Activation of 1-nitropyrene by nitroreductase increases the DNA adduct level and mutagenicity

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Abstract: 1-Nitropyrene (1-NP) is a mutagenic nitro compound in the environment. We studied correlations between the mutagenicity of 1-NP for three strains of *Salmonella typhimurium*, the activity of bacterial nitroreductases and the amount of 1-NP-derived DNA adducts. Bacterial strains used in this study were *S. typhimurium* strains TA98, nitroreductase-less mutant TA98NR and YG1021 carrying a nitroreductase-producing plasmid. The mutagenicity of 1-NP was measured using the Ames assay, and the nitroreductase activities of these strains were assayed by quantification of 1-aminopyrene produced from 1-NP. The DNA adducts were measured by the ^{32}P-postlabeling method. Among the three bacterial strains, strain YG1021 was the highest in mutagenicity of 1-NP, the nitroreductase activity and the DNA adduct level. However, *S. typhimurium* strain TA98NR had the lowest values of these three parameters. Nitroreductase activity, DNA adduct level and mutagenicity were strongly correlated with each other. These results indicate that bacterial nitroreductase plays an important role in forming the DNA adducts, and that the higher the adduct level the higher the level of mutagenicity. J. Med. Invest. 44 : 193-198, 1998

Key Words : *Salmonella typhimurium, 1-nitropyrene, nitroreductase activity, DNA adduct, mutagenicity*

INTRODUCTION

We are exposed to many nitro compounds in our daily life by smoking or by inhalation of polluted air. Nitro compounds exhibit mutagenicity following the formation of an active *N*-hydroxy arylamine intermediate produced by reduction of their nitro residues (1).

1-Nitropyrene (1-NP) is one of the nitro compounds found in airborne particles, diesel exhaust, emissions from kerosene heaters, waste water from gasoline stations, used crankcase oil, tea and grilled chicken (2). This compound has been shown to be mutagenic in bacteria and mammalian cells and carcinogenic for animals (2-4). Reductively activated 1-NP forms a major DNA adduct, *N*-(deoxyguanosin-8-yl)-1-aminopyrene (dG-C8-AP) (5). The major adduct causes −2 deletion of a GC or CG pair within a CGCGCGCG hot-spot sequence upstream of the *hisD3052* mutation in *Salmonella typhimurium* strain TA98 (6, 7). Howard *et al*. (5) demonstrated a linear correlation between the concentration of dG-C8-AP in *S. typhimurium* DNA and the number of revertant colonies. Watanabe *et al*. showed that new *S. typhimurium* strains, which possessed 50-fold higher nitroreductase activity than the original strains, were more sensitive to the mutagenicity of 1-NP than the original strains (8). Thus, nitroreduction of 1-NP by nitroreductase is considered to be an essential step for expression of the mutagenic activity. However, there are few studies which demonstrate the correlation between the nitroreductase activity, the amount of DNA adducts and the mutagenicity of 1-NP in the same system.

The present study was initiated to elucidate the correlation between bacterial nitroreductase activity, the amount of a major DNA adduct generated from 1-NP and the mutagenicity of the compound in bacteria using three strains of *S. typhimurium*. Strain TA98 has been used as a standard strain for detection of frameshift mutations (3). Strain TA98NR is resistant to the mutagenicity of nitrofuran because this strain does not produce a nitroreductase which can metabolize this mutagen (3). Strain YG1021 contains a plasmid carrying the nitroreductase-encoding gene (8, 9).

MATERIALS AND METHODS

Materials.

1-NP and 1-aminopyrene (1-AP) were purchased from Aldrich Chemical Co., Milwaukee, WI, and 1-NP was purified by silica gel column chromatography with benzene as the elutant. The purity of 1-NP was determined by high-performance liquid chromatography (10) and was found to be more than 99.99%. Nutrient broth No.2 (Oxoid) was from Unipath Ltd., Hampshire, England. Proteinase K and hypoxanthine were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

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Silica gel, xanthine oxidase, calf thymus DNA, glucose 6-phosphate (G 6-P), spermidine and apyrase were purchased from Sigma Chemical Co., St. Louis, MO. Reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH) and glucose 6-phosphate dehydrogenase (G 6-PDH) were obtained from Oriental Yeast Co., Ltd., Tokyo, Japan. Lysozyme was obtained from Seikagaku Kogyo Co. Ltd., Tokyo. Micrococcal nuclease and spleen phosphodiesterase were purchased from Worthington Biochemical Co., Freehold, NJ. T4 polynucleotide kinase and nucleoside 3,5-bisphosphate of every base were purchased from Pharmacia Biotech, Uppsala, Sweden. [γ-^{32}P] adenosine 5'-triphosphate (7,000 Ci/mmol) was obtained from ICN Radiochemicals, Irvine, CA. Polyethyleneimine (PEI)-cellulose sheet (Polygram CEL 300 PEI) was purchased from Machery-Nagel GmbH, Düren, Germany. X-ray film was obtained from Fuji Photo Film Co. Ltd., Kanagawa, Japan. All other chemicals used were reagent or higher grade.

Mutagenicity Test.

The Ames assay was performed once for each experiment with two plates per sample by the preincubation method (11) to determine the mutagenicity of 1-NP in the three *S. typhimurium* strains. The numbers of spontaneous revertants (31, 11 and 40 for strains TA98, TA98NR and YG1021, respectively) were subtracted.

Preparation of Cell-free Extracts of S. typhimurium

Bacterial cells were harvested by centrifugation from an overnight culture, washed with saline three times and suspended in saline. The cells were disrupted by sonic oscillation (Kubota Insonator Model 200) at 200 watts for 5 min seven times in an ice bath. Cell debris was removed by centrifugation at 9,000 x g for 20 min at 4°C and the supernatant was used as an enzyme source for the nitroreductase assay. The protein concentration of the supernatant was determined by the method of Lowry *et al*. (12) with bovine serum albumin as a standard.

Nitroreductase Activity

The reaction mixture (total volume 1 ml) consisted of 50 mM sodium phosphate buffer (pH 7.4), 1 mM NADH, 1 mM NADPH, 2 mM G 6-P, 3 mM $MgCl_2$, 1 U of G 6-PDH per ml and 60 μM 1-NP. The mixture was purged with argon gas for 30 min in an ice bath. The enzyme solution was added to the mixture, which was then incubated anaerobically at 37°C for 30 min. Extraction with the same volume of dichloromethane was performed three times and the combined organic phase was evaporated. The extract was dissolved in 1.5 ml of methanol and the amount of 1-AP produced from 1-NP was determined by measuring the fluorescence (emission 420 nm, excitation 365 nm) with an auto fluorescence spectrometer (Hitachi F3010). The specific activity of the nitroreductase was expressed as nmol of 1-AP produced per minute per mg of protein.

DNA Adduct Formation and DNA Extraction from S. typhimurium.

1-NP dissolved in 25 ml of DMSO, 25 ml of the culture of each *S. typhimurium* strain and 125 ml of 0.1 M sodium phosphate buffer (pH 7.4) were mixed, then incubated with shaking at 37°C for 20 min. The concentrations of 1-NP were 10 μg/ml for strain YG1021 and 500 μg/ml for strains TA98 and TA98NR. The cells were collected and suspended in 6 ml of 0.15 M NaCl-0.1 M EDTA (pH 8.0) containing lysozyme at a final concentration of 2.0 mg/ml. After additional incubation at 37°C for 20 min, 6.0 ml of 0.1 M Tris-1% sodium dodecyl sulfate-0.1 M NaCl was added to the mixture. Then, phenol extraction and ethanol precipitation were performed to obtain bacterial DNA. The DNA solution was treated with 200 μg of RNase A and 33.4 U of RNase T1 per ml at 37°C for 30 min and further incubated with 500 μg of proteinase K per ml for 30 min. After phenol extraction and ethanol precipitation, the DNA yielded was used for the ^{32}P-postlabeling assay.

DNA Adduct Formation of 1-NP by Xanthine Oxidase.

A reaction mixture consisting of 50 mM potassium phosphate buffer (pH 5.8), 500 μg of hypoxanthine per ml, 2 mg of calf thymus DNA per ml and 40 μM 1-NP was purged with argon gas for 30 min in an ice bath. After the addition of xanthine oxidase at a final concentration of 0.1 U/ml, the mixture was incubated at 37°C for 1 hr under anaerobic conditions. The modified DNA for the ^{32}P-postlabeling assay was precipitated after treatment with RNase A, RNase T 1 and proteinase K as described above.

^{32}P-Postlabeling Assay

The experimental procedures of the ^{32}P-postlabeling assay were according to the butanol extract method (13) with some modifications. ^{32}P-labeled samples were applied on a PEI-cellulose sheet and developed overnight in 0.9 M sodium phosphate (pH 6.8) to remove normal nucleotides. DNA adducts were developed with 3.6 M lithium formate-8.5 M Urea (pH 3.5), 1.2 M LiCl-0.5 M Tris-HCl-8.0 M Urea (pH 8.0) and 0.9 M sodium phosphate (pH 6.0) as D 3, D 4 and D 5, respectively. To determine the total nucleotide number, labeled samples were chromatographed with 0.25 M LiCl. Positions of adducts on the cellulose sheet were determined by exposure to an X-ray film. Radioactivity of adducts on the PEI-cellulose sheet was measured with a liquid scintillation counter (Aloka LSC-602) to calculate the relative DNA adduct level.

Statistical Analysis

The correlation coefficient was calculated by the formula of Pearson with Stat View 4.5 (Abacus Concepts, Inc.).

RESULTS

Mutagenicity of 1-NP

The mutagenicity of purified 1-NP for the three *S. typhimurium* strains was examined using the Ames assay.

The highest mutagenicity was observed in strain YG1021. In contrast, the mutagenic activity was very low in strain TA98NR (Fig 1). The number of revertants increased dose-dependently in all three strains. The numbers of revertants of strains YG1021 and TA98NR were 18.6- and 0.18-fold higher, respectively, than strain TA98 (Table 1).

Nitroreductase Activity

The amount of 1-AP was measured with a fluorescence spectrophotometer after incubation of 1-NP with a cell-free extract of each *S. typhimurium* strain (Fig 2). The amount of 1-AP produced by incubation with extracts from strains YG1021 and TA98 increased protein-dose dependently. Strain YG1021 possessed 31.8-fold higher specific activity than strain TA98 and the specific activity of strain TA98NR was 1/10 that of TA98 (Table 1).

The qualification and quantification of DNA adducts

Figure 3A shows an autoradiogram of ^{32}P-postlabeled DNA adducts formed in the mixture of 1-NP, calf thymus DNA and xanthine oxidase after anaerobic incubation. The major adduct was dG-C8-AP (5). Figures 3 B, C and D show autoradiograms of DNA adducts in the three *S. typhimurium* strains incubated with 1-NP. The spots of DNA adducts from strains YG1021 (B) and TA98 (C) appeared at the same position as the DNA adduct, dG-C8-AP (A). The spot was not observed in strain TA98NR treated with 1-NP after exposure for 8 hr at -80°C (D). The amount of dG-C8-AP adduct was 97.2-fold more in strain YG1021 than in strain TA98 (Table 1).

The Correlation Coefficients

Correlation coefficients between the mutagenicity and the nitroreductase activity, the mutagenicity and the DNA adduct level, and nitroreductase activity and the DNA adduct level were 0.999898, 0.999986 and 0.999873, respectively. Positive correlations were observed between the mutagenicity and the nitroreductase activity, between the mutagenicity and the DNA adduct level at $P<0.01$, and between the nitroreductase activity and the DNA adduct level at $P<0.05$.

DISCUSSION

1-NP is a mutagenic and carcinogenic pollutant which is frequently found in the environment (2, 3). The metabolic pathway of 1-NP in *S. typhimurium* cells has been studied extensively, and it has been shown that nitroreduction of 1-NP is an essential step for expressing its mutagenicity(5, 14). However, there are few studies which demonstrate the correlation between the nitroreductase activity of *S. typhimurium* cells, the level of DNA adducts of 1-NP metabolites in the bacterial cells and the mutagenicity of 1-NP for bacteria. Therefore, we initiated this study to clarify these correlations.

S. typhimurium strain YG1021 was the most sensitive and strain TA98NR was the least sensitive to the mutagenicity of 1-NP among the three strains studied (Fig 1, Table 1). Tokiwa and Ohnishi (2) found that the numbers of revertant colonies from strains TA98 and TA98NR were 467±25 and 87±52 per nmol of 1-NP, respectively, without S9 mix. Watanabe *et al*. (8) reported that the mutagenicity of 1-NP for strain YG1021 without

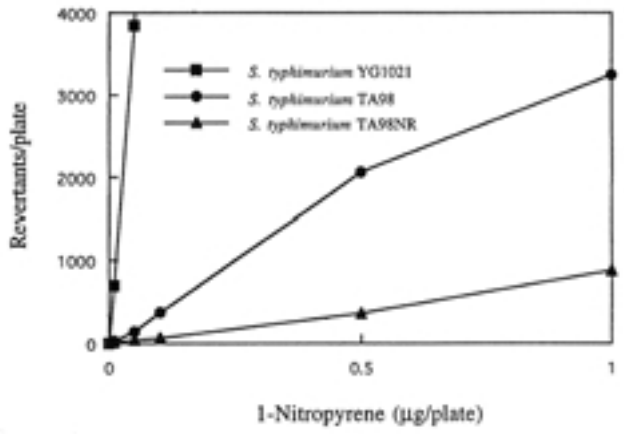

Fig.1. Mutagenicity of 1-NP for *S. typhimurium* strains TA98, TA98NR and YG1021.

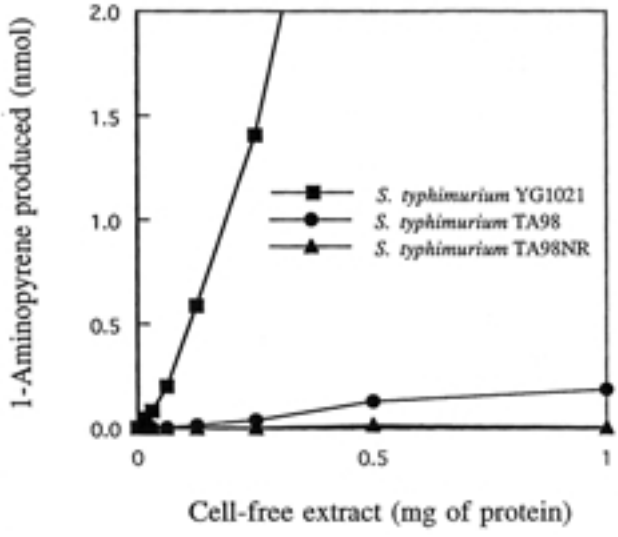

Fig.2. Nitroreductase activity of *S. typhimurium* strains TA98, TA98NR and YG1021. 1-NP was incubated with a cell-free extract of each bacterial strain at 37°C for 30 min. 1-AP produced from 1-NP was detected with a fluorescence spectrometer.

Table 1. Nitroreductase activity, the DNA adduct level and the mutagenicity of 1-NP in *Salmonella typhimurium* strains TA98, TA98NR and YG1021

Strain	Nitroreductase activity (pmol/min/mg)	DNA adduct level (adducts/10^8nucleotides/mg of 1-NP)	Mutagenicity (revertants/nmol of 1-NP)
YG1021	400.6	31.1	19,000
TA98	12.7	0.32	1,020
TA98NR	1.3	<0.15	180

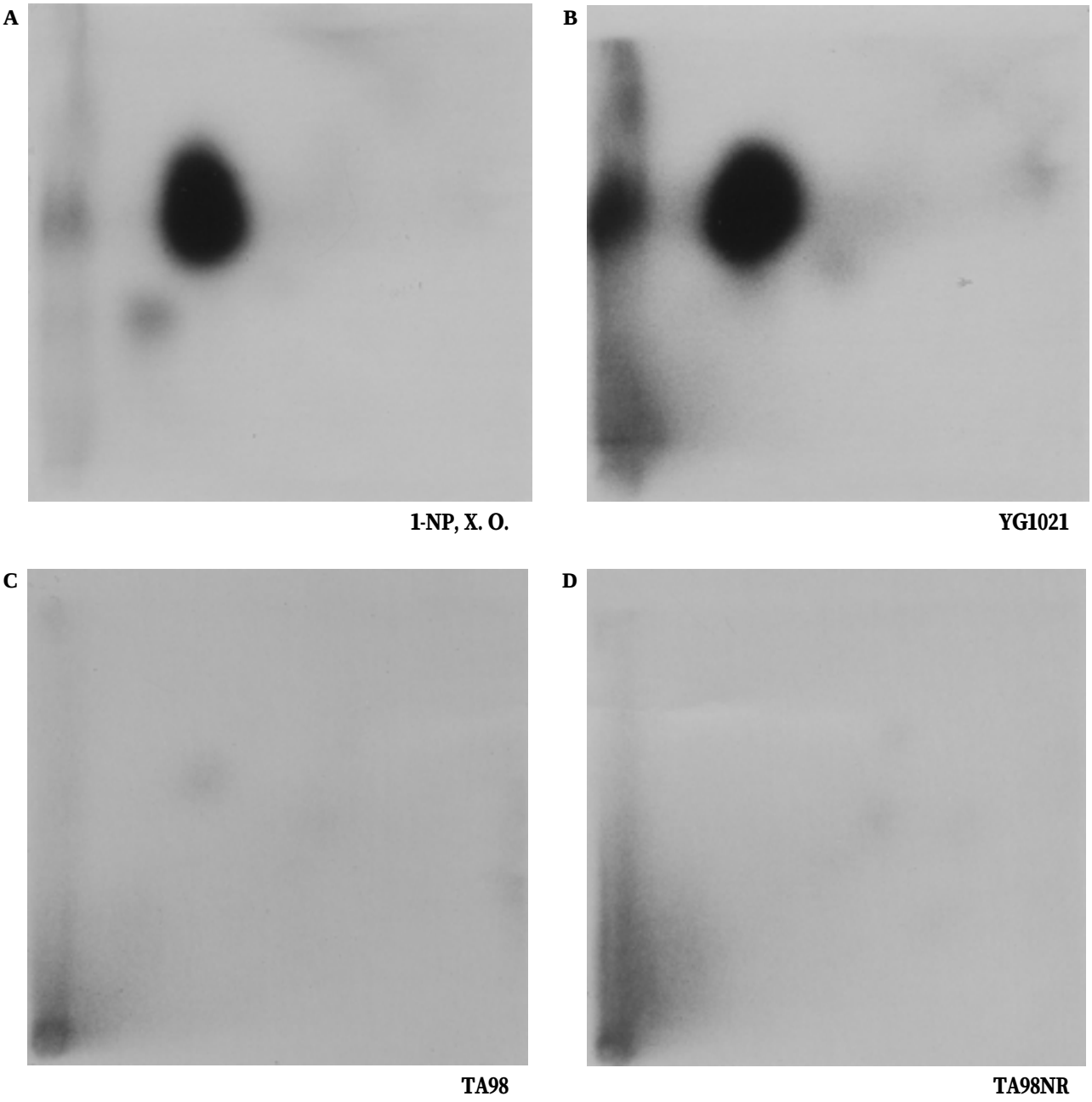

Fig.3. Autoradiograms of ^{32}P-labeled DNA adducts. A shows an autoradiogram of DNA adducts formed by anaerobic incubation of 1-NP with calf thymus DNA and xanthine oxidase. The X-ray film was exposed for 5 min at room temperature. B, C and D show autoradiograms of DNA adducts formed in *S. typhimurium* strains YG1021, TA98 and TA98NR, respectively. The X-ray film of strain YG1021 was exposed for 1 hr at -80°C, and the films of strains TA98 and TA98NR were exposed for 8 hr at -80°C.

liver S9 mix was 24,700 revertants per nmol of 1-NP. Einistö *et al*., working with the same three strains as in this study, showed that the numbers of revertants per nmol of 1-NP for strains TA98, TA98NR and YG1021 without S9 mix were 514, 12 and 12,172, respectively (15). Although the number of revertant colonies in these reports were different from those obtained in this experiment, the tendency of sensitivity of the three strains to 1-NP was similar. These differences may result from different conditions of preincubation of these tester strains and their cultures (16).

The nitroreductase activity of *S. typhimurium* strain YG1021 was the highest and that of strain TA98NR was the lowest among these three strains (Fig 2). The high nitroreductase activity of strain YG1021 was caused by the overproduction of this enzyme in response to plasmid pYG216 (8), which was made by recombination of the *S. typhimurium* strain TA1538 nitroreductase gene with the plasmid pBR322. A large number of copies (15 to 20 per cell) of this plasmid (17) may result in the overproduction of nitroreductase. However, the low nitroreductase activity of strain TA98NR may be related to the resistance to nitrofuran, which shows its mutagenicity after nitroreduction, like 1-NP(3). Byrant *et al*. found that in *S. typhimurium* strain TA98NR a "classical nitroreductase", which is one of the two nitroreductases of *S. typhimurium* strain TA98, was deleted (18). The correlation coefficient between the mutagenicity and the nitroreductase activity

was very high. These results indicate that nitroreductase plays an important role in expressing the mutagenicity of 1-NP in bacterial cells. This indication is in accordance with the findings of Oda *et al*. (19), who demonstrated high sensitivity of a nitroreductase-overexpressing strain to 1-NP using the *umu* test.

We used the ^{32}P-postlabeling method to detect DNA adducts derived from 1-NP in *S. typhimurium* strains TA98, TA98NR and YG1021 because this method is highly sensitive for detecting DNA adducts of non-radiolabeled mutagens or carcinogens even in a small quantity of DNA (20). In strains TA98 and YG1021, the DNA adduct spots were detected at the same position, by thin layer chromatography, as dG-C8-AP, which was made by *in vitro* incubation of 1-NP with xanthine oxidase and calf thymus DNA (Fig 3 A, B, C). No DNA adduct spot was detected after exposure for 8 hr at -80°C in strain TA98NR (Fig 3 D). The DNA adduct level was highest in strain YG1021 and lowest in strain TA98NR among the three strains (Table1). These results indicate that larger amounts of 1-NP are transformed to dG-C8-AP in *S. typhimurium* cells producing larger amounts of nitroreductase. Correlation coefficients between the nitroreductase activity and the DNA adduct level and between the mutagenicity and the DNA adduct level were also very high. These results suggest that the nitroreductase activity is important for formation of the DNA adduct, and that the DNA adduct plays an important role in expression of the mutagenicity.

Salmeen *et al*. reported that the amount of 1-AP and the number of revertants were higher under anaerobic conditions than under aerobic conditions and that for strain TA98 the ratio of mutagenicities (anaerobic/aerobic) was comparable with the ratio of the 1-NP reduction rates under anaerobic and aerobic incubation(14). Similarly, Lee *et al*. demonstrated that benzo[a]pyrene inhibited the nitroreduction of 1-NP to *N*-hydroxy-1-AP and the formation of dG-C8-AP and exhibited a more antagonistic effect on the mutagenicity of 1-NP in strain TA98 than in strain TA98NR (21). These findings are consistent with our suggestion that nitroreductase is correlated with the DNA adduct formation and expression of the mutagenicity of 1-NP.

In this study, we demonstrated strong correlations between the bacterial nitroreductase activity, the DNA adduct level and the mutagenicity of 1-NP in bacteria, suggesting that nitroreductase is important for the formation of dG-C8-AP and that the adduct generated leads to the expression of the mutagenicity of 1-NP in bacterial cells.

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