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Mechanisms underlying the varied mammary carcinogenicity of the environmental pollutant 6-nitrochrysene and its metabolites (–)-[*R,R*]- and (+)-[*S,S*]-1,2-dihydroxy-1,2-dihydro-6-nitrochrysene in the rat

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Abstract

The mechanisms that can account for the remarkable mammary carcinogenicity of the environmental pollutant 6-nitrochrysene (6-NC) in the rat remain elusive. In our previous studies, we identified several 6-NC-derived DNA adducts in the rat mammary gland; one major adduct was derived from (±)-*trans*-1,2-dihydroxy-1,2-dihydro-6-nitrochrysene (1, 2-DHD-6-NC). In the present study, we resolved the racemic (±)-1, 2-DHD-6-NC into (–)-[*R,R*]- and (+)-[*S,S*]-1,2-DHD-6-NC and compared their *in vivo* mutagenicity and carcinogenicity in the mammary glands of female transgenic (BigBlue F344 × Sprague-Dawley)F1 rats harboring *lacI/cII* and Sprague-Dawley rats, respectively. Both [*R,R*]- and [*S,S*]-isomers exerted similar mutagenicity and carcinogenicity but were less potent than 6-NC. Additional *in vivo* and *in vitro* studies were then performed to explore possible mechanisms that can explain the higher potency of 6-NC than 1, 2-DHD-6-NC. Using ELISA, we found that neither 6-NC nor 1, 2-DHD-6-NC increased the levels of several inflammatory cytokines in plasma obtained from rats 24 h after treatment. In MCF-7 cells, as determined by immunoblotting, the effects of 6-NC and 1, 2-DHD-6-NC on protein expression (p53, Akt, p38, JNK, c-myc, bcl-2, PCNA, and ERβ) were comparable; however, the expressions of AhR and ERα proteins were decreased by 6-NC but not 1, 2-DHD-6-NC. The expression of both receptors was decreased in mammary tissues of rats treated with 6-NC. Our findings suggest that the differential effects of 6-NC and 1, 2-DHD-6-NC on AhR and ERα could potentially account for the higher carcinogenicity of 6-NC in the rat mammary gland.

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The authors declare no competing financial interest.

Supporting Information Available: Figure S1. Comparative profiling of rat inflammation cytokines in plasma obtained from rats treated with DMSO, 6-NC or 1, 2-DHD-6-NC for 24 h and 6-NC for 72 h *via* intramammary injection. The plasma levels of TNFα, IL-6, IFNγ, IL-1α, IL-1β, MCP-1, Rantes and MIP were measured using the rat inflammation ELISA Strips. This information is available free of charge via the Internet at <http://pubs.acs.org/>."

Keywords

nitro-PAH; mammary carcinogenesis; stereoisomerism; aryl hydrocarbon receptor; estrogen receptor

Introduction

The environmental pollutant 6-nitrochrysene (6-NC) is a potent mammary carcinogen and mutagen in rats.^{1, 2} The International Agency for Research on Cancer classified 6-NC as a Group IIA carcinogen (probably carcinogenic to humans).³ 6-NC belongs to the class of nitropolycyclic aromatic hydrocarbons (nitro-PAH), a subgroup of PAHs produced mainly from incomplete combustion of organic compounds such as diesel, gas, kerosene and liquid petroleum. 6-NC is known to be metabolically activated to yield reactive electrophilic species which can react with DNA and result in the formation of DNA adducts.⁴⁻⁶ Our previous studies have shown that the metabolite ($\pm$)-*trans*-1,2-dihydroxy-1,2-dihydro-6-hydroxylaminochrysene (1,2-DHD-6-NHOH-C) is responsible for the formation of the DNA adduct 5-(deoxyguanosin-N²-yl)-1,2-dihydroxy-1,2-dihydro-6-aminochrysene [5-(dG-N²-yl)-1,2-DHD-6-AC] detected in the mammary gland of rats treated with 6-NC (Figure. 1).⁶ Among all the metabolites tested, 1, 2-DHD-6-NHOH-C exhibited the most similar *in vitro* mutation profile to that of 6-NC *in vivo*.⁷ Based on these findings, we proposed that 1,2-DHD-6-NHOH-C is the putative ultimate mutagen/carcinogen of 6-NC^{7, 8} and 1, 2-DHD-6-NC, the immediate nitro reduction precursor of 1,2-DHD-6-NHOH-C, is the proximate carcinogen of 6-NC.

The racemic 1, 2-DHD-6-NC can be resolved into [*R,R*]- and [*S,S*]-1, 2-DHD-6-NC.⁹ [*R,R*]- isomer was the major enantiomeric metabolite of 6-NC and was more mutagenic than [*S,S*]-isomer in the BigBlueTM *lacI* mammary epithelial cell *in vitro*.⁹ The varied *in vitro* mutagenicity between [*R,R*]- and [*S,S*]-1, 2-DHD-6-NC encouraged us to hypothesize that the stereochemistry of dihydrodiol might affect the mutagenic and carcinogenic activities of 6-NC. To test this hypothesis, in the present study, we compared the mutagenicity and carcinogenicity of [*R,R*]- and [*S,S*]-1, 2-DHD-6-NC *in vivo* in the rats. Our results showed that the mutagenicity and carcinogenicity of [*R,R*]- and [*S,S*]-1,2-DHD-6-NC were similar *in vivo*. Moreover, neither [*R,R*]- nor [*S,S*]-1,2-DHD-6-NC was more potent than the parent carcinogen 6-NC.

We further performed *in vitro* and *in vivo* experiments to explore the possible mechanisms that can account for the higher activity of 6-NC than 1, 2-DHD-6-NC. We compared the inflammation cytokine profiles in the plasma of rats treated with 6-NC or 1, 2-DHD-6-NC and the effects of these two compounds on several molecular targets including p53, p38, JNK, Akt, c-myc, bcl-2, PCNA, ER α , ER β and AhR.¹⁰ Our results showed that 6-NC but not 1, 2-DHD-6-NC induced a marked degradation of AhR and ER α , suggesting that 6-NC could interfere with the AhR-ER α signaling pathway which could potentially account, at least in part, for the varied carcinogenicity between 6-NC and 1,2-DHD-6-NC in the rat mammary gland.

Material and Methods

Chemicals

Caution—6-NC, 1, 2-DHD-6-NC, [*R,R*]- and [*S,S*]-1,2-DHD-6-NC are hazardous and should be handled carefully. 6-NC was synthesized using the procedure described in the literature.¹¹ The racemic ($\pm$)-1, 2-DHD-6-NC was synthesized and resolved as reported

previously.^{9, 12} Dimethylsulfoxide (DMSO) was purchased from Aldrich-Sigma Chemical Co. (St. Louis, MO).

Animal and treatment for mutagenesis assay

Female (BBR × Sprague-Dawley)F1 rats were produced and maintained as described previously.⁸ A total of 112 female (BBR × CD)F1 rats were randomly assigned to 4 treatment groups. There were 16 rats in the *[R,R]*- and *[S,S]*-1,2-DHD-6-NC treated groups and control (DMSO) and 40 rats in the 6-NC treated group. At 30 days of age, the animals received two consecutive intramammary injections of 6-NC, *[R,R]*-1, 2-DHD-6-NC, *[S,S]*-1, 2-DHD-6-NC in DMSO as vehicle or DMSO only. The route of carcinogen administration and the dose were established in our previous bioassays.^{13, 14} Briefly, the animals received the first dose of carcinogen (2.04 μmol/100 μL DMSO) by injection into the three right thoracic nipples; the corresponding areas of on the left side were treated with DMSO alone. The animals received same treatments on the next day in the inguinal nipples. Control rats were treated on both sides with DMSO. The total dose of carcinogen was 12.3 μmol (2.04 μmol/100 μL × 6) per rat. Animals treated with *[R,R]*- or *[S,S]*-1, 2-DHD-6-NC were sacrificed after 8 and 36 weeks. Rats treated with 6-NC or DMSO were euthanized at 2, 4, 8 and 36 weeks. The mammary glands were examined macroscopically for any gross lesions or abnormalities. Mammary gland (tumors, non-involved and normal tissues) and liver were snapped frozen in liquid nitrogen and stored at -80°C for mutagenesis assays.

The cII mutagenesis assay

Mammary tissue from rats treated with *[R,R]*- and *[S,S]*-1, 2 DHD-6-NC, 6-NC and DMSO was processed for mutagenesis assay as described previously.⁸ Briefly, the tissue homogenate was centrifuged and the nuclei was separated and digested with protease K and RNase (Fisher Scientific, Pittsburgh, PA). The mixture was placed onto 0.45 μ pore-size membranes and dialyzed overnight. After extraction, the DNA was packaged into phage particles using a Transpack phage packaging extract (Stratagene, La Jolla, CA) and then incubated with the appropriate host strain of *E. coli* and assayed for plaque-forming units and mutants.¹⁵

Carcinogenesis assay

Ninety-six Sprague-Dawley rats were obtained from Charles River (Kingston, NY) and randomly assigned into 4 groups of 24 rats each. At an age of 30 days, animals were treated with two consecutive intramammary injections of carcinogen 6-NC, *[R,R]*- 1, 2-DHD-6-NC, *[S,S]*-1, 2-DHD-6-NC or vehicle (DMSO) as described above. Rats were weighed prior to carcinogen administration, weekly during the first month, and then monthly until termination (32 weeks) after the second injection. Animals were palpated beginning at week 5. At necropsy, the mammary glands were examined macroscopically for any gross lesions or abnormalities. The tissues were fixed in 10% buffered formalin solution, processed for paraffin sections and stained with hematoxylin and eosin. Histological diagnosis of mammary tumors was based on criteria outlined by Russo et al.¹⁶

Profiling of inflammatory cytokines

Inflammation is recognized as a causative factor for several types of cancers.¹⁷ Thus, a short-term bioassay was conducted to obtain rat plasma for profiling the inflammation cytokines. A total of 12 female Sprague-Dawley rats (30 days old) were assigned into 3 groups and treated with DMSO (*n* = 3), 6-NC (*n* = 6) or racemic 1, 2-DHD-6-NC (*n* = 3) as described in mutagenesis assay. Animals were sacrificed after 24 h except 3 animals treated with 6-NC were sacrificed after 72 h. The plasma levels of TNFα, IL-6, IFNγ, IL-1α, IL-1β,

MCP-1, Rantes and MIP were measured using the rat inflammation ELISA Strips (Signosis, Sunnyvale, CA) according to the manufacturer's instructions.

Cell treatment and immunoblotting

In this *in vitro* study, we examined the effects of 6-NC and 1, 2-DHD-6-NC on several molecular targets which are critical in the development of breast cancer. MCF-7 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in IMDM media with 10% FBS as described before.¹⁸ Treatments were initiated after cells were seeded and incubated for at least 24 h. Cells were treated with 6-NC or 1, 2-DHD-6-NC at 1, 3, and 10 PM. Cells treated with vehicle (DMSO) were used as control. At the end of treatment (24 h), cells were washed with PBS (3×) and the whole-cell proteins were extracted using Cell Lysis buffer (Cell signaling Technology, Beverly, MA) according to the manufacturer's instructions. The concentration of protein was determined using Bio-Rad DC™ protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins (30 µg/sample) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (SDS-PAGE) and transferred to PVDF membrane. Membrane was stained with Ponceau S dye (Aldrich-Sigma Chemical Co.) to ensure equal loading of proteins. After standard immunoblotting procedures, proteins were visualized using the Pierce® ECL or SuperSignal® West Femto ECL system (Thermo Scientific Inc. USA).

The following antibodies were used: rabbit phospho-p53 (ser15) (p-p53), p53, phospho-AKT, AKT, phospho-p38 MAPK (p-p38 MAPK), p38 MAPK, phospho-JNK (p-JNK), JNK, Bcl-2 were obtained from Cell Signaling Technology (Beverly, MA); c-myc, PCNA, AhR (H-211), ERα (H-184) and ERβ (H-150) from Santa Cruz Biotechnology, Inc. (CA, USA); mouse monoclonal antibody β-actin from Sigma-Aldrich (St. Louis, MO). Secondary anti-mouse or anti-rabbit IgG, conjugated with horse radish peroxidase (HRP) were purchased from Cell Signaling Technology (Beverly, MA).

Animal treatment and immunohistochemistry

To examine the effect of 6-NC on the expressions of AhR and ERα proteins in rat mammary tissues, seven Sprague-Dawley rats at 30 days old were administered one dose of 6-NC *via* intramammary injection ($12 \times 2.04 \mu\text{mol/nipple}/50 \mu\text{L}$ DMSO, 3 animals) or oral gavage ($50 \mu\text{mol}/500 \mu\text{L}$ trioctanoin, 4 animals).¹⁴ Animals treated intramammary with DMSO ($n = 3$) or orally with trioctanoin ($n = 4$) alone were used as negative control groups. Animals were sacrificed after 24 h.

Portions of the mammary tissues were fixed in formalin, embedded in paraffin blocks and sectioned. Immunohistochemistry was performed using an indirect immunoperoxidase method. Briefly, 5 µm sections were heated to 60°C, deparaffinized in xylene, rehydrated in graded alcohols, and rinsed with water. The tissue sections were then subjected to a standard microwave antigen retrieval procedure before being blocked by hydrogen peroxide. Slides were incubated sequentially with diluted polyclonal antibodies AhR (1:200, H-211, Santa Cruz) or ERα (1:100, H-184, Santa Cruz) overnight at 4°C and then with biotinylated goat anti-rabbit secondary antibodies (1:1200 dilution, Vector Laboratories, Inc) for 40 min at room temperature. After washing, sections were incubated at streptavidin peroxidase (BoehringerMannheim, Indianapolis, IN) and visualized by incubating with 3, 3'-diaminobenzidine-tetrahydrochloride solution (DAKO Corporation, Carpinteria, CA) for 1 min. The slides were thoroughly washed with tap water and counterstained with a modified Harris hematoxylin (Fisher Scientific, Fairlawn, NJ).

A total of seven 6-NC treated and seven vehicle treated rat mammary tissues were stained. To quantitate AhR expression in cytoplasm, we utilized an H-SCORE which is the product

of the staining intensity (range: 0–3) and the percentage of positive cells.¹⁹ To quantitate ER α expression in nucleus, three random and geographically separate 400 $\times$ fields were identified and all nuclear positive cells within those fields were individually counted.

Immunoblotting of rat mammary gland

For analysis of AhR and ER α proteins from rat mammary tissues, frozen tissues were randomly selected from both vehicle and 6-NC treated groups ($n=3$ for each group) and minced to $\sim 1\text{mm}^3$ on dry ice and placed in ice-cold RIPA buffer with protease inhibitors (Roche Diagnostics). The homogenates were centrifuged at 14,000 $\times g$ for 1 h and the protein concentrations of supernatant were determined as described above. Protein (100 μg /sample) were resolved on 7.5% SDS-PAGE, transferred to nitrocellulose membrane and processed according to standard western blotting procedures. For AhR, two mouse monoclonal AhR antibodies (RPT-1 and RPT-2) from Abcam Inc. (Cambridge, MA) and polyclonal rabbit AhR antibody (H-211) from Santa Cruz were used. Polyclonal rabbit antibody ER α (H-184) from Santa Cruz was used to detect ER α protein.

Statistical methods

The two-sided Fisher's exact test was used to compare tumor incidence, multiplicity and volume across the different treatments. The calculations were performed using R version 2.14.0 (www.r-project.org). The student t -test was used to compare the mutant fractions, cytokines profiles, immunohistochemistry staining and Western blotting between groups.

Results

Mutagenesis

DNA extracted from mammary tissues was analyzed for mutations, and the results are shown in Table 1. Mammary gland from rats treated with 6-NC exhibited the highest mutant fraction (MF) of all groups. The MF was 3.8 ± 1.1 and 4.4 ± 1.0 for 2 and 4 weeks after administration of 6-NC, respectively. The MF increased up to eight weeks after administration of 6-NC and was unchanged after that. Therefore, results for 8 and 36 week groups were combined and the MF (in units of $\text{pfu} \times 10^{-5}$) was 8.1 ± 4.4 . For both the $[R,R]$ -1,2-DHD-6-NC and $[S,S]$ -1,2-DHD-6-NC -treated animals, no differences in the MFs after 8 and 36 weeks were noted and results from the 8 and 36 week groups were combined. The difference in the MF between $[R,R]$ - and $[S,S]$ -1,2-DHD-6-NC -treated animals was not statistically significant. For vehicle controls no difference was observed in MF between groups at different time points and results were combined. In all cases where the rats were treated with 6-NC or enantiomers, the increase in MF over that in controls was significant ($p < 0.05$).

Carcinogenesis

The accumulated incidence of palpable mammary tumors during the course of the bioassay is shown in Figure 2. Palpable mammary tumors induced by 6-NC were first observed at week 9 followed by $[R,R]$ -1,2-DHD-6-NC at week 15 and $[S,S]$ -1,2-DHD-6-NC at week 23. At necropsy (32 weeks), tumor incidence reached 92% for 6-NC, 46% for $[R,R]$ - and 38% for $[S,S]$ -isomer. 6-NC treatment led to a significantly higher tumor incidence than both enantiomers ($p < 0.05$); the differences between $[R,R]$ -1,2-DHD-6-NC and $[S,S]$ -1,2-DHD-6-NC in the induction of histologically classified adenocarcinoma was not significant (Figure 2 insert).

The results of mammary tumors induced by 6-NC, $[R,R]$ -, $[S,S]$ -1,2-DHD-6-NC or DMSO are summarized in Table 2. Mammary adenocarcinoma was the most common histological

type in 6-NC, *[R,R]*-1, 2-DHD-6-NC and *[S,S]*-1, 2-DHD-6-NC treated rats. 6-NC induced significantly more adenocarcinoma than did *[R,R]*- or *[S,S]*-1, 2-DHD-6-NC ($p < 0.001$); 6-NC also induced higher fibroadenoma than *[S,S]*-1,2-DHD-6-NC ($p < 0.05$). The difference between *[R,R]*- and *[S,S]*-1, 2-DHD-6-NC treated groups was not significant for any of the histological tumor type. In addition, no statistical differences between *[R,R]*- and *[S,S]*-1, 2-DHD-6-NC was observed in tumor multiplicity (Table 1) or tumor volume (data not shown). In the DMSO treated rats, no tumors were observed. No lesions were observed in any organs other than the mammary gland. Tumors were located on both thoracic and inguinal glands. One animal in 6-NC treated group was sacrificed unscheduled and the tumor tissue was not collected for histological examinations. Three animals in 6-NC-treated group were sacrificed before 32 weeks due to the ulceration of tumors.

Inflammation cytokine profiling

Rats were treated with DMSO, 6-NC, or 1, 2-DHD-6-NC and plasma were obtained after 24 and 72 h. The plasma levels of TNF α , IL-6, IFN γ , IL-1 α , IL-1 β , MCP-1, Rantes and MIP were measured and expressed as the OD at 450 nm. No significant differences were observed between groups treated with 6-NC for 24 or 72 h (supplementary data, Figure S1). The results also showed that there were no significant differences in the inflammation cytokines profiles between 6-NC, 1, 2-DHD-6-NC and DMSO (control) treatments (Supplementary data, Figure S1).

Immunoblotting analysis in MCF-7 cells

We examined the protein expressions of p-p53 (ser15), p53, p-AKT, AKT, p-p38 MAPK, p38 MAPK, p-JNK, JNK, c-myc, bcl-2, PCNA, AhR, ER α and ER β in MCF-7 cells after treatments with 6-NC or 1, 2-DHD-6-NC (10, 3 and 1 PM) for 24 h. Figure 3, a representative immunoblotting analysis, shows that both 6-NC and 1, 2-DHD-6-NC had no significant effect on p-p53, p53, p-Akt, Akt, p-JNK, JNK, c-myc, bcl-2, PCNA and ER β ; however, both compounds induced p-p38 MAPK protein but had no effect on the levels of total p38 MAPK. It was also noted that 6-NC induced reductions of both AhR and ER α proteins, while 1, 2-DHD-6-NC had no effect on these receptors.

Effect of 6-NC on AhR and ER α protein expressions in rat mammary tissue

To further determine if the *in vitro* findings in MCF-7 cells could be replicated *in vivo*, we examined the expression of AhR and ER α proteins in mammary tissues of rats treated with 6-NC using immunohistochemistry and immunoblotting analysis. Rat mammary tissues were obtained from Sprague-Dawley rats (30 days old) treated with 6-NC or vehicle and sacrificed after 24 h. Figures 4A-C show the representative immunohistochemical staining pictures of AhR protein with intensity from 1–3, respectively. Figure 4D represents the quantitative immunohistochemical analysis (by H-SCORE) of AhR protein present in the cytoplasm of mammary tissues. The H-SCORE of 6-NC treated mammary tissues ($n = 7$) was significantly lower than those of vehicle treated ($n = 7$) ($p < 0.001$, t-test). Immunoblot analysis of AhR protein expression using the protein lysate extracted from these rat mammary tissues was, however, not successful.

Figures 4E shows a representative immunohistochemical staining picture of ER α protein. All nuclear ER α positive cells within three randomly selected fields were counted. The quantitative analysis of ER α is shown in Figure 4F. There appeared to be less ER α protein in 6-NC treated group than control group but there was no statistical significance between the groups when analyzed by a number of different endpoints (average, min, max, and median values). All of the endpoints yield similar results (less protein in 6-NC treated group as compared to control group), but none of them showed a significant treatment effect.

Figure 4G shows immunoblotting analysis of ER α protein in tissues treated with 6-NC (animal# 1–3) or vehicle (animal# 4–6) for 24h ; protein lysate obtained from MCF-7 was used as positive control (lane 1). ER α protein expression was quantitatively determined by normalization of ER α band density to β -actin band density to control for protein loading errors (Figure 4H). Although there was some inter-animal variability, the results clearly show a decreased expression of ER α in 6-NC treated animals ($p < 0.05$).

Discussion

In the present study, we compared the mutagenic and carcinogenic activities of the putative proximate carcinogens [*R,R*]- and [*S,S*]-1,2-DHD-6-NC. The results showed that the [*R,R*]- and [*S,S*]-isomers exerted similar mutagenic effects in the *in vivo* system, despite the higher mutagenic activity of the former in the mammary epithelial cells derived from a *lacI* (BigBlueTM) Fisher 344 rat.⁹ Assuming that the two enantiomers have similar absorption and distribution in the body following intramammary administration, the apparent inconsistency between *in vitro*⁹ and *in vivo* mutagenicity (this study) may be, in part, due to differences in the levels of metabolic activation/detoxification and DNA repair systems, *in vitro* vs. *in vivo* and the fact that *in vivo*, a mixture of cell types, may be involved in metabolism and accumulation of mutations induced by the isomers.

With identical route of administration and dose, [*R,R*]- and [*S,S*]-1,2-DHD-6-NC yielded comparable carcinogenicity which is consistent with the results of mutagenesis assay. However, this finding is novel and different from numerous bulky aromatic compounds including chrysene, where the (–)-[*R,R*] enantiomer of the bay region dihydrodiol displays much greater tumorigenic activity than the (+)-[*S,S*] enantiomer.^{20–23} In contrast to unsubstituted polycyclic dihydrodiols, which form dihydrodiol epoxides as the ultimate DNA reactive species, the chiral centers of 1, 2-DHD-6-NC are remote from the reactive hydroxylamino group, which is the site of DNA binding. Our results clearly indicate that stereochemistry appears to have no effect on the mutagenicity/carcinogenicity of 1, 2-DHD-6-NC under the conditions of the bioassay performed here.

6-NC induced more tumors than [*R,R*]- or [*S,S*]-1,2-DHD-6-NC or any of its metabolites tested so far.¹⁴ This observation suggests that besides genotoxic metabolites derived from 6-NC, other factors may also contribute to overall carcinogenic activity of 6-NC. Reactive oxygen species (ROS) are involved in tumor initiation but more so in tumor promotion. ROS can stimulate cytokines release from inflamed cells which in turn, may lead to increase ROS generation.²⁴ Recent studies have shown that several NO₂-PAHs can induce the expression of cytokines in lung and bronchial epithelial cells.^{25, 26} Cytokines are the major determinants of inflammatory responses and expression of inflammatory cytokines is a characteristic of inflammatory disorder which is contributed to several disease conditions including carcinogenesis.^{17, 24} Although the effect of 1, 2-DHD-6-NC on the formation of ROS was not determined in this study, we found that neither 6-NC nor 1, 2-DHD-6-NC increased the levels of inflammation cytokines in plasma. Based on our results it appears that inflammatory cytokines may not contribute to the different carcinogenicity of 6-NC and 1, 2-DHD-6-NC. These results appear to be in line with our previous report demonstrating that 6-NC has no effect on the basal level of oxidative DNA damage measured as 8-OH-dG.¹⁴

Earlier studies conducted in our laboratories have shown that human breast adenocarcinoma MCF-7 cells can metabolize 6-NC to generate DNA adducts derived primarily from nitroreduction of 1, 2-DHD-6-NC;²⁷ however, in the same system, no accumulation of p53 protein was detected.¹⁸ Landvik et. al.¹⁰ reported that the molecular mechanisms underlying the mutagenic and carcinogenic effect of numerous nitro-PAHs could be due to the

activation of various intracellular signaling pathways related to cell proliferation/apoptosis and suggested that the lack of functional p53 or inability to activate signaling pathways that can induce apoptosis may help cells survive with DNA damage, thereby increasing mutagenic and carcinogenic potential. In this study, we found that no distinct p53 phosphorylation or accumulation was induced in MCF-7 cells after exposure to either 6-NC or 1, 2-DHD-6-NC for 24 h. Phosphorylation (activation) of p38 MAPK can be achieved by estrogen signaling mediated by a G-protein coupled receptor known as GPR3.^{28, 29} However, in this study, we found that both 6-NC and 1,2-DHD-6-NC induced similar responses in p-p38 MAPK and total p38 MAPK. Thus, we considered this effect may not contribute to the differential carcinogenic activities between 6-NC and 1, 2-DHD-6-NC.

The AhR is a ligand-activated basic-helix-loop-helix-PAS (bHLH-PAS) transcription factor that functions as an intracellular mediator of xenobiotic signaling pathways. Studies have shown that AhR can interact with ER α signaling pathway which is known to have critical role in mammary carcinogenesis.^{30, 31} The AhR mediates a variety of biological effects and is activated by structurally diverse compounds including PAH and NO₂-PAH.^{32, 33} The best characterized AhR ligand is 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). Both estrogenic and anti-estrogenic activities of TCDD have been reported.³⁴ In addition to induce the expression of *CYP1* family of estrogen-metabolizing genes, TCDD initiates a AhR-mediated degradation of ER α and suppress estrogen signaling.³⁵ In contrast, an ER-dependent gene expression induced by TCDD was also reported in murine uterus upon ovariectomy.³⁶ Studies conducted in rat models indicate that the susceptibility to mammary tumors likely correlates with the age or the differentiation state of the mammary gland at the time of TCDD exposure.^{37, 38} However, the relationship between exposure to AhR ligands and breast cancer risk is still unclear. 6-NC is known to induce the expression of *CYP1* and therefore being considered as a AhR agonist.³² We found that similar to TCDD, 6-NC induced a dose-dependent reduction of AhR and ER α proteins in MCF-7 cells, but this effect was not observed with 1, 2-DHD-6-NC. Consistent with this observation, 6-NC treatment resulted in decreases of AhR and ER α proteins in rat mammary tissues *in vivo*. Several published studies have shown that the degradation of AhR and ER α by AhR-ligand binding is through a proteasome-dependent mechanism.^{35, 39–41} Although 6-NC is an AhR agonist, the binding activity of its metabolite 1,2-DHD-6-NC to AhR has not been determined yet. Based on the varied responses in AhR and ER α induced by 6-NC and 1,2-DHD-6-NC, both may not bind to AhR in the same way.

Despite the well-documented crosstalk between AhR and ER α signaling pathways, the underlying molecular mechanisms that contribute to carcinogenesis remain largely elusive. Binding of 6-NC to AhR may contribute to carcinogenesis through both genomic and non-genomic pathways. For example, 6-NC may regulate the expressions *CYP1* family enzymes that metabolize estrogens and thus influence the ER α signaling pathways or its downstream targets. An example of non-genomic response induced by 6-NC is the degradation of AhR and ER α . Although genomic and non-genomic responses mediated by the AhR are species and tissues specific, our findings suggest that in addition to genotoxicity, differences in the effects of 6-NC and 1, 2-DHD-6-NC mediated by AhR/ER α pathways may play a role in the varied mammary carcinogenicities between 6-NC and 1, 2-DHD-6-NC. Future studies will examine the downstream signaling pathways of AhR and ER α in the development of 6-NC-induced rat mammary tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

6-NC	6-nitrochrysene
nitro-PAH	nitropolycyclic aromatic hydrocarbons
1,2-DHD-6-NC	(±)- <i>trans</i> -1, 2-dihydroxy-1,2-dihydro-6-nitrochrysene
1,2-DHD-6-NHOH-C	(±)- <i>trans</i> -1, 2-dihydroxy-1,2-dihydro- <i>N</i> -hydroxy-6-aminochrysene
<i>N</i>-OH-6-AC	<i>N</i> -hydroxy-6-aminochrysene
BBR	Big Blue™ rat
MF	mutant fraction
aryl hydrocarbon receptor	AhR
estrogen receptor	ER

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Fig. 1.
Metabolic activation pathways of 6-NC.

Fig. 2.

Percentage of incidence of palpable mammary tumors in female Sprague-Dawley rats following intramammary administration of 6-NC (□), [R,R]- 1, 2-DHD-6-NC (▲), [S,S]-1, 2-DHD-6-NC (△) or DMSO (vehicle, ■). Figure 2 insert presents tumor incidence induced by 6-NC and its dihydrodiol metabolites.

Fig. 3.

Representative immunoblotting analysis of phospho-p53 (ser15), p53, phospho-Akt, Akt, phospho-p38 MAPK, p38 MAPK, phospho-JNK, JNK, Bcl-2, c-myc, PCNA, AhR, ER α and ER β in MCF-7 cells after exposure to 1, 3, and 10 μ M of 6-NC or 1, 2-DHD-6-NC for 24 h. Cells treated with DMSO alone were used as negative control. β -Actin was used as a loading control.

Fig. 4. (A–C) Immunohistochemical staining pictures of AhR in representative rat mammary tissues with intensity ranging from 1 to 3, respectively. Magnification 400×. Staining for AhR is brown in color, while the cell nuclei, counterstained with hematoxylin, appear blue. (D) Quantitative analysis of AhR immunostaining by H-SCOREs. Mammary tissues of rats were obtained 24 h after treatment with 6-NC or vehicle (mean $\pm$ S.D., $n = 7$). The effect of treatment in AhR is significant (* $p < 0.001$, t-test). (E) A representative immunostaining for ERα in rat mammary tissue. The staining was primarily detected in the nuclei of epithelial cells. (F) Quantitative analysis of ERα by counting nuclear immunostaining positive cells in mammary tissues of rats obtained 24 h after treatment with 6-NC or vehicle (mean $\pm$ S.D., $n = 7$). Although less ERα in 6-NC treated group, there is no significant treatment effect. (G) Immunoblotting analysis of ERα in cell lysate (100 Pg) extracted from rat mammary tissues obtained 24 h after treatment with 6-NC (animal#4–6) or vehicle (animal#1–3). Protein lysate obtained from MCF-7 was used as positive control (lane 1). β-Actin was used as a loading control. (H) Quantitative analysis of ERα in Figure G (mean $\pm$ S.D.). Band density was normalized to β-actin for quantification purpose. * $p < 0.05$.

Table 1

Mutant fractions in the mammary gland from female (BBR x CD)F1 transgenic rats treated with 6-NC, [R,R]- and [S,S]-1,2-DHD-6NC or vehicle (control).

	[R,R]-1,2-DHD-6NC ^a	[S,S]-1,2-DHD-6NC ^a	6-NC (2 weeks)	6-NC (4 weeks)	6-NC (8+36 weeks) ^a	Control ^b
MF ^c	2.4±1.1 ^{*,#}	3.6±1.1 ^{*,#}	3.8±1.1 [*]	4.4±1.0 [*]	8.1±4.4 [*]	1.3±1.1

* Denotes a $p < 0.05$ in treated versus control group;

denotes a $p > 0.05$ between [R,R]- and [S,S]-1,2-DHD-6-NC groups.

^a Combination of results from rats euthanized at 8 and 36 weeks after treatment as the MFs from these groups were not different.

^b control=untreated rats + untreated side of rats treated with [R,R]- or [S,S]-1,2-DHD-6-NC.

^c mutant fraction $\times 10^5$, mean $\pm$ standard deviation

Table 2

Mammary tumors in rats treated with 6-NC, [R,R]- or [S,S]-1,2-DHD-6-NC^a.

compound	no. of animals	No. of rats with mammary tumors				Mammary tumor multiplicity ^b			
		fibroadenoma	adenoma	adenocarcinoma	others	fibroadenoma	adenoma	adenocarcinoma	others
1. 6-NC	23 ^c	8(35) ^d	2(9)	19(83)	3(13) ^e	0.39±0.58(9) ^f	0.17±0.65(4)	1.39±1.20(32)	0.13±0.29(3)
2.[R,R]-1,2-DHD-6NC	24	5(21)	3(13)	5(21)	0(0)	0.29±0.82(7)	0.17±0.67(4)	0.21±0.50(5)	0±0
3.[S,S]-1,2-DHD-6NC	24	2(8)	2(8)	5(21)	2(8) ^g	0.08±0.28(2)	0.17±0.64(4)	0.25±0.53(6)	0.08±0.28(2)
4. Control (DMSO)	24	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0±0

^aTreatment was initiated at 30 days of age. The chemicals were administered *via* two consecutive intramammary injections in the right nipples; DMSO was injected into the left nipples of the same animals to serve as an internal control. Group 4 received only DMSO in both right and left nipples. Animals were observed for tumor induction up to 32 wk prior to sacrifice.

^b6-NC: all tumors occurred on the right side; [S,S]-: two tumors occurred on the left side; [R,R]-: three tumors occurred on the left side.

^cOne animal was sacrificed without tumor tissue collection.

^dNumbers in parentheses represent percentage.

^eTwo rats each had one DCIS and one rat had one spindle cell carcinoma.

^fNumbers are mean ± standard deviation; numbers in parentheses represent the total number of mammary tumors.

^gTwo rats each had one DCIS.