

# Mutagenic and Carcinogenic Significance and the Possible Induction of Lung Cancer by Nitro Aromatic Hydrocarbons in Particulate Pollutants

Hiroshi Tokiwa,<sup>1</sup> Nobuyuki Sera,<sup>1</sup> Akio Nakashima,<sup>2</sup> Koichi Nakashima,<sup>3</sup> Yoichi Nakanishi,<sup>4</sup> and Nobuaki Shigematu<sup>4</sup>

<sup>1</sup>Fukuoka Institute of Health and Environmental Sciences, Dazaifu, Fukuoka, Japan; <sup>2</sup>Department of Internal Medicine, Saiseikai Shimonoseki General Hospital, Shimonoseki, Japan; <sup>3</sup>Department of Nutrition, Faculty of Science of Life, Kyushu Women's University, Kitakyushu, Japan; <sup>4</sup>Research Institute for Diseases of the Chest, Faculty of Medicine, Kyushu University, Fukuoka, Japan

Studies of genotoxicity and carcinogenicity of nitro aromatic hydrocarbons focus on their high mutagenicity for bacteria and mammalian cells. Nitrobenzo[*a*]pyrenes (NBPs) and related nitroazaarenes also are extraordinarily mutagenic. 3-Nitro-6-azabenz[*a*]pyren-*N*-oxide was found to be a more potent mutagen than 1,8-dinitropyrene. Mutagenicity of NBPs was associated with the position of substitution of the nitro function when nitrogen dioxide (NO<sub>2</sub>) was substituted at the third position on the benzo[*a*]pyrene (BP) structure, as in 3,6-dinitrobenzo[*a*]pyrene but not in 1,6-diNBP. The NBPs were reduced by a rat liver postmitochondrial fraction to nitroso- and subsequently to amino-derivatives. Therefore, tumoral action in rats was induced at significant levels by subcutaneous injection of 3,6-diNBP, but no tumors were observed in rats given 1,6-diNBP. Carcinogenic nitropyrenes were detected in the resected lung of a patient with lung cancer. It is suggested that the presence of nitropyrenes and the resulting tumor were due to exposure to by-products of combustion of heavy oil. The patient was a nonsmoker and farmer who had bred chickens for 40 years. He used heavy oil for heating the chicken house. Similarly, a group of Chinese people at high risk of developing lung cancer was selected to determine the initiator of lung cancer. Lung cancers were obtained from six Chinese female nonsmokers who were living in Fuyuan County, China. Polycyclic aromatic hydrocarbons were detected in resected lung specimens; they were benzo[*k*]fluoranthene, BP, benzo[*g,h,i*]perylene, and pyrene. These cases were associated with exposure to soot from combustion of coal usually used for heating and cooking indoors. — Environ Health Perspect 102(Suppl 4):107–110 (1994).

Key words: nitropyrenes, dinitrobenzo[*a*]pyrenes, carcinogenicity, mutagenicity, lung cancer

## Introduction

Chemical mutagens and carcinogens present in the urban air differ according to the source of the pollutant, and are formed secondarily by reaction of polycyclic aromatic hydrocarbons (PAHs) with gaseous pollutants such as nitrogen dioxide and sulfur dioxide (1). Crude extracts prepared from particulate pollutants normally induce mutagenic activity in *Salmonella typhimurium* TA98 at a frequency of from 50 to 500 revertants/mg of extracts of particulates (2). This mutagenic potency corresponds to revertants per pmole of dinitropyrenes (diNPs), well known to be extraordinarily mutagenic.

The behavior of nitro aromatic hydrocarbons (nitro-PAHs) in the environment is related to combustion of fossil fuels. It is,

therefore, thought that nitro-PAHs are being produced continuously and spread in urban air. This speculation is based on the fact that most nitro-PAHs are detected at substantial levels in airborne and diesel emission particulates (3). On the other hand, it is important that respiratory tract and lung cancer occurs among both smokers and nonsmokers. The aim of this study was to clarify whether nitro-PAHs in urban air were involved in the induction of lung cancer.

## Materials and Methods

### Chemicals

Dinitrobenzo[*a*]pyrene (DiNBP) and their related compounds were synthesized by Fukuhara et al. (4). DiNPs (three isomers) and dinitrofluoranthene (diNFs) (two isomers) were prepared as reported previously (5).

### Animals for Carcinogenicity Test

A total of 184 male six-week-old F344/DuCrj rats with body weights of 111 to 125 g were purchased from Charles River Japan Inc. (Atsugi, Japan). The rats were

divided into four groups consisting of 84 rats for a 3,6-diNBP-treated group, 44 for a 1,6-diNBP-treated group, 40 for a benzo[*a*]pyrene (BP)-treated group, and 20 given a mixture of equal volumes of beeswax and tricapylin. The rats were subcutaneously injected with the chemical dissolved in an equal volume of beeswax and tricapylin by the method of Stanton et al. (6).

### Determination of Mutagen in Lung Specimens

Lung specimens were obtained after surgical resection from one Japanese subject and six Chinese subjects who had lung cancer. Also, 54 other lung specimens from Japanese subjects were examined to determine the values of chemical deposits as the background. To determine the mutagen, the specimens were dried at 20°C for a week in a dark room and weighed. The amounts of material used for analysis were 1517 mg for case 1 (Japanese male); 880 mg for case 2; 1200 mg for case 3; 900 mg for case 4; 1100 mg for case 5; 1300 mg for case 6; and 1200 mg for case 7 (cases 2–7 were all Chinese females).

This paper was presented at the Symposium on Risk Assessment of Urban Air: Emissions, Exposure, Risk Identification and Risk Quantitation held 31 May–3 June 1992 in Stockholm, Sweden.

Address correspondence to Hiroshi Tokiwa, Department of Environmental Health, Kyushu Women's University, Jiyugaoka, Yahatanishi-ku, Kitakyushu 807, Japan.

**Table 1.** Mutagenicity of nitroarenes and their related compounds.

Nitroarene	Revertants/ nmole for	
	TA98	YG1024
3-Nitro-6-azabenz[ <i>a</i> ]pyrene- <i>N</i> -oxide	396,000	9,580,000
1,8-Dinitropyrene	296,000	6,190,000
3-Nitro-6-cyanobenz[ <i>a</i> ]pyrene	174,000	1,230,000
3,6-Dinitrobenz[ <i>a</i> ]pyrene	137,000	1,640,000
1,6-Dinitropyrene	126,000	4,120,000
3,7-Dinitrofluoranthene	123,000	6,600,000
1-Nitro-6-azabenz[ <i>a</i> ]pyrene	105,000	820,000
3-Nitro-6-azabenz[ <i>a</i> ]pyrene	104,000	2,290,000
3,9-Dinitrofluoranthene	104,000	1,910,000
1,3-Dinitropyrene	85,900	1,190,000
1-Nitro-6-cyanobenz[ <i>a</i> ]pyrene	43,100	348,000
1-Nitro-6-azabenz[ <i>a</i> ]pyrene- <i>N</i> -oxide	36,100	606,000
3-Nitrofluoranthene	4,670	57,000
3,4-Dinitrofluoranthene	4,120	52,000
4-Nitropyrene	2,700	56,400
3-Nitrobenz[ <i>a</i> ]pyrene	1,370	32,000
2,6-Dinitrophenanthrene	730	1,230
1-Nitrobenz[ <i>a</i> ]pyrene	650	35,000
1-Nitropyrene	470	2870

Abbreviations: TA 98, *Salmonella typhimurium his<sup>-</sup>* strain; YG1024, *Salmonella typhimurium his<sup>-</sup>* carrying a plasmid of the acetyltransferase gene transferred into cells of TA98 (8).

**Table 2.** Nitro-reduction of nitrobenz[*a*]pyrene in the presence of rat liver S-100 fraction.

Chemical	Mutagenicity, rev/μg	Cofactor	Oxygen	Products, pmole/ mg protein <sup>b</sup>	
				Nitroso	Amino
1-Nitrobenz[ <i>a</i> ]pyrene <sup>a</sup>	2,200	NADPH	-	15±17	20±18
			+	5±0	5±0
			NADH	-	15±6
3-Nitrobenz[ <i>a</i> ]pyrene <sup>a</sup>	4,600	NADPH	-	47±15	38±41
			+	6±1	6±1
			NADH	-	47±15
1,6-Dinitrobenz[ <i>a</i> ]pyrene <sup>a</sup>	4,000	NADPH	-	25±20	58±19
			+	6±0	6±0
			NADH	-	25±20
3,6-Dinitrobenz[ <i>a</i> ]pyrene <sup>a</sup>	401,000	NADPH	-	242±64	847±102
			+	89±15	112±15
			NADH	-	100±11
			+	143±11	57±4

<sup>a</sup> 1-Nitrobenz[*a*]pyrene, 3-nitrobenz[*a*]pyrene, 1,6-dinitrobenz[*a*]pyrene, or 3,6-dinitrobenz[*a*]pyrene in 2.5 μM quantities dissolved in 50 mM phosphate buffer, pH 7.4 was exposed to nitrogen gas for 20 sec and reacted with rat liver S-100 at the final concentration of 1 mg of protein per ml in the presence of 50 μM NADPH or 5 μM NADH as a cofactor. The mixtures were incubated at 37° C for 20 min and treated with chloroform and methanol (1:1, vol/vol). <sup>b</sup> Products were developed on a plate of silica gel and analyzed by high-pressure liquid chromatography.

**Preparation of Rat Liver Postmitochondrial Fraction**

The enzymatic reduction of 1- and 3-nitrobenz[*a*]pyrene, and 1,6- and 3,6-diNBP was performed in the presence of rat liver postmitochondrial fraction on the basis of the method of Poirier and Weisburger (7) and Djuric et al. (8).

**Mutagenicity Test**

The mutagenicity test used was the *Salmonella* microsome test described previously (9).

**Results**

**Mutagenicity of Nitro-PAHs and Their Related Compounds**

The mutagenicity of major nitro-PAHs for strain TA98 is summarized according to the order of their potency (Table 1). In addition to diNPs and diNFs, NBP and their related compounds showed high mutagenicity. 3-Nitro-6-azabenz[*a*]pyrene (N-6-ABPO), 3-nitro-6-cyanobenz[*a*]pyrene (N-6-CBP), and 3-nitro-6-azabenz[*a*]pyrene (N-6-ABP)

as well as diNPs and diNFs were potent mutagens. Nitro derivatives with the substitution at the third position on the BP structure mutated strain TA98 strongly. Therefore, there was a structure-activity relationship between mutagenic potency and the nitro substitute. With the exception of 1-nitropyrene (NP) and 2,6-dinitrophenanthrene, the nitro-PAHs were activated by *O*-acetyltransferase; the mutagenicity of these chemicals was greatly enhanced in strain YG 1024, which carries a plasmid of the acetyltransferase gene in TA98.

**Structure-Activity Relationship of NBPs**

The enzymatic reduction of 1- and 3-NBP and 1,6- and 3,6-diNBP was investigated in the presence of rat liver postmitochondrial fraction (S-100). Incubation was under anaerobic conditions. The reduction products were determined by the appearance of 1-nitrosobenzo[*a*]pyrene and 1-aminoBP for 1-NBP, of 3-nitrosoBP and 3-aminoBP for 3-NBP, of 1-nitroso-6-NBP and 1-amino-6-NBP for 1,6-diNBP, and of 3-nitroso-6-NBP and 3-amino-6-NBP for 3,6-diNBP. In the presence of rat liver S-100, 3-NBP and 3,6-diNBP were reduced to nitroso- and amino-derivatives stronger than 1-NBP and 1,6-diNBP more readily (Table 2). Reduction of 3,6-diNBP yielded 10 times or more of the nitroso- and amino-derivatives compared to reduction of 1,6-diNBP. The ability of the reduction was for diNBP than for mono-NBPs. As the first step of the reduction of 3,6-diNBP, 3-nitroso-6-NBP was produced and was further reduced to 3-amino-6-NBP via a 3-hydroxylamino-6-NBP intermediate and finally to 3,6-diaminoBP. Figure 1 is an outline of the pathway of reduction.

**Carcinogenicity of 3,6-diNBP in Rats**

Table 3 shows the incidence of tumors in rats observed for 100 weeks after injection of chemical. Fourteen rats (70%) given 1000 mg of 3,6-diNBP per rat developed subcutaneous tumors by 35 weeks. The earliest appearance of the tumors was on day 183 in the 3,6-diNBP-treated rats. Similarly, animals given 200, 40, and 5 mg per rat developed tumors at the rate of 38, 24, and 4.7%, respectively, at the local site. The incidence of tumors was dose-dependent. However, no tumors were induced in any rats given 1,6-diNBP. In the rats given 3,6-diNBP and BP as a positive control, more BP-treated rats than 3,6-diNBP-treated rats developed tumors after injection of the minimum dose of 8 μg.

### Association between Initiation of Human Lung Cancer and Mutagen in Particulates

We examined human lung cancer tissues for the presence of mutagens or carcinogens and their metabolites and investigated the association between these chemicals and the possible induction of lung cancer. The findings in lung specimens from a Japanese subjects and six Chinese subjects are presented. None of the patients were cigarette smokers or had an occupational history of exposure to special pollutants as coke workers.

The Japanese patient was a 64-year-old male farmer. In 1983, he had a chest X-ray because of recurrence of coughing raising much sputum and a slight fever. Consolidation in the lower lobe of the right lung was found. By bronchoscopic brushing and biopsy, squamous cell carcinoma was diagnosed. The tumorous right lung was then resected. Macroscopically, the tumor was grayish white, solid, and hard with irregular foci of hemorrhage. It measured 55 × 66 × 40 mm in size with cavity formation and bronchial invasion. The histological features of the resected lung tumors led to the diagnosis of keratinizing squamous cell carcinoma (10).

The six Chinese patients were also non-smokers and consisted of a female cook and five female farmers who lived in Fuyuan County, China. Histological features of the resected right or left lung led to the diagnosis of adenocarcinoma for cases 2, 3, and 5; squamous cell carcinoma for cases 4 and 7; and small cell carcinoma for case 6 (Table 4). To determine the mutagen in lung specimens, the materials were extracted with dichloromethane or acetone-hexane mixture. The crude extracts were divided into three fractions, acidic, basic, and neutral. Only the neutral fraction was mutagenic for strain TA98 or TA100, giving 12.2 revertants for strain TA98 without S9 mix for case 1 and 0.01, 1.8, 0.01, 0.2, and 0.01 revertants for TA100 with S9 mix for cases 2, 3, 4, 5, 6, and 7, respectively, per µg of dry lung sample. The active fractions eluted on a high-performance liquid chromatography (HPLC) column and were

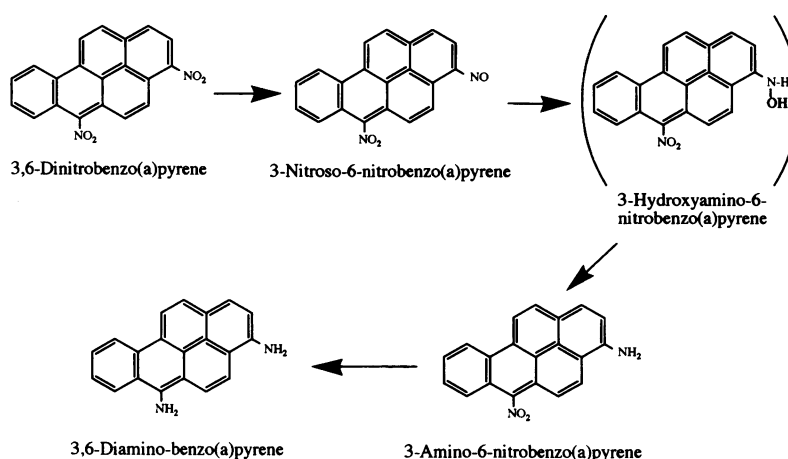


Figure 1. Reduction of 3,6-dinitrobenzo[a]pyrene.

Table 3. Carcinogenicity of 3,6-dinitrobenzo[a]pyrene compared to benzo[a]pyrene in rats.

Chemical	Total dose		No. of rats injected	No. of rats with subcutaneous tumors (%)
	µg / rat	µM / rat		
3,6-diNBP	1000	2.92	20	14 (70)
	200	0.59	21	8 (38)
	40	0.12	21	5 (24)
	8	0.02	21	1 (4.7)
1,6-diNBP	1000	2.92	11	0
	200	0.59	11	0
	40	0.12	11	0
	8	0.02	11	0
BP	1000	3.97	10	10 (100)
	200	0.79	10	6 (60)
	40	0.16	10	5 (50)
	8	0.03	10	2 (18)
Control			20	0

Abbreviations: 1,6- and 3,6-diNBP, 1,6- and 3,6-dinitrobenzo[a]pyrene; BP, benzo[a]pyrene.

analyzed by gas chromatography-mass spectrometry.

The major mutagens found and their concentrations are shown in Table 5. 1-NP, 1-nitro-3-hydroxypyrene, 1,3-diNP, and chrysenes were detected in the lung specimen of case 1. Four PAHs consisting of benzo[k]fluoranthene, BP, benzo[ghi]perylene, and pyrene were detected in all lung specimens of cases 2, 3, 4, 5, 6, and 7. Therefore, the lung specimen of case 1 was contaminated sub-

stantially with NPs, whereas similar specimens of the other cases were contaminated mostly with PAHs. These results are dependent on the fact that deposition of the chemical in the lung differs according to the source of the environmental pollutant. There was a possibility that the Japanese patient had been exposed to combustion by-products of heavy oil used for air conditioning the chicken house for a period of time over 10 years. In contrast, Chinese specimens were obtained in Fuyuan County,

Table 4. Lung cancer of Japanese and Chinese subjects; a relationship between carcinogenesis and environmental causes.

Case no.	Japanese or Chinese	Sex	Age	Occupation	Lung cancer	Exposure to combustion products	Status after operation
1	Japanese	Male	64	Farmer	Squamous cell carcinoma	Heavy oil	Died after 30 days
2	Chinese	Female	56	Farmer	Adenocarcinoma	Coal	Died after 30 days
3	Chinese	Female	28	Farmer	Adenocarcinoma	Coal	Died after 11 months
4	Chinese	Female	46	Farmer	Squamous cell carcinoma	Coal	Died after 15 months
5	Chinese	Female	51	Farmer	Adenocarcinoma	Coal	Not clear
6	Chinese	Female	53	Cook	Small cell carcinoma	Coal	Not clear
7	Chinese	Female	49	Farmer	Squamous cell carcinoma	Coal	Not clear

**Table 5.** Concentration of mutagen and carcinogen in lung specimen as possible cancer initiation.<sup>a</sup>

Mutagen	Case no, ng/g, dry weight							
	1	2	3	4	5	6	7	Others (54 specimens)
1-Nitropyrene	0.11	0.002	ND	ND	ND	ND	ND	ND
1-Nitro-3-hydroxypyrene	0.036	ND	ND	ND	ND	ND	ND	ND
1,3-Dinitropyrene	0.095	ND	ND	ND	ND	ND	ND	ND
Chrysene	0.16	ND	ND	ND	ND	ND	ND	ND
Benzo[ <i>k</i> ]fluoranthene	ND	0.11	0.32	0.06	0.14	0.17	0.1	ND
Benzo[ <i>a</i> ]pyrene	ND	0.18	0.43	0.13	0.17	0.31	0.19	ND
Benzo[ <i>g, h, i</i> ]perylene	ND	0.14	0.76	0.18	0.2	0.24	0.13	ND
Pyrene	ND	0.29	ND	0.16	0.16	ND	ND	ND

ND, not detected.<sup>a</sup> Lung specimens were extracted with dichloromethane for case 1 and with acetone and hexane mixture for cases 2, 3, 4, 5, 6, and 7 by ultrasonification. Crude extracts were separated into three fractions, acidic, basic, and neutral, by liquid-liquid separation. To purify mutagens, the active fraction for strain TA98 or TA100 was partially purified on a column of silica gel for case 1, or, in addition, of florisil and, subsequently, alumina for cases 2, 3, 4, 5, 6, and 7. Finally, the materials were analyzed by high-pressure liquid chromatography on a Unisil C18 column.

China, and people living in this area usually use coal as fuel for heating and cooking indoors. It is therefore suggested that the Chinese subjects were mostly exposed to soot or tar from combustion of coal indoors. In fact, many deposits of dust were microscopically observed in several areas in all lung tissues.

It is presumed that in human lungs various chemicals are deposited with dust. Therefore, the 54 lung specimens with induced tumors were examined to investigate the distribution of mutagen in background, but none were detected (Table 5).

## Discussion

It is well known that most dinitro-PAHs are extraordinarily mutagenic for bacteria and mammalian cells (10). Major mutagenic dinitro-PAHs also are carcinogenic in rats and mice (10). Normally, the mutagenic potency is correlated with the carcinogenic potency in rats as demonstrated by diNPs,

diNFs, and diNBP. In reference to the NBP structure, the position of the substituted nitro function is important for inducing gene mutation; 3-N-6-ABP, 3-N-6-CBP, and 3,6-diNBP substituted at the third position were classified as the most mutagenic of these dinitro-PAHs, but mutagenicity of 1-N-6-ABP, 1-N-6-CBP, and 1-N-6-ABPO substituted at position 1 and 6 showed a little decrease. The fact that 1,6-diNBP was mutagenic and noncarcinogenic at tested doses may be due to the lack of actual enzymes for reduction in rat liver cytosol or to the lack of products of the hydroxylamino intermediate that bind on DNA in cells.

There is no evidence of an association between mutagenic and carcinogenic nitro-PAHs and the induction of lung cancer. The Japanese case in this study is a case of lung cancer complicated by pulmonary silicosis and pulmonary artery stenosis. The histological feature of the lung was that of keratinizing squamous cell carcinoma. It is interest-

ing that deposits of large amounts of dust were seen in several areas in the right lung, the bronchus, and the trachea. The patient was a nonsmoker and farmer who had bred many chickens over a 40-year period. It is presumed that the patient might have been exposed to combustion by-products of heavy oil used for air conditioning the chicken house for a long time. Normally, heavy oil as fuel is often used for heating in chicken houses and for drying chicken stools. It has been found that the mutagenic and carcinogenic NPs detected are inducible from combustion products of fossil fuels (11,12).

In contrast, all Chinese cases originated in exposure to combustion of coal indoors. This study found that Chinese subjects living in Fuyuan and Xuan Wei Counties, which are next to each other, usually use coal as fuel for heating and cooking indoors. In addition, lung cancer mortality since 1986 has increased to the high level of over 50%. These results are in sharp contrast with those of Japanese case.

Nitro-PAHs are ubiquitous mutagens that are induced in incomplete combustion of fossil fuels or in photochemical reaction of PAH in the environment. Therefore, it is difficult to find a high risk group that is exposed to the chemicals. Microscopically, deposition of dustlike particles was observed in interstitial tissue. These results suggest that the detected mutagens and carcinogens participated in the initiation of the cancer. This hypothesis for the induction of lung cancer might be more valid if DNA adducts for these chemicals are detected in these specimens. Nitro-PAHs are induced in and contaminate urban air pollutants wherever fossil fuels are used.

## REFERENCES

1. Tokiwa H, Nakagawa R, Morita K, Ohnishi Y. Mutagenicity of nitro derivatives induced by exposure of aromatic compounds to nitrogen dioxide. *Mutat Res* 85:195-205 (1981).
2. Tokiwa H, Kitamori S, Horikawa K, Nakagawa R. Some findings on mutagenicity in airborne particulate pollutants. *Environ Mutagen* 5:87-100 (1983).
3. Newton DL, Erickson MD, Tomer KB, Pellizzari ED, Gentry P. Identification of nitroaromatics in diesel exhaust particulate using gas chromatography/negative ion chemical ionization mass spectrometry and other techniques. *Environ Sci Technol* 16:206-213 (1981).
4. Fukuhara K, Hakura A, Sera N, Tokiwa H, Miyata N. 1- and 3-Nitro-6-azabenz[*a*]pyrenes and their N-oxides; highly mutagenic nitrated azaarenes. *Chem Res Toxicol* 5:149-153 (1992).
5. Nakagawa R, Horikawa K, Sera N, Koderia Y, Tokiwa H. Dinitrofluoranthene; induction, identification, and gene mutation. *Mutat Res* 191:85-91 (1987).
6. Stanton MF, Miller E, Wrench C, Blackwell, R. Experimental induction of epidermoid carcinoma in the lungs of rats by cigarette smoke condensate. *J Natl Cancer Inst* 49:867-877 (1972).
7. Poirier LA, Weisburger JH. Enzymic reduction of carcinogenic aromatic nitro compounds by rat and mouse liver fractions. *Biochem Pharmacol* 23:661-669 (1974).
8. Djuric Z, Potter DW, Heflich RH, Beland FA. Aerobic and anaerobic reduction of nitrated pyrenes *in vitro*. *Chem Biol Interact* 59:309-324 (1986).
9. Sera N, Kai M, Horikawa K, Fukuhara K, Miyata N, Tokiwa H. Detection of 3,6-dinitrobenzo[*a*]pyrene in air particulates. *Mutat Res* 263:27-32 (1991).
10. Tokiwa H, Ohnishi Y. Mutagenicity and carcinogenicity of nitroarenes and their sources in the environment. *CRC Crit Rev Toxicol* 17:23-60 (1986).
11. Rosenkranz HS, McCoy EE, Sanders DR, Butler M, Kiriazides DK, Mermelstein R. Nitropyrenes: Isolation, identification, and reduction of mutagenic impurities in carbon black and toners. *Science* 209:1039-1043 (1980).
12. Rosenkranz HS, Mermelstein R. Mutagenicity and genotoxicity of nitroarenes; all nitro-containing chemicals were not created equal. *Mutat Res* 114:217-267 (1983).