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Micronucleus induction and chromosomal aberration of 1- and 3-nitroazabenzo[*a*]pyrene and their *N*-oxides

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Nitro-azabenzo[*a*]pyrenes, 1- or 3-nitro-azabenzo[*a*]pyrene and their *N*-oxides are nitrated derivatives of azabenzo[*a*] pyrene (ABP) containing nitrogen in the 6-position of benzo[*a*]pyrene (B[*a*]P). The nitro-ABP-*N*-oxides (ABPOs) were formed by reaction of ABP with excess HNO_3. These derivatives were noteworthy as potent mutagens for *Salmonella* strains, and were present in fine particles of diesel particulates. In this study, micronucleus induction in mice and chromosomal aberrations due to means of Chinese hamster lung fibroblast (CHL) cells were investigated to determine genotoxicity in order to define the relationship with the mutagenic potency of these derivatives. The induction of micronucleus polychromatic erythrocytes (MNPCEs) was dependent on the dose response of 10–40 mg for 3-*N*-6-ABP, and of 10–40 mg for 1-*N*-6-ABP, and in addition, 1- and 3-*N*-6-ABPOs markedly induced MNPCEs in a dose range of 10–400 mg and from 1 to 80 mg, respectively, when the compound was intraperitoneally administrated in two mice at each dose. The results show that of the four compounds, 3-*N*-6-ABPO demonstrated a marked increase in MNPCEs. On the other hand, chromosomal aberrations of the four compounds were investigated by the duplicate tests using CHLs. The results after a 48 h treatment induced aberrations of the chromatid type, chromatid breaks and exchanges for 1- and 3-*N*-6-ABP, and mainly chromatid exchanges for 1- and 3-*N*-6-ABPO. The frequency of chromosomal aberrations associated with nitro substitution on the ABPO structure. Chromosomal aberrations of nitro derivatives of ABPO substituted at the 3-position on the structure were more potent than those at the 1-postion. *N*-oxide derivatives have been found to be reduced to anion radicals much more easily than azaB[*a*]P and its nitro derivatives. This suggests that the electrochemical reduction of the chemicals plays an important role in the metabolic activation of nitrated B[*a*]P derivatives.

Introduction

The mutagenicity and genotoxicity of various polycyclic aromatic hydrocarbons (PAHs) and their nitrated derivatives have been widely investigated for bacterial and mammalian cells (Tokiwa *et al.*, 1986), but those of azabenzo[*a*]pyrene (ABP) and its *N*-oxide derivatives containing nitrogen in the parent compound have not been clarified. According to our previous results, nitrated ABP and its *N*-oxides were found to be potent mutagens as well as nitrated derivatives of most PAHs for *Salmonella* tester strains (Sera *et al.*, 1992). In airborne particulate matter, the nitrated ABPs were present in a semivolatile phase of the fine particle matter, mostly as combustion products from diesel and gasoline emissions (Sera *et al.*, 1994). These semivolatile organics were adsorbed onto the XAD-4 resin, and chemicals were purified on a silica gel-packed column. The presence of these chemicals was chemically-ignored for mutagens from the organic phase of particulate matter. However, the behavior of mutagens inhaled into the lung alveolar is important to evaluate carcinogenic action and biochemical reactions due to by phagocytosis in alveolar macrophages (Tokiwa *et al.*, 1999).

It has been reported that particulate air pollution was associated with lung cancer mortality and cardiopulmonary, and in particular, exposure to fine particles in air pollutants resulted in increased mortality and an increase in respiratory symptoms and respiratory hospitalizations (Pope *et al.*, 1995). Of the various mutagens present in fine particles, nitrated ABP and its *N*-oxides may play an important role in mutagenesis in lung cells and also may be associated with declines of lung function because the chemicals are more mutagenic and genotoxic than other nitroarenes.

In the present study, the genotoxic activity of 1- and 3-*N*-6-ABP or ABPO were determined for induction of micronuclei in polychromatic erythrocytes in mice, and chromosomal aberrations in Chinese hamster lung fibroblast (CHL) cells.

Materials and methods

Chemicals

1- and 3-*N*-6-ABP and 1- and 3-*N*-6-ABPO were synthesized according to the methods mentioned in a previous report (Fukuhara *et al.*, 1992).

Micronucleus induction in mice

Eight-week-old ddY mice were used for determination of micronuclei. The animals were intraperitoneally treated with the test chemicals suspended in olive oil or dimethylsulfoxide (DMSO) at dose levels of 10–150 mg/kg for 1- and 3-*N*-6-ABP, and 25–400 mg/kg and 3–50 mg/kg for 1- and 3-*N*-6-ABPO, respectively. Twenty four hours after inoculation the animals were then killed by cervical dislocation. The test was replicated with the same age mice, and the mean results were calculated. The femoral marrow cells were flushed out with fetal bovine serum and smeared on clean slides. Some of the smeared cells were fixed with methanol for 5 min and stained according to the May–Grünwald–Giemsa technique. Other preparations were stained with acridine orange. The acridine orange (0.24 mM in 1/15 M of Sörensens phosphate buffer pH 6.8) was used as a working solution. The fixed slides were rinsed in the buffer three times for 1–3 min each time. The preparations were mounted with the same buffer, and sealed with Balsam paraffin or another suitable medium. Observations were made within 24 h. The slides were analyzed by one microscopist from coded slides; 1000 polychromatic and normochromatic erythrocytes with or without micronuclei were scored per animal. The ratio of polychromatic to normochromatic cells was simultaneously recorded by counting the number of cells until the score for one cell type reached 1000.

Chromosomal aberration test in vitro

The chromosomal aberration test was carried out according to the protocol previously reported by Matsuoka *et al.* (1991, 1993). Briefly, CHLs were

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cultured in Eagle's minimal essential medium (EMEM; Gibco BRL) supplemented with 10% heat-inactivated calf serum. In the direct method, cells were seeded at a density of 2.2×10^4 cells per dish with 5 ml of medium. On the third day, the cells were treated with each compound for 6, 24, or 48 h and then harvested. In the metabolic activation method, the cells were treated for 6 h with 0.5 ml of rat liver S9 mix, 2.5 ml of medium and 0.015 ml of the test compound dissolved in DMSO, 3 days after seeding. The final S9 concentration was 5%. The S9 fraction, prepared from the livers of male SD rats pretreated with phenobarbital and 5,6-benzoflavone, was purchased from Kikkoman. After the treatment, the reaction mixture was replaced with fresh medium and cells were harvested after an additional culture for 18 h. Chromosome preparation cultures were made and stained with Giemsa solution. The test was repeated three times and the data were summarized as the mean number of chromosome aberrations.

The slides were coded, but not scored, blind. One observer scored for aberrations. The number of cells with chromosomal aberrations among 100 well-spread metaphases was recorded. The types of aberrations were divided into six groups: chromatid and chromosome gaps (ctg), chromatid breaks (ctb), chromatid exchanges (cte), fragmentation (frg), chromosome breaks (csb) and chromosome exchanges (cse). In our criteria, a gap was a chromatic lesion whose length was longer than or equal to the width of a chromatid, suggesting a discontinuity at the DNA level. Thus, the evaluation of results was carried out including gaps in the present study. The incidence of polyploid cells among the 100 metaphases was also recorded. Solvent-treated cells served as negative controls.

Our historical database showed that the frequency of CHL cells with structural or numerical aberrations in both untreated and solvent-treated negative controls did not exceed 4%. We therefore decided a result was positive (+) if the frequency of aberrant cells or polyploidy was ~2-fold greater than that of the control, that is, ≧10%. The uncertain range between negative and positive, i.e. 5–9%, we termed inconclusive (±). Overall evaluation for each chemical was made by judging individual results in different dose groups. When the outcome was evaluated as inconclusive, the experiment was repeated.

Statistical analysis was conducted using the Cochran–Armitage test.

Results

Incidence of micronucleus polychromatic erythrocytes (MNPCEs) with 1- and 3-N-6-ABP, and 1- and 3-N-6-ABPO

The chemical structures of 1- or 3-*N*-6-ABP, and 1- or 3-*N*-ABPO are illustrated in Figure 1. In experimental nitration, 6-azaB[*a*]P formed 1-*N*-6-ABP and 3-*N*-6-ABP at rates of 50 and 37%, respectively, when it was nitrated under excess HNO_3. In order to determine the micronucleus induction, mice were inoculated intraperitoneally with 1- and 3-*N*-6-ABP or ABPO suspended in olive oil or DMSO as described in the Materials and methods. MNPCEs were scored after staining using the May–Grünwald–Giemsa technique for the preparation from chemically-treated mice using DMSO, and by the acridine orange technique for the preparation using olive oil as the solvent. With DMSO as a solvent, the spontaneous induction of micronuclei was 31 (0.51%) per 6000 PCE. The induction of MNPCE in mice treated with 1- and 3-*N*-6-ABP was dose-dependent at dose levels from 10 to 40 mg, corresponding to 3.8 to ~31 nM 1- and 3-*N*-6-ABP/kg of mouse (Figure 2). On the other hand, the micronucleus induction of 3-*N*-6-ABPO in mice was more potent than that of 1-*N*-6-ABPO (Figure 3): MNPCEs in mice treated with 3-*N*-6-ABPO were dose-dependent at smaller doses (from 10 to 80 mg, corresponding to 2 to ~16 nM 3-*N*-6-ABPO), and MNPCEs in mice treated with 1-*N*-6-ABPO were observed at larger doses (from 20 to 400 mg, corresponding to 7.7 to ~248 nM 1-N-6-ABPO/kg of mouse). Of the four compounds, therefore, micronucleus induction of 3-*N*-6-ABPO was clearly observed at dose levels $<$50 mg (16 nM) (Figure 3). The results indicate that only 3-*N*-6-ABPO showed a marked induction of the appearance of micronuclei.

Chromosomal aberrations of 1- and 3-N-6-ABP or ABPO

Chromosomal aberration tests for 1- and 3-*N*-6-ABP or ABPO were carried out using the direct method for 6, 24 and 48 h, and the results after 48 h of treatment are summarized in Tables I and II. No cytotoxicity was seen in almost all derivatives: there was no clear decrease in surviving cells and analyzable metaphases. Both 1- and 3-*N*-6-ABP induced chromosomal aberrations in the absence of exogenous metabolic activation after continuous treatment for 48 h (Table I). The 48 h treatments of both compounds induced potent chromatid exchanges. Generally, dose–response relationships were not clearly shown. This phenomenon could be explained by the insolubility of these chemicals in the culture medium, although we did not analyze the concentration of chemicals in it. The chromosome aberrations induced by both 1- and 3-*N*-6-ABP were mainly of the chromatid type, such as chromatid

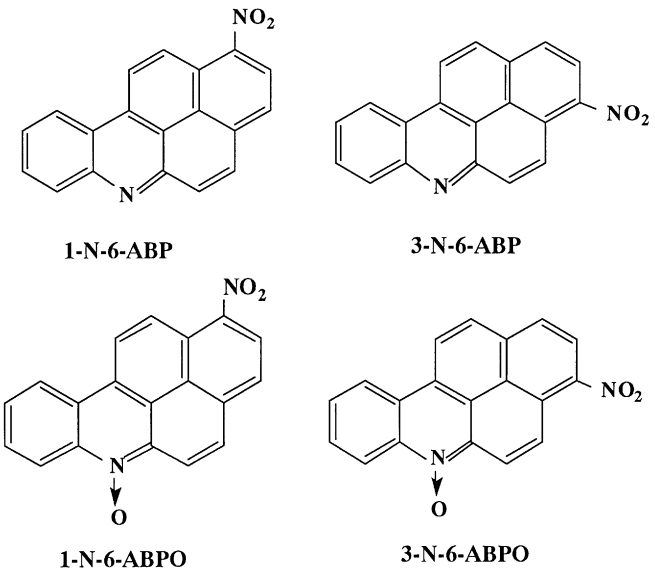

Fig. 1. Chemical structure of nitro ABP and its *N*-oxide.

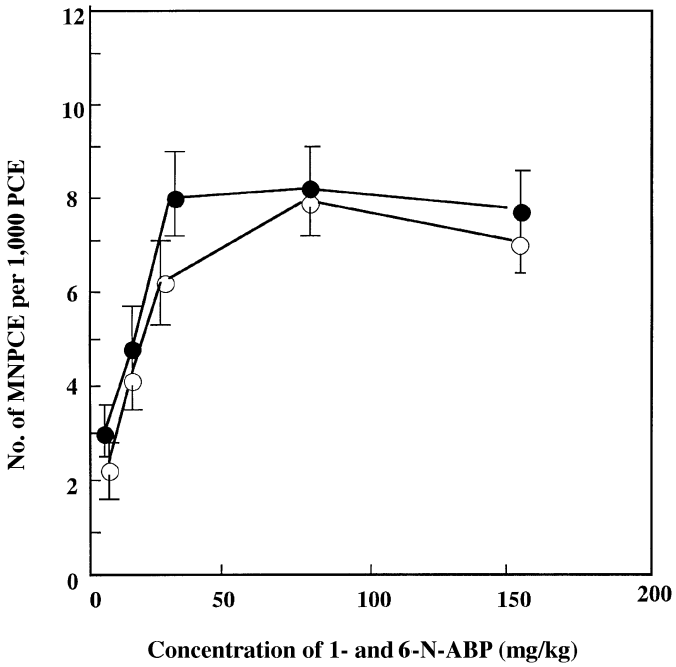

Fig. 2. Dose–response curves of micronucleous induction of 1-*N*-6-ABP (open circles) and 3-*N*-6-ABP (closed circles).

breaks and exchanges (Table I). The results of chromosomal aberrations for 1- and 3-*N*-6-ABPO are summarized in Table II, showing that chromatid exchanges were mainly induced. The frequency of chromatid breaks and exchanges, and chromosome breaks were found in 3-*N*-6-ABPO rather than in 1-*N*-6-ABPO, but their dose-dependency for aberrations was not defined except for chromatid exchanges: chromatid exchanges were found at a significant level with a dose of 0.2 mg for 1-*N*-6-ABPO, and both 0.01 and 0.005 mg for 3-*N*-6-ABPO/kg of mouse. The induction of chromatid exchanges for both chemicals was potent, corresponding to the same magnitude at the smaller dose of 0.04 μg/ml of mitomycin C as a positive control. These positive results were not found using the metabolic activation method (Table III). In the presence of the rat liver S9 preparation, chromosome aberrations of 1- and 3-*N*-ABP, or ABPO were lower than seen for the 48 h treatment using the direct method. This fact indicates that chromosome aberrations of these chemicals were not activated by the rat liver S9 preparation, whereas those of benzo[*a*]pyrene (B[*a*]P), as a positive control, were dependant on the metabolic activation (Table III).

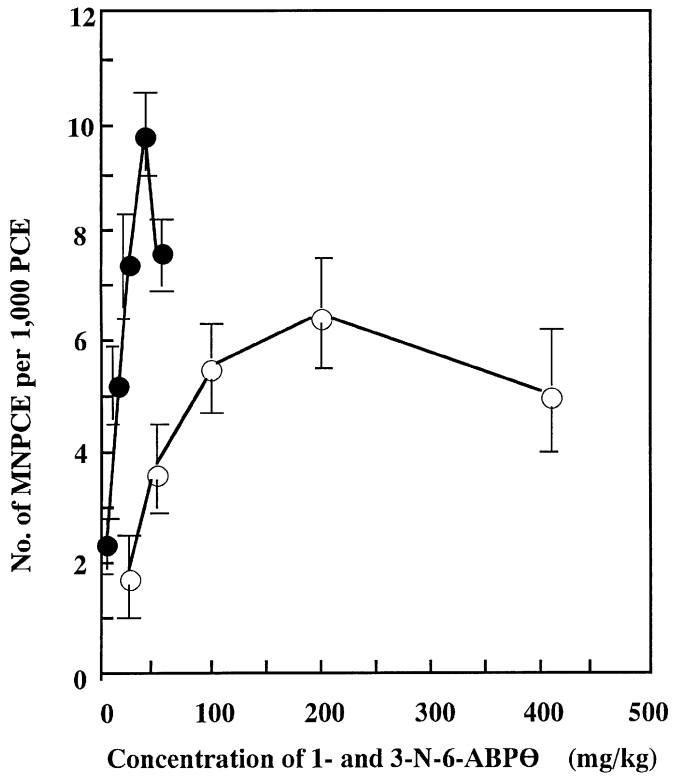

Fig. 3. Dose–response curves of micronucleous induction of 1-*N*-6-ABPO (open circles) and 3-*N*-6-ABPO (closed circles).

Discussion

In this study, the results of micronucleus induction in mouse femoral marrow cells and chromosomal aberrations in CHLs showed the same structure–activity relationship for nitro-substitution of B[*a*]P, as in the results for *Salmonella* mutagenicity (Sera *et al.*, 1992). The results for micronucleus induction were revealed to be more potent in nitro derivatives substituted at the 3-position than at the 1-position in ABP *N*-oxides but not ABP. The induction resulted in a marked increase in *N*-oxide derivatives of 3-*N*-6-ABPO. Basically, 6-azaB[*a*]P and its *N*-oxides were inactive without the S9 mix for TA98 and TA100, while they showed moderate mutagenicity with the S9 mix (Sera *et al.*, 1992). As for chemical properties, it was suggested that the increase in hydrophilicity of the azaarenes compared to the deaza analogue was due to the decrease in mutagenicity (Fukuhara *et al.*, 1992). Micronucleus induction of 3-*N*-6-ABPO was potent as observed in a previous study with 3,7- and 3,9-dinitrofluoranthenes (Tokiwa *et al.*, 1988). This result may be due to nitroreduction of nitroreductase and acetyltransferase in the *in vivo* test, i.e., the reduction ability of the derivative substituted at the 3-position of the nitro function was more mutagenic in *Salmonella* strains and tumorigenic in rats than for the substitution at the 1- or 6-position (Horikawa *et al.*, 1998). In addition, nitroreduction has been demonstrated to be more potent in 3,6-dinitroB[*a*]P than in 1,6-dinitroB[*a*]P (Sera *et al.*,1991, 1992).

On the other hand, chromosomal aberrations of nitro azaB[*a*]P and its *N*-oxides were mainly observed as chromatid breaks and exchanges. These aberrations were similar to the results for 3,7- and 3,9-dinitrofluoranthenes (Tokiwa *et al.*, 1988). However, chromosome aberrations of nitro azaB[*a*]P and its *N*-oxides were not dependant on metabolic activation of rat liver S9 preparation whereas those of B[*a*]P were significantly induced in the presence of rat liver S9. These results suggest that there was a possibility of metabolic activation due to nitroreductase being present in CHL cells,

Table I. Frequency of chromosomal aberrations induced by 1- and 3-*N*-6-ABP

Chemical	Dose (mg/ml)	Cells observed	Polyploid (%)	Cells with chromosome aberrations (%)						Total	Judgement
				ctg	ctb	cte	frg	csb	cse		
1-*N*-6-ABP	0.023	100	0	0	1	9	0	1	0	11	+
	0.045	100	0	0	3	18[a]	0	1	0	20[a]	+
	0.09	100	1	1	2	21[a]	0	2	1	24[a]	+
3-*N*-6-ABP	0.023	100	0	0	1	9	0	1	0	11	+
	0.045	100	0	0	3	18[a]	0	2	0	21[a]	+
	0.09	100	1	1	2	22[a]	0	2	1	25[a]	+
Mitomycin C	0.04[b]	100	1	0	10[a]	12[a]	0	0	0	17[a]	+
DMSO		100	1	1	0	0	0	0	0	2	−

ctg, chromosome and chromatid gaps; ctb, chromatid breaks; cte, chromatid exchanges; frg, fragmentation; csb, chromosome breaks; cse, chromosome exchanges.
[a]$P \geqq 0.01$.
[b]μg/ml.

Table II. Frequency of chromosomal aberrations induced by 1- and 3-*N*-6-ABPO

Chemical	Dose (mg/ml)	Cells observed	Polyploid (%)	Cells with chromosome aberrations (%)						Total	Judgement
				ctg	ctb	cte	frg	csb	cse		
1-*N*-6-ABPO	0.05	100	0	0	1	5	0	1	0	7	+
	0.1	100	0	0	1	9	0	1	0	11	+
	0.2	100	0	1	1	10[a]	0	1	1	13[a]	+
3-*N*-6-ABPO	0.0025	100	0	0	2	10	0	2	1	13	+
	0.005	100	0	0	1	19[a]	0	2	0	21[a]	+
	0.01	100	0	1	2	39[a]	0	2	1	42[a]	+
Mitomycin C	0.04[b]	100	1	0	10	13[a]	0	0	0	18[a]	+
DMSO		100	2	1	0	0	0	0	0	2	−

ctg, chromosome and chromatid gaps; ctb, chromatid breaks; cte, chromatid exchanges; frg, fragmentation; csb, chromosome breaks; cse, chromosome exchanges.
[a]$P \geqq 0.01$.
[b]μg/ml.

Table III. Chromosomal aberrations induced by 1- and 3-*N*-6-ABP, and 1-and 3-*N*-6-ABPO in the presence of S9 mix

Chemical	Dose (mg/ml)	Cells observed	Polyploid (%)	Cells with chromosome aberrations (%)						Total	Judgement
				ctg	ctb	cte	frg	csb	cse		
1-*N*-6-ABP	0.023	100	0	0	0	0	0	0	0	0	−
	0.045	100	0	1	0	1	0	0	0	2	−
	0.09	100	0	2	1	1	0	0	0	4	±
3-*N*-6-ABP	0.023	100	0	2	0	0	0	0	0	2	−
	0.045	100	0	1	0	1	0	0	1	3	−
	0.09	100	0	0	1	2	0	1	0	4	±
1-*N*-6-ABPO	0.05	100	0	0	0	0	0	0	0	0	−
	0.1	100	0	1	1	1	0	0	0	3	−
	0.2	100	0	1	2	2	0	0	0	5	±
3-*N*-6-ABPO	0.0025	100	0	1	0	0	0	0	0	1	−
	0.005	100	0	1	2	2	0	0	0	5	±
	0.01	100	0	2	1	2	0	1	1	7	±
B[*a*]P	0.01	100	0	6	27[a]	34[a]	0	1	0	37[a]	+
DMSO		100	0	0	0	0	0	0	0	0	−

ctg, chromosome and chromatid gaps; ctb, chromatid breaks; cte, chromatid exchanges; frg, fragmentation; csb, chromosome breaks; cse, chromosome exchanges; ±, inconclusive.
[a]$P \geqq 0.01$.

as well as *Salmonella* mutagenicity (Sera *et al.*, 1992), but not in rat liver microsomes.

As for the chemical properties of the four derivatives, two reversible waves involving a one-electron transfer process were observed (Fukhara *et al.*, 1992). The first half-wave redox potential of nitrated 6-ABPs, such as 1- or 3-*N*-6-ABP, was higher than that of mononitroB[*a*]Ps, such as 1- or 3-nitroB[*a*]P, and, in addition, that of their *N*-oxides was also higher: 1- and 3-*N*-6-ABPO were reduced to anion radicals much more easily than mononitroB[*a*]P (Fukhara *et al.*, 1992). As indicated previously, the mutagenicity of 3-*N*-6-ABP and 3-*N*-6-ABPO for *Salmonella* strains TA98 and YG1024, an acetyltransferase-rich mutant of TA98, showed 348 and 7670, and 1260 and 30 500 revertants/ng without the S9 mix, respectively. The 3-*N*-6-ABP was more mutagenic than 1-*N*-6-ABP (352 and 2750 revertants/ng, respectively), and 3-*N*-6-ABPO was more mutagenic than 1-*N*-6-ABPO (115 and 1930 revertants/ng, respectively). It has also been found that the mutagenicity and tumorigenicity of 3,6-dinitroB[*a*]P were more potent than those of 1,6-dinitroB[*a*]P (Horikawa *et al.*, 1998; Sera *et al.*, 1991): the difference in the activity may cause metabolic pathways dependent on nitro reduction (Sera *et al.*, 1992). There was also an association between biological activity and the electrochemical reduction of related compounds.

On the other hand, nitro-azaB[*a*]P was detected from semivolatile materials passed through a 0.45 μm filter on airborne and diesel particulate matter (Sera *et al.*, 1994). It is likely that related compounds may be present as a coating chemical on elemental carbon or as a semivolatile form involved with smaller particles inhaled into lung alveoli, and were deposited in lung tissues (Tokiwa *et al.*, 1993, 1998). Normally, most organic chemicals are identified by gas-chromatography and mass spectrometry, but these compounds could be directly determined by mass spectrometric analysis after purification using liquid column chromatography. The compounds were detected on the column at an oven temperature programmed from 80 to 310°C (Sera *et al.*, 1994). Under these conditions, 1- and 3-*N*-6-ABP or ABPO were detected at the levels from 0.8 to 7.7 ng/g of material. In our earlier study on the ABPs and ABPOs, the production of 8-hydroxyguanosine as a result of oxidative damage in cellular DNA, resulted in a marked increase compared with other related nitroarenes, although this was *in vivo* data from formed mouse lungs (unpublished data). These results suggest that these ABPs and ABPOs generated oxygen free radicals (or hydroxyl radicals) in the mouse lung,

and led to mutation at the deoxyguanosine residue in the DNA. These results indicate that the ABPs and ABPOs induced not only bacterial mutation and genotoxic action *in vitro*, but also pulmonary injury as a result of oxidative damage.

The sources and formation of these chemicals in the environment have not been chemically-revealed in the present study. However, these are serious mutagens that elevate cardiopulmonary and lung cancer incidence.

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