA pooled from four rats at each dose of MNNG. The dotted line represents the background level of 0.087 μmole N7-MedGp mole^{-1} dGp detected in untreated, control animals.

in DNA from the pyloric mucosa of rats exposed to MNNG doses lower than those previously investigated. At the highest dose, N7-MedGp levels were approximately 1.5-fold higher than previously observed at similar doses (Kobori *et al.* 1988) whereas at the lowest dose they were approximately 10-fold higher than those found in control, untreated, animals. A non-linear dose–response relationship was observed in both target and non-target tissues (figure 2), confirming previous results for O^6-methylguanine (O^6-MeG) obtained not only in the rat stomach following exposure to MNNG (Kobori *et al.* 1988, Zaidi *et al.* 1993b) but also in the kidney and liver following oral administration of *N*-nitrosodimethylamine (NDMA) to rats (Pegg and Hui, 1978) or via the drinking water (Lindamood *et al.* 1984). The sharp rise in dose-responsiveness observed when the dose of MNNG was increased from 0.62 to 4.1 mg kg^{-1} day^{-1} may have resulted from the saturation of detoxification and DNA repair mechanisms (Kawachi *et al.* 1970, Hoel *et al.* 1983).

N7-MedGp levels were lower in wbc than those in the pyloric mucosa at each of the MNNG doses (and time points) investigated (figures 2 and 3), probably because the amount of MNNG available to methylate other tissues was significantly reduced by the thiol-catalysed decomposition undergone by MNNG in the stomach (Lawley and Thatcher 1970, Kobori *et al.* 1988). Bianchini and Wild (1994) similarly demonstrated that rat wbc N7-methylguanine (N7-MeG) levels, generated by a variety of other methylating agents, were lower than those determined in target organ DNA. As routine biopsy of internal organs may not be feasible for epidemiological studies, more accessible tissues such as wbc are being increasingly used as surrogate sources of DNA. The lack of a consistent ratio

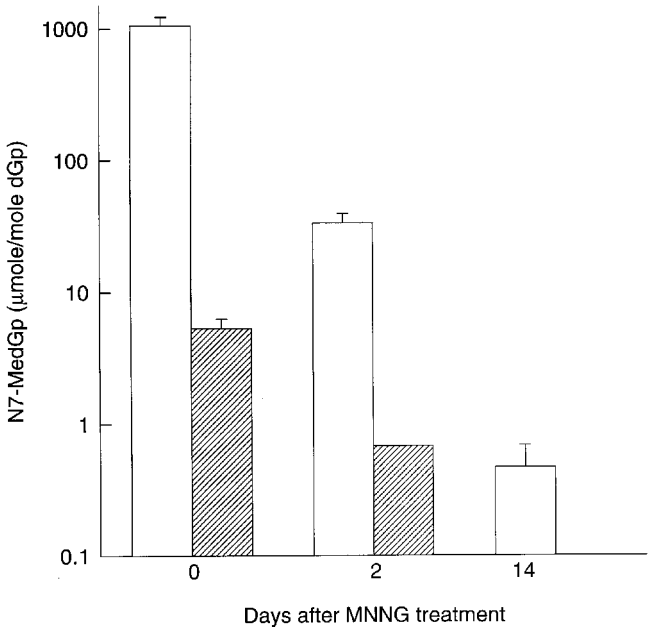

Figure 3. The persistence of N7-MedGp after cessation of exposure of MNNG in the drinking water. N7-MedGp levels were determined in pyloric mucosa (open bar) and wbc (shaded bar) DNA of rats exposed to 40 µg MNNG ml^{-1} for 3 weeks after which the animals received normal drinking water for a further 2–14 days.

between adduct levels in DNA from wbc and the pylorus, which in this case varied from 0.005 to 0.09, suggests that measurement of adduct levels in wbc in humans may not adequately reflect stomach adduct levels. Furthermore, as adduct levels in wbc may reflect exposure from other sources (e.g. smoking), wbc adduct levels may not be a valid marker of exposure within the stomach.

The presence of N7-MedGp in pyloric DNA of untreated animals, in this study, suggests that these animals are exposed to as yet unidentified methylating agent(s). Thus, the lack of a dose response in adduct levels in wbc DNA between 0.06 and 0.6 mg MNNG kg^{-1} day^{-1} may reflect exposure to such agents. Bianchini and Wild (1994) have also reported the presence of N7-MeG in liver, oesophageal, colonic and lung DNA from rats not exposed to methylating agents. There are two possible explanations, which are not mutually exclusive, for the presence of N7-MedGp in control DNA. Firstly, methylating agents e.g. NDMA may be present in commercial sources of the rodent diet (Peto *et al.* 1991), and secondly, *N*-nitroso compounds may be formed in the stomach under normal physiological conditions from dietary precursors or from endogenous sources of nitrosating agents (Bartsch and Montesano 1984).

In this study, N7-MedGp was lost from both wbc and pyloric mucosa DNA at a rate which was higher than that expected to result from simple chemical instability. The half-life of N7-MeG in DNA *in vitro* at 37 °C and pH 7 is approximately 6 days (Margison *et al.* 1973) but within 48 h after the end of MNNG treatment, 87 and 97% of the adducts had been lost from wbc and pylorus DNA respectively (figure 3). The adduct half-life in either tissue could not be accurately estimated but may be lower than that observed in rat colon (31 h) and hamster forestomach

(25–35 h) DNA (Herron and Shank 1981, Likhachev *et al.* 1983). Loss of N7-MeG from DNA may result therefore from an active repair mechanism, e.g. *N*-methylpurine-DNA glycosylase (Roy *et al.* 1996) or through cell loss, e.g. by cell sloughing and cell turnover. The relative contribution of one or both of these two processes to the removal of N7-MeG lesions from pyloric DNA cannot precisely be determined. However, the turnover time for pyloric mucosal cells in the rat stomach, which has been reported to be 68–156 h (Stevens and Leblond 1953), can help to explain the rapid loss of both N7-MedGp and other adducts e.g. 92% of aristolochic acid I adducts were removed in 7 days (Fernando *et al.* 1993) from the pyloric mucosa. Herron and Shank (1981, 1982) also reported that, in the colon, reduction in adduct levels may be mediated primarily by cell sloughing and cell turnover as the rate of repair of N7-MeG and O^6-MeG adducts was similar although they are repaired by different mechanisms. In contrast, while the loss of alkylated white blood cells, such as neutrophils, could explain the rapid removal of N7-MedGp from wbc DNA in this study, similar high rates of N7-MeG loss have been observed in rat lymphocytes (Degan *et al.* 1988) and benzo(*a*)pyrene adducts can persist in the rat lymphocyte DNA for up to 17 days after exposure (Ross *et al.* 1990). The rapid loss of N7-MedGp probably reflects the presence of an active repair system, and indeed, human lymphocytes have been demonstrated to contain DNA glycosylase activity capable of excising both N7-MeG and N3-methylguanine (Singer and Brent 1981). Interestingly, N7-MeG appears to be more persistent in human wbc (van Delft *et al.* 1992).

N7-MedGp was still detectable in the DNA of the pylorus 14 days after the final dose at levels approximately 10-fold higher than the untreated controls. This suggests that within the stomach, a subpopulation of methylated cells persists within the pyloric mucosa. This is in agreement with the findings of Zaidi *et al.* (1995) who identified a group of non-parenchymal cells in the upper half of the pyloric mucosa in which O^6-MeG was detectable by immunohistochemistry up to 7 days after MNNG exposure. In contrast to epithelial cells, these methylated cells, possibly fibroblasts, were not exfoliated and appeared to be less effective at mediating O^6-MeG repair. These cells may then accumulate DNA damage and levels of DNA damage may reflect cumulative exposure over a period of weeks (or possibly longer) rather than recent exposure which adduct measurement within epithelial cells would reflect.

As far as we can ascertain, levels of N7-MeG in human stomach tissue have not yet been reported. O^6-MeG, however, has been detected in human stomach tissue at levels ranging from 0.02 to 0.12 (Umbenhauer *et al.* 1985), 0.05–3.3 (Hall *et al.* 1991), 0.06–0.26 (Povey *et al.* unpublished data) µmole O^6-MeG mole^{-1} deoxyguanosine (dG). Assuming an O^6-MeG/N7-MeG of approximately 0.1 (Saffhill *et al.* 1985), then the N7-MeG levels in these individuals would have ranged from 0.2 to 33 µmole N7-MedG mole^{-1} dG. In this study, the lowest MNNG dose (6 µg MNNG kg^{-1} day^{-1}) resulted in an adduct level of 1.1 µmole N7-MedG mole^{-1} dG, suggesting that human exposure to methylating agents is of a similar magnitude. This is consistent with previous appraisals of human exposure to both endogenous and exogenous sources of *N*-nitroso compounds, estimated to be between 0.25 (Bartsch and Montesano 1984) and 1 µg kg^{-1} day^{-1} (Wild and Montesano 1991). Although it is not known whether tumours can be induced at these low MNNG levels, it is interesting to note that rats exposed to 0.01 µg NDMA ml^{-1} in drinking water had an increased incidence of liver tumours when

compared with untreated control animals (Peto *et al.* 1991). In addition, levels of O^6-MeG in liver DNA have been measured under conditions similar to those employed in this NDMA tumorigenesis study (Souliotis *et al.* 1995). After 21 days of NDMA administration, using doses similar to those used in our study (73–370 μg kg^{-1} day^{-1}), levels of O^6-MeG in liver DNA ranged from 0.18 to 0.45 μmole O^6-MeG $mole^{-1}$ dG. Assuming an O^6-MeG/N7-MeG of approximately 0.1, then the levels of N7-MeG would have been approximately 1.8–4.5 μmole N7-MeG $mole^{-1}$ dG, i.e. following similar exposure to two different methylating agents target tissue adduct levels were of a similar magnitude.

In summary, the ^{32}P-postlabelling assay for N7-MedGp has been demonstrated to be capable of accurately quantitating adduct levels in the target tissue arising from exposure to environmentally relevant levels of methylating agents but N7-MeG levels in wbc DNA may not accurately reflect the level of damage occurring within the target tissue. Loss of N7-MeG adducts from the DNA of both the pylorus and wbc is rapid and levels may therefore reflect only recent exposure. However, adducts may persist within certain cell types within the target tissue and these cells may accumulate measurable levels of DNA damage over a longer period.

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