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Genotoxicity of Glycidamide in Comparison to 3-*N*-Nitroso-oxazolidin-2-one

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Acrylamide (AA) is generated by thermal processing of foods, depending on processing conditions and precursor availability. AA is not genotoxic by itself but becomes activated to its genotoxic metabolite glycidamide (GA) via epoxidation, mediated primarily by cytochrome P450 2E1. In the Comet assay in V79 cells and in human lymphocytes, GA induced DNA damage down to 300 μM concentration (4 h). After post-treatment with the DNA repair enzyme formamidopyrimidine−DNA−glycosylase (FPG), DNA damage became already detectable at 10 μM (4 h). By comparison, the *N*-nitroso compound 3-*N*-nitroso-oxazolidin-2-one (NOZ-2) is a much stronger genotoxic agent, significantly inducing DNA damage already at 15 min (3 μM). Post-treatment with FPG in this case did not enhance response. GA induced DNA damage in V79 cells rather slowly, with first response detectable at 4 h. The *hPRT* forward mutation test encompasses 5 days of expression time during which also repair can take place. GA-induced hPRT mutations only became detectable at concentrations of 800 μM and above. This is 80-fold higher than the lowest significant response to GA in the Comet assay (10 μM with FPG). In contrast, NOZ-2 was as effective in the hPRT test as in the Comet assay (3 μM). These results demonstrate substantial differences in the genotoxic potency of GA and NOZ-2. Whereas NOZ-2 is a pontent genotoxic mutagen, GA in comparison shows only low genotoxic and mutagenic potential, presumably as a result, at least in part, of preferential N7-G alkylation.

KEYWORDS: Glycidamide; *N*-nitroso-oxazolidinones; Comet assay; *hPRT* gene mutation assay; DNA strand break persistence

INTRODUCTION

Foods may contain compounds with genotoxic potential, normally present at trace levels. A group of such compounds, the so-called "process-related toxicants", can be formed during the processing and preparation of food from foodborne or environmental precursors.

Acrylamide (AA) can be formed in substantial amounts during the heating of foods, mainly from asparagine in the presence of reducing sugars (*1, 2*). As shown in animal studies, AA undergoes metabolic activation to glycidamide (GA) by cytochrome P 450 (CYP) 2E1 (*3*). AA itself is not or only marginally genotoxic and does not show appreciable reactivity toward DNA (*4*–*8*). Thus, genotoxic effects related to AA exposure are to be ascribed to GA. GA interacts with DNA bases, predominantly by forming N7 adducts with guanine and, to a much lower extent, N3 adducts with adenine (*6*). A significant increase of AA-induced DNA damage in HepG2 cells has been reported after ethanol-mediated induction of CYP2E1 (*8*). AA has been shown to be carcinogenic in rodents, inducing tumors in the thyroid, the mammary gland, or the mesothelial cells of the scrotum (*9*), and has been rated as a class 2 carcinogen, being probably carcinogenic in humans. Mitigation of AA exposure from food according to the as low as reasonably achievable (ALARA) principle is considered to be a priority for consumer protection (*5*).

Our previous findings together with results from other groups (*7, 8*) lend support to the assumption that AA exerts its genotoxic activity via the formation of GA. GA induces DNA damage in human blood lymphocytes dose-dependently at ≥300 μM (4 h) as measured in the Comet assay under standard conditions. In contrast, AA was inactive even at very high (mM) exposure concentrations (*4*). Similar results with GA were obtained in V79 cells, Caco-2 cells, and primary hepatocytes from rats (*10*). Postexposure of the cellular DNA with the DNA repair enzyme formamidopyrimidine−DNA−glycosylase (FPG) resulted in detection of DNA strand breakage already at 80-fold lower concentrations (*11*). FPG recognizes apurinic and

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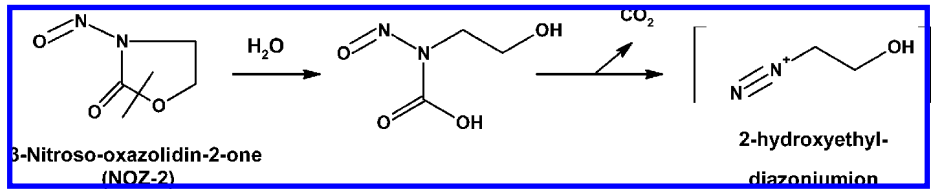

Figure 1. Hydrolytic activation of NOZ-2.

apyrimidinic sites as well as ring-opened purines (*12*), introducing strand breaks at such DNA modifications that can all derive from initial N7-guanine adduct formation.

Human AA uptake via food has been assessed between 0.4 and 0.9 μg/kg of body weight (bw) (*5*). In terms of risk assessment, bioavailability and biotransformation, including activating and detoxifying pathways, need to be taken into account. Moreover, the mutagenic potency of GA-induced DNA lesions as well as their persistence in the genome of the target cell should be considered. In our previous studies, we found AA inactive in the hypoxanthine−phosphoribosyl-transferase (*hPRT*) mutagenicity test with V79 cells, even at cytotoxic concentrations. In contrast, GA was mutagenic, an effect that became however only detectable at rather high incubation concentrations (800 μM) (*4*).

The *N*-nitroso compound 3-nitrosooxazolidin-2-one (NOZ-2) was used as a model compound for a group of heterocyclic *N*-nitrosamines presumably formed from amino acids in the presence of dietary aldehydes and nitrosating agents.

NOZ-2 is mutagenic in *Salmonella typhimurium* (*13*) and was found to be carcinogenic in rodents (*13–15*). When NOZ-2 was given orally to rats, the liver and forestomach were identified as target organs for tumor induction (*13, 14*).

It is assumed that NOZs have genotoxic potential because they are expected to be transformed in DNA alkylating intermediates via hydrolytic ring opening. For NOZ-2 such a reaction leads to the generation of 2-hydroxyethyldiazonium ions (**Figure 1**) (*16*), DNA-alkylating electrophiles that also are inferred to be formed from the carcinogen *N*-nitrosodiethanolamine (NDELA) after hydroxylation of the α-carbon atom (*17, 18*). On reaction with DNA, nucleophilic centers, preferably N7 (*19*) and O^6 of guanine (*18, 20*) among other sites, are 2-hydroxyethylated. Another possible alkylating site is the phosphodiester bond at the DNA−sugar−phosphate backbone. This would result in the formation of DNA phosphotriesters. Whereas alkyl phosphotriesters are very stable, 2-hydroxyethyl phosphodiesters are unstable, creating DNA strand breaks by rapid spontaneous decomposition (*21–23*).

In the present study we compared the time- and concentration-dependent induction and disappearance of DNA lesions induced by GA and NOZ-2 in V79 cells using the Comet assay with and without additional treatment of the DNA with FPG. In addition, the mutagenic potency of GA and NOZ-2 was investigated in the *hPRT* test.

MATERIALS AND METHODS

Chemicals. NOZ-2 was synthesized according to the method given in ref *24*; FPG was provided by A. R. Collins, University of Oslo, Norway; GA (2,3-epoxypropanamide) was prepared according to the method of Payne and Williams (*25*). 6-Thioguanine was obtained from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany).

All chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

Cell Culture, Incubation with Test Compound, and Postincubation. V79 cells (Chinese hamster lung fibroblasts) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) and 1 mM sodium pyruvate.

Cell suspensions were obtained from the monolayer by treatment with trypsin (0.5%) after washing with phosphate-buffered saline (PBS). V79 cells (1×10^6 cells/mL) were incubated with test compounds (dissolved in 0.1% DMSO) for 15 min−24 h at 37 °C in incubation medium (culture medium without FCS).

In postincubation experiments, V79 cells were first incubated with the test compounds (dissolved in 0.1% DMSO) for 4 h in incubation medium. The incubation medium was removed, and the cells were washed twice with PBS and postcultivated for 1, 2, 4, or 8 h in DMEM, supplemented with 10% FCS and 1 mM sodium pyruvate.

Cytotoxicity. Following (post-)incubation, the cell suspension (50 μL) was mixed with trypan blue solution (50 μL, 0.5% in PBS) and microscopically checked for membrane integrity. Viability was expressed as percentage of total cells (absolute viability in percent). Only cell suspensions with viabilities >80% were used for determination of DNA damage and *hPRT* mutagenicity to avoid artifacts resulting from cell death.

Alkaline Single-Cell Gel Electrophoresis (SCGE, Alkaline Comet Assay). Aliquots of cell suspension (70000 cells) were mixed with 65 μL of low melting point agarose and distributed onto a slide precoated with a layer of normal melting point agarose. The agarose layer was covered with a coverslip and kept at 4 °C to allow solidification of agarose. After removal of the coverslip, slides were immersed in lysis solution for 1 h at 4 °C. After the cells had been washed three times with enzyme buffer, cells were incubated for 30 min at 37 °C with either 50 μL of enzyme buffer or FPG dissolved in enzyme−buffer as described (*26*). After DNA unwinding (pH >13, 20 min, 4 °C), horizontal gel electrophoresis (Bio-Rad Sub Cell GT) was performed at 4 °C for 20 min (25 V, 300 mA). After the slides had been washed three times with 0.4 M Tris (pH 7.5), they were stained with ethidium bromide. Microscopic analysis was done with a Zeiss Axioskop 2, equipped with filter set 15 (excitation, BP 546/12; emission, LP 590). Slides were analyzed by computerized image analysis (Perceptive Instruments, Haverhill, U.K.), scoring 2 × 50 cells per slide (2 gels/slide). DNA migration was expressed as mean relative tail intensity (TI %), defined as relative rate of Comet head intensity to tail intensity (*27*). TI of cells was quantified by computer-assisted microscopy using *Comet II* software (Perceptive Instruments).

***hPRT* Gene Mutation Assay with V79 Mammalian Cells.** Cells were maintained in DMEM, supplemented with 10% FCS, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C under 5% CO_2 and 95% saturated atmospheric humidity.

The *hPRT* assay was performed according to the method of Bradley et al. (*28*), with slight modifications as outlined in ref *6*. Briefly, cells (1×10^6) were seeded into 75 cm^2 cell culture flasks with DMEM. After 24 h, medium was replaced by medium containing test compound, predissolved in DMSO (final concentration = 1%). After 24 h of incubation, medium was exchanged to DMEM. Mutations were expressed during a period of 5 days. Mutant frequencies (MF; mutants/10^6 cells) were determined in triplicate, and cloning efficiencies (CE) were scored in duplicate (240 cells per plate) as described (*29*). MF was calculated from the number of detected mutants multiplied with a correction factor F derived from CE (F = 240/number of scored colonies; MF = mutants × F). The mutagenic potential of substances was compared by calculated D_{3C} values (concentration that induced a 3 times increase in mutagenicity).

Cytotoxicity was monitored in cell suspension by determination of trypan blue exclusion as described above.

Statistics. Data were statistically analyzed using a one-sided paired student t test and two-sided ANOVA. Normal distribution was ascertained by Anderson−Darling test and standardized normal distribution (Gaussian).

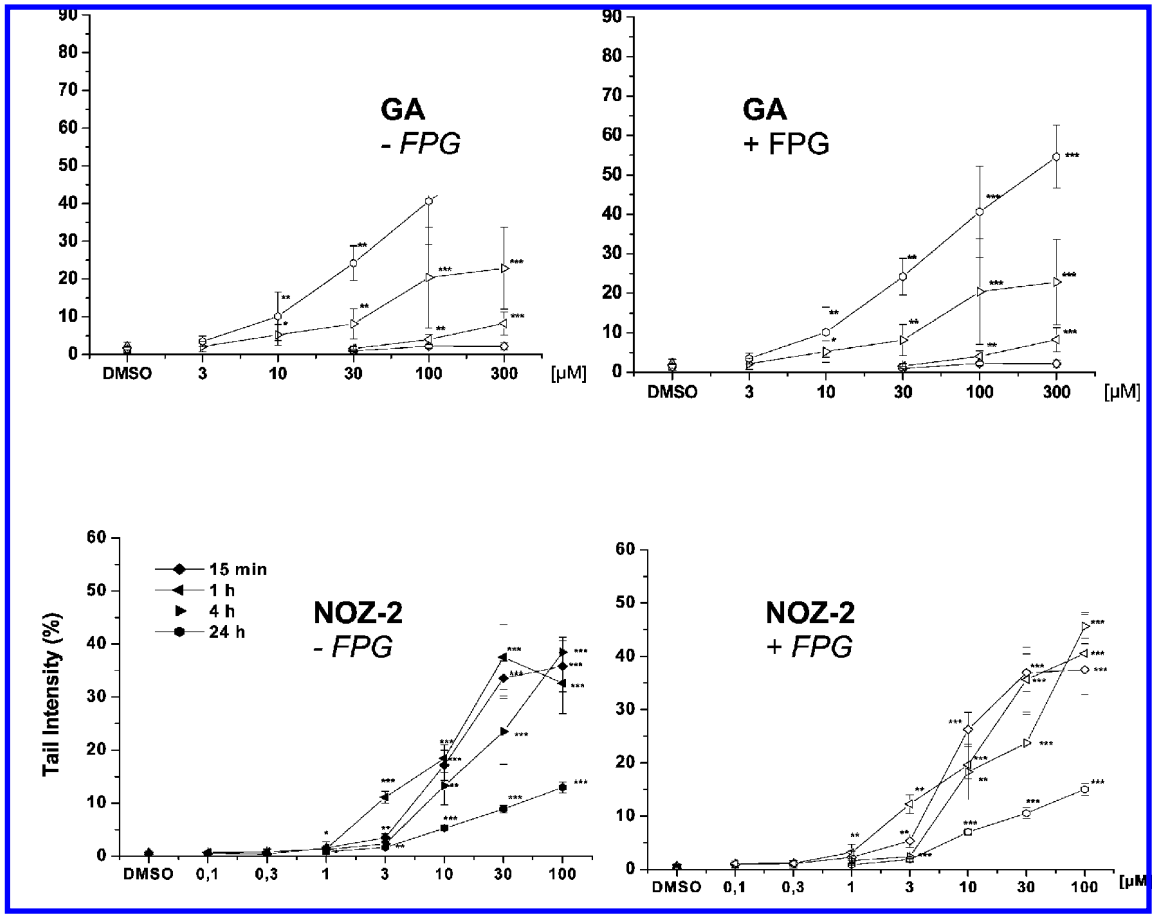

Figure 2. Induction of DNA strand breaks in V79 cells after incubation with GA (3−300 μM; 15 min−24 h) and NOZ-2 (0.1−100 μM; 15 min−24 h) [control, DMSO; ∗, $p < 0.5$; ∗∗, $p < 0.05$; ∗∗∗, $p < 0.005$ (two-sided ANOVA); values represent means and SD of three experiments; $n = 3$].

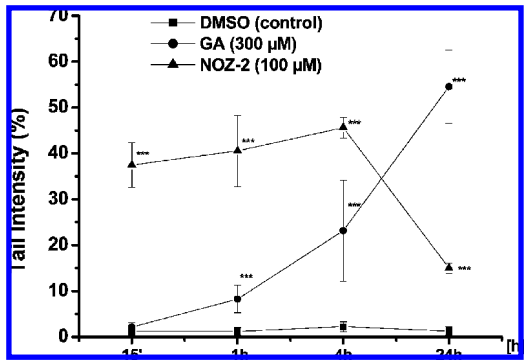

Figure 3. Time dependency of DNA strand break induction in V79 cells after incubation with GA (300 μM) and NOZ-2 (100 μM; 15 min−24 h) [control, DMSO; ∗, $p < 0.5$; ∗∗, $p < 0.05$; ∗∗∗, $p < 0.005$ (two-sided ANOVA); values represent means and SD of 3 experiments; $n = 3$].

RESULTS AND DISCUSSION

Induction of DNA Strand Breaks (SCGE). As investigated in the alkaline Comet assay in V79 cells with and without FPG treatment of DNA, GA was concentration- (**Figure 2**) and time- (**Figure 3**) dependently active. Under the conditions tested, GA-induced DNA strand breaks became detectable after 1 h of incubation time. Without FPG treatment, strand breaks became significant at 300 μM after 4 h and at 100 μM after 24 h. These findings corroborate data reported earlier for V79 cells (*10*) and human blood lymphocytes (*4*). After additional FPG treatment of lymphocyte−DNA, a significant increase of DNA strand breaks, compared to the Comet assay without FPG treatment, was observed with GA (10 μM, 4 h) (**Figure 2**). A similar result has also been reported for lymphocytes from human whole blood (*11*). The pattern of DNA lesions recognized by FPG is known to reflect oxidative DNA damage as exemplified by the primary formation of 8-hydroxy-/8-oxoguanine, leading to the generation of formamidopyrimidines and apurinic/apyrimidinic sites. A similar array of DNA lesions is known to result from N7-G alkylation. Therefore, a strongly enhanced response is observed after GA exposure in the Comet assay after FPG treatment. This is in line with the observed enhancement of DNA strand breaks in human lymphocytes after FPG treatment as discussed in ref *11*.

NOZ-2 showed maximum DNA strand breakage already after 15 min at ≥3 μM. Further FPG treatment did not enhance strand breakage significantly (**Figure 2**). Genotoxicity initially induced by NOZ-2 was found to persist over a time period of 4 h. At later time points (after 24 h), it was found to consistently decrease (**Figure 3**).

The results demonstrate that strand break induction kinetics of GA and NOZ-2 are markedly different. Whereas NOZ-2 induced maximal genotoxic response within a few minutes, GA-induced genotoxicity appeared much more slowly with a first response after 1 h, reaching maximum response only after 24 h (**Figure 3**).

In contrast to GA-induced DNA damage, FPG had no enhancing effect on strand breakage induced by NOZ-2. This can be reconciled with the hypothesis that NOZ-2 may preferentially induce alkylation at the phosphodiester bonds of the DNA backbone. Such hydroxyethyl phosphotriesters are expected to rapidly decay, resulting in spontaneous single-strand break induction (21). This process likely interferes with the detection of FPG-sensitive N7 adducts. O^6 hydroxyethylation of guanine is also expected to occur, but these lesions are not recognized by FPG.

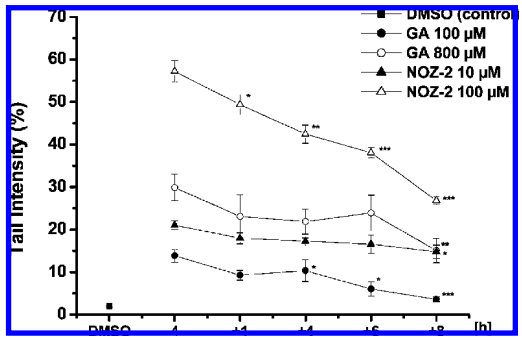

Figure 4. Disappearance kinetics of DNA strand breaks in V79 cells with GA (100 and 800 μM) and NOZ-2 (10 and 100 μM) with FPG treatment (incubation time, 4 h followed by a 1−8 h postincubation of treated cells in cell culture medium) [control, DMSO; ∗, $p < 0.5$; ∗∗, $p < 0.05$; ∗∗∗, $p < 0.005$ (two-sided ANOVA); values represent means and SD of three experiments; $n = 3$].

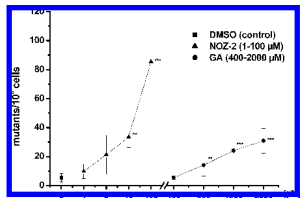

Figure 5. Induction of *hPRT* mutations in V79 cells by GA adapted from ref *4* and NOZ-2 (incubation time, 24 h, 5 days of mutation expression time; mutations are selected by treatment of cells with 6-thioguanine) [control, DMSO ∗∗, $p < 0.05$; ∗∗∗, $p < 0.005$ (two-sided ANOVA); values represent means and SD of three experiments; $n = 3$].

Disappearance of DNA Strand Breaks. Disappearance of DNA strand breaks was measured after 4 h of treatment at concentrations that have been found to induce significant DNA damage in the Comet assay (100 μM). Within 8 h, strand breaks induced by GA (100 μM) decreased by 80%, probably as a result of efficient base excision repair (BER). In contrast, strand breakage induced by NOZ-2 (10 μM) decreased much more slowly within the same time period. This differential persistence is especially evident when the decrease kinetics of GA (100 μM) are compared with those of NOZ-2 (10 μM) (**Figure 4**).

With respect to lowest concentrations found to be mutagenic in the hPRT test, DNA strand breaks induced by GA (800 μM) were reduced by about 40%, whereas those induced by NOZ-2 (10 μM) were only marginally reduced.

Induction of *hPRT* Mutations in V79 Cells. In the *hPRT* gene mutation assay, the ability of NOZ-2 and GA to induce gene mutations at the *hPRT* locus in V79 Chinese hamster lung fibroblasts was investigated. As reported earlier (*4*), GA induced mutations in V79 cells at 800 μM (**Figure 5**). The fact that such a relatively high GA concentration was needed to exert significant mutagenic effects might reflect the slow onset and sluggish rate of DNA damage induction. This corresponds also to the rather fast disappearance of GA-induced strand breaks at submutagenic concentrations after the cells had been challenged, most probably as a result of repair processes. At the lowest mutagenic concentration (800 μM) DNA strand breaks were more persistent.

NOZ-2 significantly induced *hPRT* mutations already at 10 μM (MF, 20 ± 4; D_{3C}, 7 μM) (**Figure 5**). This is the same concentration range as reported earlier for another directly hydroxyethylating agent, α-acetoxy-NDELA (*11*). Similar potency had been observed for the potent preactivated carcinogen benzo[*a*]pyrene−dihydrodiol-epoxide (*11*). Thus, in comparison to GA, NOZ-2 was mutagenic at 80-fold lower concentrations. V79 cells are deficient in O^6-methylguanine−methyl-transferase activity (*30*). It might therefore be inferred that strongly promutagenic DNA adducts, such as O^6-hydroxyethylguanine, known to be generated in addition to N7 adducts from NOZ-2−DNA interaction, will persist. This adduct may well be primarily responsible for the strong *hPRT* mutagenicity of NOZ-2, in addition to other minor DNA adducts that might not or only slowly be repaired under the experimental conditions. A correlation between low carcinogenic and mutagenic potential and a low ratio of O^6-methylguanine to N7-methylguanine adduct formation rate was also discussed for other DNA alkylating compounds primarily forming adducts with the N7 of guanine such as dimethyl sulfate (*31–33*).

O^6-G adduct formation was shown for carcinogenic epoxides, for example, styrene 7,8-oxide, ethylene oxide, or propylen-oxide (*34–36*), but not for GA. Further studies should focus on GA-induced formation of these highly promutagenic lesions.

Conclusion. Our data demonstrate that GA-induced DNA damage rather slowly becomes detectable, the earliest after 1 h of exposure. In addition, DNA damage is efficiently reduced after the cells are challenged with GA, probably due to effective base excision repair. These observations are in line with the relatively low mutagenic activity of GA.

In contrast, NOZ-2 is a rapidly acting, potent genotoxic agent and mutagen with a potency similar to that of other preactivated potent carcinogens such as α-acetoxy-NDELA or B[a]P-7,8-dihydrodiol 9,10-epoxide.

For GA, if formed from AA, induction of significant mutagenicity seems to be primarily a phenomenon associated with generation of DNA N7-G adducts at high concentrations, expected to override repair processes. Compounds that preferentially induce N7-G alkylation have largely been found to have low mutagenic/carcinogenic potential. However, local generation of GA concentrations sufficiently high to generate genotoxicity as might be the case for tissues with significant CYP 450 activity, such as gut and liver, is not very probable, given the fact that such organs have not been found to be targets for tumor induction. Overall, AA was found to induce tumors in rats at relatively high dosages of 0.5–2 mg/kg of bw over a lifetime (*9*). Therefore, the search for a practical threshold for AA/GA-induced genotoxicity in vivo appears to be promising to support risk assessment of dietary AA exposure in the future.

ABBREVIATIONS USED

AA, acrylamide; α-A-NDELA, α-acetoxy-*N*-nitrosodiethanolamine; ALARA, as low as reasonably achievable; AP sites, apurinic and apyrimidinic sites; B[a]P, benzo[*a*]pyrene; BER, base excision repair; CE, cloning efficiency; CYP 450, cytochrome P450; D_{3C} values, concentration that induced a 3 times increase in mutagenicity; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FPG, formamidopyrimidine−DNA−glycosylase; GA, glycidamide; *hPRT*, hypoxanthine−phosphoribosyl-transferase; MGMT, O^6-methylguanine−methyl-transferase; MF, mutant frequency; NDELA, *N*-nitrosodiethanolamine; N7-G, N7-guanine; NOZ-2, 3-nitrosooxazolidin-2-one; NOZs, *N*-nitroso-oxazolidinones; 8-oxo-G, 8-oxoguanine; PBS, phosphate-buffered saline; SCGE, single-cell gel electrophoresis; TI, tail intensity.

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