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Adduct formation and repair, and translesion DNA synthesis across the adducts in human cells exposed to 3-nitrobenzanthrone

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ABSTRACT

3-Nitrobenzanthrone (3-nitro-7*H*-benz[*d*,*e*]anthracen-7-one, 3-NBA) is a potent environmental mutagen that is found in diesel exhaust fumes and airborne particulates. It is known to produce several DNA adducts, including three major adducts *N*-(2′-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-ABA), 2-(2′-deoxyadenosin-N^6-yl)-3-aminobenzanthrone (dA-N^6-C2-ABA), and 2-(2′-deoxyguanosin-N^2-yl)-3-aminobenzanthrone (dG-N^2-C2-ABA) in mammalian cells. In the present study, we measured the quantity of the formation and subsequent reduction of these adducts in human hepatoma HepG2 cells that had been treated with 3-NBA using LC–MS/MS analysis. As a result, dG-C8-*N*-ABA and dG-N^2-C2-ABA were identified as major adducts in the HepG2 cells, and dA-N^6-C2-ABA was found to be a minor adduct. Treatment with 1 μg/mL 3-NBA for 24 h induced the formation of 2835 ± 1509 dG-C8-*N*-ABA and 3373 ± 1173 dG-N^2-C2-ABA per 10^7 dG and 877 ± 330 dA-N^6-C2-ABA per 10^7 dA in the cells. The cellular DNA repair system removed the dG-C8-*N*-ABA and dA-N^6-C2-ABA adducts more efficiently than the dG-N^2-C2-ABA adducts. After a 24-h repair period, 86.4 ± 11.1% of the dG-N^2-C2-ABA adducts remained, whereas only 51.7 ± 2.7% of the dG-C8-*N*-ABA adducts and 37.8 ± 1.7% of the dA-N^6-C2-ABA adducts were present in the cells. We also evaluated the efficiency of bypasses across these three adducts and their mutagenic potency by introducing site-specific mono-modified plasmids into human cells. This translesion DNA synthesis (TLS) assay showed that dG-C8-*N*-ABA blocked DNA replication markedly (its replication frequency was 16.9 ± 2.7%), while the replication arrests induced by dG-N^2-C2-ABA and dA-N^6-C2-ABA were more moderate (their replication frequencies were 33.3 ± 6.2% and 43.1 ± 7.5%, respectively). Mutagenic TLS was observed more frequently in replication across dG-C8-*N*-ABA (30.6%) than in replication across dG-N^2-C2-ABA (12.1%) or dA-N^6-C2-ABA (12.1%). These findings provide important insights into the molecular mechanism of 3-NBA-mutagenesis.

1. Introduction

3-Nitrobenzanthrone (3-nitro-7*H*-benz[*d*,*e*]anthracen-7-one, 3-NBA, CAS 17117-34-9, Fig. 1) is a powerful mutagenic aromatic nitro ketone that is found in diesel exhaust fumes and airborne particles [1]. Its atmospheric washout by rainfall is associated with the 3-NBA contamination of surface soil [2–4]. In the Ames *Salmonella typhimurium* (TA98) assay, the mutagenicity of 3-NBA was found to be as high as that of 1,8-dinitropyrene, the strongest mutagen that has been reported so far [1]. 3-NBA induces micronucleus formation in mouse peripheral blood reticulocytes [1,5] and causes DNA strand breaks in human cell comet assays [6–8]. Further studies have demonstrated that 3-NBA is mutagenic in mammalian assay systems [5,9] and induces lung tumors in experimental animals [10]. Therefore, 3-NBA is regarded as an environmental risk factor for human lung cancer in urban areas (see review [11]).

The covalent bonding of DNA to nitrated polycyclic aromatic hydrocarbons (nitro-PAH) results in events that are critical for mutation and cancer initiation [12]. Nitro-PAH acquires

Abbreviations: 3-NBA, 3-nitrobenzanthrone; ABA, aminobenzanthrone; dA-N^6-C2-ABA, 2-(2′-deoxyadenosin-N^6-yl)-3-aminobenzanthrone; dG-C8-*N*-ABA, *N*-(2′-deoxyguanosin-8-yl)-3-aminobenzanthrone; dG-N^2-C2-ABA, 2-(2′-deoxyguanosin-N^2-yl)-3-aminobenzanthrone; MRM, multiple reaction monitoring; NER, nucleotide excision repair; *N*-Aco-ABA, *N*-acetoxy-3-aminobenzanthrone; *N*-OH-ABA, *N*-hydroxy-3-aminobenzanthrone; SI, stable isotope; XP, xeroderma pigmentosum; XPA, XP patient who belongs to complementation group A.

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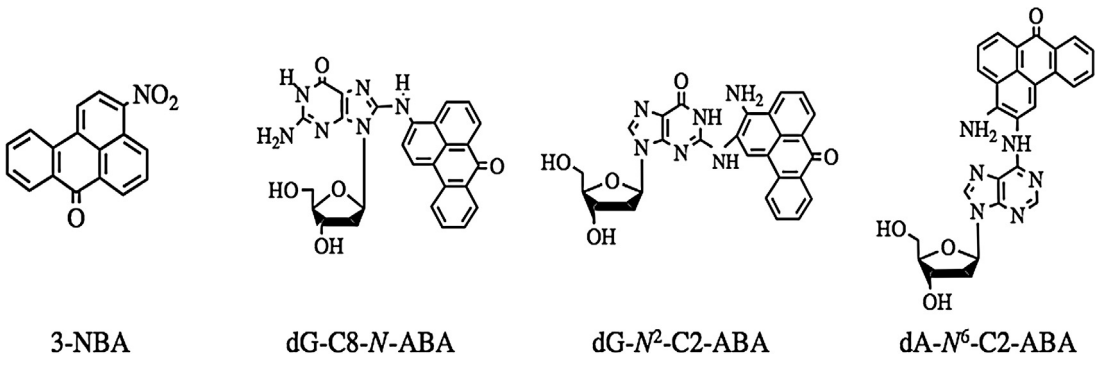

Fig. 1. Chemical structures of 3-NBA and its DNA adducts.

carcinogenicity by being metabolized into reactive electrophiles. It has been shown that the metabolism of 3-NBA is primarily mediated through cytosolic nitroreductases, followed by activation by *N*-acetyltransferase and sulfotransferases, which leads to the formation of reactive intermediates that can form covalent bonds with DNA [13–18]. Using the ^{32}P-postlabeling and high performance liquid chromatography equipped with tandem mass spectrometry (LC–MS/MS) method, a number of bulky aromatic DNA adducts, including three major adducts, *N*-(2′-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-ABA), 2-(2′-deoxyadenosin-N^6-yl)-3-aminobenzanthrone (dA-N^6-C2-ABA), and 2-(2′-deoxyguanosin-N^2-yl)-3-aminobenzanthrone (dG-N^2-C2-ABA), have been detected in cells and tissues treated with 3-NBA (Fig. 1) [5,6,8,10,13–15,17,19–29].

The nucleotide excision repair (NER) pathway is the major mechanism by which bulky adducts are removed, and the efficiency of their removal is known to depend on the chemical structures of the adducts [30]. In proliferating cells, residual adducts arrest progression at replication forks, form replication gaps, or cause DNA disconnection, leading to apoptosis [30]. To prevent replication being blocked, cells possess a translesion DNA synthesis (TLS) mechanism [30]. TLS is performed by low stringency polymerases, each of which displays a different specificity for adducted substrate DNA bases. In mammalian cells, these polymerases include Polη, ι, κ, and Rev1 in the Y-family and Polζ in the B-family [31–37]. The ability of TLS polymerases to extend a DNA strand over adducted bases and their ability to insert the correct bases opposite the adducted bases affect the risk of mutation. Human Polη inserts incorrect bases opposite benzo[*a*]pyrene (B[*a*]P)-adducted guanine (B[*a*]P-dG) residues; *i.e.*, in an error-prone manner, whereas it efficiently bypasses UV-induced cyclobutane pyrimidine dimers in an error-free manner *in vitro* [38,39]. The efficiency of adduct bypasses during TLS and the associated risk of mutagenesis depends on the chemical structures of the DNA adducts involved.

To elucidate the molecular mechanism responsible for 3-NBA-induced mutagenesis in human cells, we used LC–MS/MS to quantify the formation of dG-C8-*N*-ABA, dG-N^2-C2-ABA and dA-N^6-C2-ABA adducts in human hepatoma HepG2 cells that had been treated with 3-NBA, as well as the subsequent reduction of these adducts through the cellular repair process. We also evaluated the efficiency of bypasses across these adducts and the frequency of mutations induced by the adducts by introducing site-specific mono-modified plasmids into human cells.

2. Materials and methods

2.1. Cell culture

Human hepatoma HepG2 cells obtained from the RIKEN Cell Bank (Wako, Japan) and the SV40-transformed human fibroblast cell line XP2OS(SV) [40] were cultured in RPMI1640 medium (Sigma–Aldrich Japan, Tokyo) supplemented with 10% fetal bovine serum (JRN BIOSCIENCES, KS, USA) in a 5% CO_2 atmosphere at 37 °C. The XP2OS(SV) was originated from a xeroderma pigmentosum (XP) patient who belonged to complementation group A (XPA) [40], and this cell line is incapable of performing NER.

2.2. 3-NBA treatment and genomic DNA extraction

The cells were exposed to 3-NBA, as reported previously [28]. Briefly, HepG2 cells (80% confluent) on a 75 cm^2 dish were washed with phosphate-buffered saline (PBS; pH 7.5) (Takara Bio Co., Kyoto, Japan) and incubated in RPMI1640 containing various concentrations of 3-NBA for the indicated time periods. 3-NBA was dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan). The DMSO concentration was not higher than 1% (v/v). Cellular DNA was extracted and digested with the micrococcal nuclease/spleen phosphodiesterase method, as described previously [41]. In order to evaluate the efficiency of adduct removal by the cellular DNA repair mechanism, HepG2 cells were treated with 0.01 μg/mL of 3-NBA for 1 h. The cells were washed with PBS, and then, in order to allow the cells to repair their DNA, they were cultured for the indicated time period in RPMI1640 medium without fetal bovine serum to inhibit cell proliferation. To measure cell viability, HepG2 cells cultured in a 96-well microtiter plate were exposed to various concentrations of 3-NBA for the indicated time periods. Living cells were stained with Neutral Red (Wako Chemical Co., Osaka, Japan; 50 g/mL) and fixed with 1% formalin containing 1% $CaCl_2$ for 1 h. The Neutral Red was extracted from the stained cells with 50% ethanol containing 50% acetic acid, and their OD_{540} values were measured with a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was calculated as the ratio of the OD_{540} of the 3-NBA-exposed cells to that of the solvent control cells.

2.3. Adduct quantification with LC/ESI-MS/MS

The adduct quantification analysis was performed using the Shimadzu HPLC system (Shimadzu, Kyoto, Japan), which consists of LC-10ADvp dual pumps, an SIL-10ADvp autosampler, a Shim-pack FC-ODS column (4.6 mm × 150 mm, 3 μm, Shimadzu), and an SPD-10 ADvp UV–Vis detector, as described previously [41]. The HPLC mobile phases A and B were water and methanol, respectively. The HPLC flow rate was set at 0.4 mL/min. The HPLC gradient started at 40% B, was increased linearly to 80% B over 20 min, and returned to the initial conditions over 1 min, which were maintained for a further 20 min. The HPLC system was interfaced with a Quattro Ultima Pt tandem quadrupole mass spectrometer with an electrospray interface (Waters-Micromass, Milford, MA, USA). The temperature of the electrospray source was maintained at 130 °C, and the desolvation temperature was maintained at 380 °C. Nitrogen was used as the desolvation gas (700 L/h), and the cone gas flow rate was set to 30 L/h. The capillary voltage was set at 3.5 kV. The collision cell pressure and collision energy were set to 3.8×10^{-3} mbar and 10 eV, respectively. The adducts were analyzed by MS/MS using multiple reaction monitoring (MRM). The ion transition was set at $[M+H]^+$ to $[M+H-116]^+$, and the [M+H] of dG-C8-*N*-ABA and dG-N^2-C2-ABA were set at *m/z* 511.2, and that of dA-N^6-C2-ABA was set at *m/z* 495.2. Fifty μL samples were injected in each experiment. The absorbance at 254 nm was also monitored with a UV–Vis detector to monitor DNA digestion, and the peak areas of dG and dT were used for peak normalization, as described previously [41]. The adduct standards were prepared as described previously [29,42]. The stable isotope-labeled (SI-labeled) internal adduct standards were also synthesized as described previously [29,42] with ^{13}C and ^{15}N labeled 2′-deoxyguanosin (U-$^{13}C_{10}$ 98%, U-$^{15}N_5$ 96–98%; Cambridge Isotope Laboratories, Andover, MA, USA) and 2′-deoxyadenosin (U-$^{13}C_{10}$ 98%, U-$^{15}N_5$ 96–98%; Cambridge Isotope Laboratories, Andover, MA, USA). The SI-labeled standards (1 nM) and the indicated concentrations of the non-SI-labeled standards were mixed and injected into the LC–MS/MS system. The [M+H] of the SI-labeled dG-C8-*N*-ABA and dG-N^2-C2-ABA were *m/z* 526.2, and that of the SI-labeled dA-N^6-C2-ABA was *m/z* 510.2. The standard curves for quantification were drawn using the peak area of the SI-labeled adduct (a_{IS}) and the peak area of the unlabeled adduct (a_{AS}). To quantify the amount of the adducts, the SI-labeled adducts