Oxidative DNA Damage Induced by an N-Hydroxy Metabolite of Carcinogenic 4-Dimethylaminoazobenzene

Shiho Ohnishi,1 Mariko Murata,1 Masakuni Degawa2 and Shosuke Kawanishi1, 3

1Department of Hygiene, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507 and 2Department of Molecular Toxicology, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526

Formation of adducts has been considered to be a major causal factor of DNA damage by carcinogenic aminoazo dyes. We investigated whether a metabolite of hepatocarcinogenic 4-dimethylaminoazobenzene (DAB) can cause oxidative DNA damage or not, using 32P-5′-end-labeled DNA fragments. The DAB metabolite N-hydroxy-4-aminoazobenzene (N-OH-AAB) was found to cause Cu(II)-mediated DNA damage, including 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation. When an endogenous reductant, β-nicotinamide adenine dinucleotide (NADH) was added, the DNA damage was greatly enhanced. Very low concentrations of N-OH-AAB could induce DNA damage via redox reactions. Catalase and a Cu(I)-specific chelator inhibited the DNA damage, suggesting the involvement of H2O2 and Cu(I). A typical OH scavenger did not inhibit the DNA damage. The main reactive species are probably DNA-copper-hydroperoxo complexes. We conclude that oxidative DNA damage may play an important role in the carcinogenic processes of DAB, in addition to DNA adduct formation.

Key words: Azobenzene — DNA damage — Copper — Hydrogen peroxide

There is ample evidence for the carcinogenicity of 4-dimethylaminoazobenzene (DAB) in experimental animals. DAB induced lung tumors and hepatomas in mice and liver tumors in rats. In dogs it produced bladder tumors following oral administration. DAB has also been tested by s.c. injection in mice, and the results are suggestive of local and hepatic carcinogenicity. The International Agency for Research on Cancer (IARC) has assessed that DAB is possibly carcinogenic to humans (group 2B). DAB is metabolized to N-methyl-4-aminoazobenzene (MAB) through N-demethylation. MAB is metabolized to 4-aminoazobenzene (AAB) through demethylation or to N-hydroxy-N-methyl-4-aminoazobenzene (N-OH-MAB) through N-hydroxylation, followed by further transformation to N-hydroxy-4-aminoazobenzene (N-OH-AAB). N-Hydroxylation is believed to be a step leading aminoazo dyes to proximate carcinogenic or mutagenic metabolites. Watanabe and Hashimoto reported that N-OH-AAB elicited higher levels of unscheduled DNA synthesis (UDS) than AAB, suggesting higher DNA damaging activity of the N-hydroxy derivative than that of the corresponding mother aminoazo dye. It was also reported that N-OH-AAB dyes showed greater mutagenicity than the mother AAB dyes, without S-9 treatment. It is generally accepted that covalent binding of these metabolites with DNA is a major carcinogenic factor. N-(Deoxyguanosin-8-yl)-4-aminoazobenzene was also obtained from mice or rats given an i.p. dose of AAB. It was regarded as an adduct formed by reaction of deoxyguanosine with a metabolite of AAB after N-hydroxylation and esterification. On the other hand, N-OH-AAB and N-OH-MAB are reported to generate H2O2 and O2. Administration of 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB) which is a stronger carcinogenic derivative of DAB, increased the levels of 8-hydroxyguanine and its repair activity in rodent liver DNA. These reports suggested that oxidative DNA damage plays a part in carcinogenesis by aminoazo dyes.

To clarify the mechanism of carcinogenesis by DAB, we examined oxidative DNA damage induced by N-OH-AAB, using 32P-5′-end-labeled DNA fragments obtained from the c-Ha-ras-1 protooncogene and the p53 tumor suppressor gene. In addition, we measured the content of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in calf thymus DNA by high-performance liquid chromatography with an electrochemical detector (HPLC-ECD). It has been reported that 8-oxodG is a marker of oxidative DNA damage and that its formation can lead to DNA misreplication, resulting in mutation and cancer.

MATERIALS AND METHODS

Materials N-OH-AAB was prepared from 4-nitroazobenzene, according to the references. Restriction enzymes (SmaI, EcoRI, HindIII, ApaI, StyI and XbaI) and T4 polynucleotide kinase were purchased from New England
Biolabs (Beverly, MA). ([γ-32P]ATP (222 TBq/mmol) was obtained from New England Nuclear (Boston, MA). Alkaline phosphatase from calf intestine was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Piperidine was purchased from Wako Chemical Industries Ltd. (Osaka). Copper (II) chloride dihydrate was purchased from Sigma Chemical Co. (Kumamoto). Calf thymus DNA, superoxide dismutase (SOD) (3000 units/mg from bovine erythrocytes) and catalase (45 000 units/mg from bovine liver) were purchased from Sigma Chemical Co. (St. Louis, MO). Nuclease P1 (400 units/mg) was purchased from Yamasa Shoyu Co. (Chiba). Methional (3-(methylthio)propanaldehyde) was purchased from Tokyo Kasei Co. (Tokyo).

Preparation of 32P-5'-end-labeled DNA fragments obtained from the p53 gene and the c-Ha-ras-1 gene DNA fragments were obtained from the human p53 tumor suppressor gene.15) Two fragments containing exons from the p53 gene were amplified by the PCR method using an Omnigene Temperature Cycling System. The PCR products were digested with SmaI and ligated into SmaI-cleaved pUC 18 plasmid, and then transferred to Escherichia coli JM 109. The plasmid pUC 18 was digested with EcoRI and HindIII, and the resulting DNA fragments were fractionated by electrophoresis on 2% agarose gels. The 5'-end-labeled 650-bp fragment (HindIII 13972-EcoRI 14621) and 460-bp fragment (HindIII 13038-EcoRI 13507) were obtained by dephosphorylation with calf intestine phosphatase and repolishylation with [γ-32P]ATP and T4 polynucleotide kinase. The 650-bp fragment was further digested with ApaI to obtain a singly labeled double-stranded 211-bp fragment (HindIII 13972-ApaI 14182). The 460-bp fragment was further digested with SryI to obtain a singly labeled double-stranded 343-bp fragment (SryI 13160-EcoRI 13507) and a 118-bp fragment (HindIII 13038-SryI 13155). A DNA fragment was also obtained from the human c-Ha-ras-1 protooncogene.16) A DNA fragment was prepared from plasmid pbcNI, which carries a 6.6-kb BamHI chromosomal DNA segment containing the human Ha-ras-1 protooncogene. A singly labeled double-stranded 341-bp fragment (XbaI 1906-AvaI 2246) was obtained according to the method described previously.17) The nucleotide numbering starts with the BamHI site.15

Detection of DNA damage The standard reaction mixture (in a microtube; 1.5 ml) contained the indicated metal ions, NADH as necessary, an ethanol solution of the indicated concentrations of N-OH-AAB, the 32P-labeled double-stranded DNA fragments and 25 μM/base of sonicated calf thymus DNA in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. After incubation at 37°C for 30 min, the DNA fragments were precipitated and dried, followed by heating at 90°C in 1 M piperidine for 20 min. By piperidine treatment, we can detect not only strand breakage, but also base damage. The DNA fragments recovered by ethanol precipitation were dissolved in formamide dye. The DNA fragments were denatured at 90°C for 2 min and immediately chilled on ice. The denatured DNA was electrophoresed on an 8% polyacrylamide/8 M urea gel in Tris borate/EDTA buffer. The autoradiogram was obtained by exposing X-ray film to the gel. The extent of DNA damage was roughly estimated by using a laser densitometer (LKB 2222 UltroScan XL).

The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the MaxamGilbert procedure19) using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

Measurement of 8-oxodG formation Calf thymus DNA fragment was incubated with N-OH-AAB and CuCl2, in the presence and absence of NADH. After ethanol precipitation, DNA was digested to the nucleosides with nuclease P1 and calf intestine phosphatase and analyzed by means of HPLC-ECD, as described previously.20)

Measurement of oxygen consumption Oxygen consumption by the reaction of N-OH-AAB with Cu(II) and NADH was measured using a Clarke oxygen electrode (Electronic Stirrer Model 300, Rank Brothers Ltd., Bottenham, Cambridge, England). The reaction was performed in phosphate buffer containing 10% (v/v) ethanol, and was started by the addition of NADH, CuCl2, or N-OH-AAB to the chamber of the oxygen electrode. Catalase was added to detect H2O2 generation resulting from oxygen consumption, 5 min after starting the reaction (data not shown).

RESULTS

Damage to 32P-labeled DNA fragment induced by N-OH-AAB in the presence of various concentrations of Cu(II) and NADH Fig. 1 shows an autoradiogram of a DNA fragment treated with N-OH-AAB plus Cu(II) in the presence and absence of NADH. Oligonucleotides were detected on the autoradiogram as a result of DNA cleavage. In the absence of N-OH-AAB, DNA damage was not observed with NADH and Cu(II) under the conditions used. N-OH-AAB alone or N-OH-AAB plus NADH did not cause DNA damage. In the presence of Cu(II), N-OH-AAB induced DNA damage. The intensity of DNA damage increased with the concentration of N-OH-AAB. When NADH was added, low concentrations of N-OH-AAB efficiently induced Cu(II)-mediated DNA damage.
The extent of DNA damage was dependent on the concentrations of both NADH and Cu(II) when the same dose of N-OH-AAB was used (Fig. 2). The increase of oligonucleotides with piperidine, treatment compared with no treatment (data not shown), suggested that N-OH-AAB induced not only strand breakage but also base modification and/or liberation.

N-OH-AAB caused no DNA damage in the presence of Mn(II), Fe(II), Co(II) or Ni(II) (data not shown).

**Effects of scavengers and bathocuproine on DNA damage** The effects of scavengers and bathocuproine on DNA damage by N-OH-AAB were investigated (Fig. 3). A typical •OH scavenger, mannitol, did not inhibit DNA damage induced by N-OH-AAB in the presence of Cu(II), whereas methionine inhibited the DNA damage. Bathocuproine, a Cu(I)-specific chelator, also inhibited the DNA damage, suggesting the involvement of Cu(I). DNA damage induced by N-OH-AAB was inhibited by catalase, but not by heated catalase. SOD showed little inhibitory effect on DNA damage. In the presence of NADH, these effects were similar (data not shown).

**Site specificity of DNA damage by N-OH-AAB** An autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensity of DNA cleavage in the human p53 tumor suppressor gene (Fig. 4A).
and the c-Ha-ras-1 protooncogene (Fig. 4B). N-OH-AAB plus Cu(II) induced piperidine-labile sites preferentially at thymine and cytosine residues. When NADH was added, a similar cleavage pattern was observed.

Formation of 8-oxodG in calf thymus DNA by N-OH-AAB in the presence of Cu(II) and the effect of NADH

Using HPLC-ECD, we measured 8-oxodG content in calf thymus DNA treated with N-OH-AAB in the presence of Cu(II) (Fig. 5A). The amount of 8-oxodG increased with the concentration of N-OH-AAB in the presence of Cu(II). The formation of 8-oxodG increased after DNA denaturation. When NADH was added, 8-oxodG formation was observed at very low concentrations of N-OH-AAB (Fig. 5B).

H$_2$O$_2$ generation by the autoxidation of N-OH-AAB in the presence of Cu(II) and NADH

Oxygen consumption
occurred during the autoxidation of N-OH-AAB in the presence of NADH and Cu(II). The addition of catalase increased dissolved oxygen, suggesting that N-OH-AAB generated H$_2$O$_2$, which was decomposed by catalase to generate molecular oxygen. When NADH was omitted, N-OH-AAB plus Cu(II) induced a little oxygen consumption. Cu(II) plus NADH did not consume molecular oxygen under the conditions used (data not shown).

**DISCUSSION**

The present study showed that N-OH-AAB, a metabolite of DAB caused oxidative DNA damage, including 8-oxodG formation, in the presence of Cu(II). In addition, the DNA damage was dramatically enhanced by the endogenous reductant NADH. To clarify the nature of the reactive species we examined the effects of scavengers on the DNA damage induced by N-OH-AAB. Both catalase and bathocuproine inhibited the DNA damage, suggesting the involvement of H$_2$O$_2$ and Cu(I). The effect of catalase on oxygen consumption confirmed that H$_2$O$_2$ was generated by the Cu(II)-mediated autoxidation of N-OH-AAB.

It is suggested that free •OH does not play an important role, because of the lack of inhibition of DNA damage by a typical •OH scavenger and the site specificity of DNA damage.$^{18, 21}$ The inhibitory effect of methional on the DNA damage can be explained by assuming that sulfur compounds are reactive with •OH and less reactive species.$^{22}$ Alternatively, the lack of inhibitory effect of •OH scavengers can be explained by the possibility that DNA damage is induced by •OH generated in very close proximity to the nucleic acid by the bound metal ion. Furthermore, because of site-specific binding of copper ions to DNA, it might be expected that site-specific DNA damage by hydroxyl radicals is generated by bound copper.

Based on these results, a possible mechanism is as follows (Fig. 6): DAB is metabolized to N-OH-AAB through several metabolic steps by demethylation and N-hydroxylation.$^4$ N-OH-AAB induces Cu(II)-mediated DNA damage through reactive oxygen species. Autoxidation of N-OH-AAB to the nitroso compound (NO-AB) via an intermediate occurs, coupled with generation of O$_2$. O$_2$ is dismutated to H$_2$O$_2$, with the reduction of Cu(II) to Cu(I). H$_2$O$_2$ interacts with Cu(I) to form DNA-copper-hydroperoxo complexes, causing DNA damage.$^{23}$ When NADH was added, NO-AB might have been reduced to the intermediate or N-OH-AAB, and again autoxidation would occur, forming a redox cycle.

An endogenous reductant such as NADH would enhance the DNA damage through excessive generation of reactive oxygen species, by forming a redox cycle. The concentration of NAD(P)H in certain tissues was estimated to be as high as 100–200 µM.$^{24}$ Several studies indicate that NADH may react nonenzymatically with some xenobiotics and mediate their reduction.$^{25–27}$ The biological importance of NADH and NADPH as nuclear reductants has been pointed out.$^{28}$

N-OH-AAB plus Cu(II) induced piperidine-labile sites preferentially at thymine and cytosine residues. Generally, site-specific and characteristic mutations have been found in human cancers as molecular mutational fingerprints associated with chemical carcinogens.$^{29}$ In this study, we did not detect N-OH-AAB-induced damage at well-known hotspots. However, we previously detected site-specific DNA damage, including hotspots, by the N-hydroxy metabolite of a heterocyclic amine, via a mechanism simi-
lar to that of N-OH-AAB.\textsuperscript{27} Further researches may reveal the correlation between the site specificity and the carcinogenic process.

Carcinogenicity of amino azo dyes have been explained in terms of the formation of DNA adducts.\textsuperscript{30} Amino azo dyes have an exocyclic amino group that is the key to their carcinogenicity, because this group undergoes biochemical N-oxidation and further conversion to reactive electrophiles.\textsuperscript{30} DNA adducts formed by covalent binding through the activated nitrogen have been identified.\textsuperscript{30} On the other hand, it has been pointed out that free radicals and subsequently formed reactive oxygen species are involved in aromatic amine carcinogenesis.\textsuperscript{30} Hirano et al. reported that administration of 3′-MeDAB increased 8-hydroxyguanine in rodent liver DNA.\textsuperscript{30}

The present study has revealed that N-OH-AAB, a metabolite of DAB, has oxidative DNA-damaging ability, and the existence of NADH enhances the damage through cycling redox reactions. It is noteworthy that a very low concentration, 0.1 \( \mu \text{M} \) N-OH-AAB induced 8-oxodG formation, suggesting that oxidative DNA damage may occur

\section*{REFERENCES}

1) IARC Working Group. Some aromatic azo compounds. In “IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man,” Vol. 8, pp. 125–146 (1975). IARC, Lyon.

2) IARC Working Group. Overall evaluations of carcinogenicity: an updating of IARC Monographs volumes 1 to 42. In “IARC Monographs on the Evaluation of Carcinogenic Risks to Humans,” Suppl. 7, 62 pp. (1987). IARC, Lyon.

3) Gupta, R. and Dani, H. M. \textit{In vitro} formation of organ-specific ultimate carcinogens of 4-dimethylaminoazobenzene and urethane by microsomes. \textit{Toxicol. Lett.}, 45, 49–53 (1989).

4) Delclos, K. B., Tarpley, W. G., Miller, E. G. and Miller, J. A. 4-Aminoazobenzene and N,N-dimethyl-4-aminoazobenzene as equipotent hepatic carcinogens in male C57BL/6xC3H/He F1 mice and characterization of N-(deoxyguanosine-8-yl)-4-aminoazobenzene as the major persistent hepatic DNA-bound dye in these mice. \textit{Cancer Res.}, 44, 2540–2550 (1984).

5) Degawa, M., Miyairi, S. and Hashimoto, Y. Electrophilic reactivity and mutagenicity of ring-methyl derivatives of N-acloxy-N-methyl-4-aminoazobenzene and related azo dyes. \textit{Gann}, 69, 367–374 (1978).

6) Watanabe, H. K. and Hashimoto, Y. Unscheduled DNA synthesis induced by 4-aminoazobenzene, N-hydroxy-4-aminoazobenzene, and their derivatives in primary cultures of rat and mouse hepatocytes. \textit{Gann}, 72, 930–936 (1981).

7) Hashimoto, Y., Watanabe, H. K. and Degawa, M. Mutagenicity of 4-aminoazobenzene, N-hydroxy-4-aminoazobenzene, 4-nitrosoazobenzene, 4-nitroazobenzene, and their ring methoxylated derivatives on \textit{Salmonella}. \textit{Gann}, 72, 921–929 (1981).

8) Hashimoto, Y., Degawa, M., Watanabe, H. K. and Tada, M. Amino acid conjugation of N-hydroxy-4-aminoazobenzene dyes: a possible activation process of carcinogenic 4-aminoazobenzene dyes to the ultimate mutagenic or carcinogenic metabolites. \textit{Gann}, 72, 937–943 (1981).

9) Nakayama, T., Kimura, T., Kodama, M. and Nagata, C. Generation of hydrogen peroxide and superoxide anion from active metabolites of naphthylamines and aminoazo dyes: its possible role in carcinogenesis. \textit{Carcinogenesis}, 4, 765–769 (1983).

10) Hirano, T., Higashi, K., Sakai, A., Tsurudome, Y., Ootsuyama, Y., Kido, R. and Kasai, H. Analyses of oxidative DNA damage and its repair activity in the livers of 3′-methyl-4-dimethylaminoazobenzene-treated rodents. \textit{Jpn. J. Cancer Res.}, 91, 681–685 (2000).

11) Kasai, H., Crain, P. F., Kuchino, Y., Nishimura, S., Ootsuyama, A. and Tanooka, H. Formation of 8-hydroxyguanosine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. \textit{Carcinogenesis}, 7, 1849–1851 (1986).

12) Tchou, J., Kasai, H., Shibusutani, S., Chun, M. H., Laval, J., Grollman, A. P. and Nishimura, S. 8-Oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. \textit{Proc. Natl. Acad. Sci. USA}, 88, 4690–4694 (1991).

13) Degawa, M. and Hashimoto, Y. Studies on N-hydroxy-4-aminoazo dyes. I. Synthesis of N-hydroxy-N-methyl-4-aminoazo dyes and the acyl derivatives, and their degradation in alkaline solution. \textit{Chem. Pharm. Bull.}, 24, 1485–1489 (1976).
14) Sato, K., Poirier, L. A., Miller, J. A. and Miller, E. C.  Studies on the N-hydroxylation and carcinogenicity of 4-aminoazobenzene and related compounds.  *Cancer Res.*, 26, 1678–1687 (1966).
15) Yamashita, N., Murata, M., Inoue, S., Hiraku, Y., Yoshinaga, T. and Kawanishi, S.  Superoxide formation and DNA damage induced by a fragment furanone in the presence of copper (II).  *Mutat. Res.*, 397, 191–201 (1998).
16) Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H. and Goeddel, D. V.  Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue.  *Nature*, 302, 33–37 (1983).
17) Kawanishi, S. and Yamamoto, K.  Mechanism of site-specific DNA damage induced by methylhydrazines in the presence of copper (II) or manganese (III).  *Biochemistry*, 30, 3069–3075 (1991).
18) Kawanishi, S., Inoue, S. and Sano, S.  Mechanism of DNA cleavage induced by sodium chromate (VI) in the presence of hydrogen peroxide.  *J. Biol. Chem.*, 261, 5952–5958 (1986).
19) Maxam, A. M. and Gilbert, W.  Sequencing end-labeled DNA with base-specific chemical cleavages.  *Methods Enzymol.*, 65, 499–560 (1980).
20) Ito, K., Inoue, S., Yamamoto, K. and Kawanishi, S.  8-Hydroxyguanosine formation at the 5′ site of 5′-GG-3′ sequences in double-stranded DNA by UV radiation with riboflavin.  *J. Biol. Chem.*, 268, 13221–13227 (1993).
21) Celander, D. W. and Cech, T. R.  Iron (II)-ethylenediaminetetraacetic acid catalyzed cleavage of RNA and DNA oligonucleotides: similar reactivity toward single- and double-stranded forms.  *Biochemistry*, 29, 1355–1361 (1990).
22) Youngman, R. J. and Elstner, E. F.  Oxygen species in paraquat toxicity: the crypto-OH radical.  *FEBS Lett.*, 129, 265–268 (1981).
23) Schweigert, N., Acero, J. L., von Gunten, U., Canonica, S., Zehnder, A. J. B. and Eggen, R. I. L.  DNA degradation by the mixture of copper and catechol is caused by DNA-copper-hydroperoxo complexes, probably DNA-Cu(DO)OH.  *Environ. Mol. Mutagen.*, 36, 5–12 (2000).
24) Malaisse, W. J., Hutton, J. C., Kawazu, S., Herchuelz, A., Valverde, I. and Sener, A.  The stimulus-secretion coupling of glucose-induced insulin release XXXV.  *Diabetologia*, 16, 331–341 (1979).
25) Naito, N., Ono, Y., Somiya, I., Inoue, S., Ito, K., Yamamoto, K. and Kawanishi, S.  Role of active oxygen species in DNA damage by pentachlorophenol metabolites.  *Mutat. Res.*, 310, 79–88 (1994).
26) Murata, M., Imada, M., Inoue, S. and Kawanishi, S.  Metal-mediated DNA damage induced by diabetogenic alloxan in the presence of NADH.  *Free Radic. Biol. Med.*, 25, 586–595 (1998).
27) Murata, M., Kobayashi, M. and Kawanishi, S.  Mechanism of oxidative DNA damage induced by a heterocyclic amine, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline.  *Jpn. J. Cancer Res.*, 90, 268–275 (1999).
28) Kukielka, E. and Cederbaum, A. I.  Ferritin stimulation of hydroxy radical production by rat liver nuclei.  *Arch. Biochem. Biophys.*, 308, 70–77 (1994).
29) Toyota, M., Ushijima, T., Akiuchi, H., Canzian, F., Watanabe, M., Imai, K., Sugimura, T. and Nagao, M.  Genetic alterations in rat colon tumors induced by heterocyclic amines.  *Cancer*, 77, 1593–1597 (1996).
30) Williams, G. M. and Weisburger, J. H.  Chemical carcinogenesis.  In “Casarett and Doull’s Toxicology: the Basic Science of Poisons,” 4th Ed., ed. M. O. Amdur, J. Doull and C. D. Klaassen, pp. 127–200 (1991). McGraw-Hill Inc., New York.
31) Ohnishi, S., Murata, M., Oikawa, S., Hiraku, Y. and Kawanishi, S.  Copper-dependent DNA damage induced by hydrazobenzene, an azobenzene metabolite.  *Free Radic. Res.*, 32, 469–478 (2000).
32) Kaneko, M., Nakayama, T., Kodama, M. and Nagata, C.  Detection of DNA lesions in cultured human fibroblasts induced by active oxygen species generated from a hydroxylated metabolite of 2-naphthylamine.  *Gann*, 75, 349–354 (1984).