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Stereoselective Metabolism of the Environmental Mammary Carcinogen 6-Nitrochrysene to *trans*-1, 2-Dihydroxy-1, 2-Dihydro-6-Nitrochrysene by Aroclor 1254-Treated Rat Liver Microsomes and Their Comparative Mutation Profiles in a *lacI* Mammary Epithelial Cell Line

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Abstract

The environmental pollutant 6-nitrochrysene (6-NC) is a powerful mammary carcinogen and mutagen in rats. Our previous studies have shown that 6-NC is metabolized to *trans*-1, 2-dihydroxy-1, 2-dihydro-6-nitrochrysene (1, 2-DHD-6-NC) in rats and in several *in vitro* systems including human breast tissues and the latter is the proximate carcinogenic form in the rat mammary gland. Since optically active enantiomers of numerous polynuclear aromatic hydrocarbon (PAH) metabolites including chrysene have different biological activities, we hypothesized that the stereochemical course of 6-NC metabolism might play a significant role in the carcinogenic/mutagenic activities of the parent 6-NC. The goal of this study is to evaluate the effect of stereochemistry on the mutagenicity of 1, 2-DHD-6-NC using the *cII* gene of *lacI* mammary epithelial cells *in vitro*. Resolution of (±)-1, 2-DHD-6-NC was obtained by either non-chiral or chiral stationary phase HPLC methods. We determined that the ratio of (–)-[*R,R*]- and (+)-[*S,S*]-1,2-DHD-6-NC formed in the metabolism of 6-NC by rat liver microsomes is 88:12. The mutation fractions and mutation spectra of [*R,R*] and [*S,S*]-enantiomers were examined. Our results showed that [*R,R*]- is significantly ($p < 0.01$) more potent mutagen than the [*S,S*]-isomer. The major types of mutation induced by the [*R,R*]-enantiomer are AT > GC, AT > TA and GC > TA substitutions; and these are similar to those obtained from 6-NC *in vivo* in the mammary gland of rats treated with 6-NC. The mutation spectra of the [*S,S*]- isomer were similar to [*R,R*]-isomer, but a higher percentage of AT > GC substitutions in the [*R,R*]- isomer was noted. Based on the results of the present study, we hypothesize that [*R,R*]- 1,2-DHD-6-NC is the proximate carcinogen of 6-NC in the rat mammary gland *in vivo* and will test this hypothesis in future study.

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Introduction

Nitropolynuclear aromatic hydrocarbons (NO₂-PAH) are widespread environmental contaminants mainly produced from incomplete combustion of nitrogenous organic compounds found in diesel, gasoline, petroleum and food (1–3). This class of compounds includes a number of nitropyrenes, nitrofluorenes, nitrofluoranthenes and 6-nitrochrysene (6-NC), which exhibit carcinogenic activity in experimental animals and thus pose a health risk to humans (1,3–5). Studies have indicated that exposure to carcinogens may contribute to the etiology of cancers (6,7); environmental pollutants that are known to induce mammary cancer in rodents must be regarded as potential human risk factors for the induction of analogous human cancers. Among all of the NO₂-PAH tested so far, 6-NC is the most potent mammary carcinogen in the rat (8). 6-NC can be metabolically activated *via* ring-oxidation, nitroreduction or a combination of both pathways (Scheme 1) that are evident in rats as well as in several *in vitro* systems including human breast cancer cell lines and human breast tissues (9,10). Ring-oxidation of 6-NC is mediated *via* cytochrome P450 monooxygenases to form nitroarene oxides which can be enzymatically hydrolyzed by epoxide hydrolase to form *trans*-1, 2-dihydroxy-1, 2-dihydro-6-nitrochrysene (1, 2-DHD-6-NC). Further nitroreduction of 1,2-DHD-6-NC yielded a very reactive electrophile *trans*-1,2-dihydroxy-1,2-dihydro-6-hydroxylaminochrysene (1,2-DHD-6-NHOH-C) which is responsible for the formation of the major DNA adducts detected in the mammary gland of rats treated with 6-NC and thus considered as a putative ultimate genotoxic metabolite of 6-NC (11).

In order to determine the ultimate mutagenic metabolite(s), we have compared the mutant fraction (MF) and mutational spectra in mammary tissues of female transgenic (Big Blue F344 × Sprague-Dawley) F1 rats treated with 6-NC with those of a number of its metabolites in the *cII* gene of *lacI* mammary epithelial cells *in vitro* (12,13). Mutational profile here refers to the distribution of the different classes of mutations. It is hypothesized that the metabolite whose mutation profile is most similar to that obtained *in vivo* in the mammary gland of rats treated with 6-NC is likely the ultimate mutagen.

For a number of polynuclear aromatic hydrocarbons (PAH) including chrysene, the (–)-[*R,R*] enantiomer of dihydrodiol displays much greater tumorigenic activity than the (+)-[*S,S*] enantiomer and the bay-region diol epoxide isomer with an [*R,S*]-diol-[*S,R*]-epoxide absolute configuration exerts high mutagenic and tumorigenic activities (14–16). This implies an important role of stereochemistry in the carcinogenic activity of PAH. Studies also showed that the stereoselectivity of the microsomal enzymes involved in the metabolic activation of these chemicals is influenced by substitution in parent PAH as well as the molecular shape of substituted PAH (16). The proximate carcinogenic metabolite 1, 2-DHD-6-NC is the metabolic precursor of the putative ultimate carcinogen 1, 2-DHD-6-NHOH-C in rats (9,17). Thus, it is of considerable interest to determine the stereoselectivity in the metabolism of 6-NC to [*R,R*]- and [*S,S*]-1, 2-DHD-6-NC. In this study, we determined the enantiomeric composition of 1, 2-DHD-6-NC formed in the metabolism of 6-NC by rat liver microsomes from Aroclor 1254-treated Sprague-Dawley rats. Enantiomeric separation of (±)-1, 2-DHD-6-NC was obtained by using either non-chiral or chiral stationary phase (CSP) HPLC methods; the absolute stereochemistry of [*R,R*]- and [*S,S*]-1, 2-DHD-6-NC was established by chiral spectral methods. The mutant fraction and mutational spectra of each enantiomer were examined and compared to that obtained in the mammary gland of rats treated with 6-NC *in vivo* (12,13).

Material and Methods

Materials

6-NC was synthesized using the procedure described by Newman and Cathcart (18). 1, 2-DHD-6-NC was synthesized as reported previously (19). (–)-Menthoxycetyl chloride, anhydrous pyridine, magnesium chloride, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP⁺, and dimethylsulfoxide (DMSO) were purchased from Aldrich-Sigma Chemical Co. (St. Louis, MO). RNase A, DMEM/F12 and Protease K were purchased from Fisher Scientific (Pittsburgh, PA). The mammary epithelial cell (MEC) line from a *lacI* (BigBlue™) Fisher 344 rat was kindly provided by David Josephy (University of Guelph, Guelph, Canada). The preparation of this line has been described previously (20).

High-performance liquid chromatography (HPLC)

Normal and reverse phase HPLC were performed with a system consisting of two Waters Model 501 solvent delivery pumps (Waters Associates, Milford, MA) and a Model 680 automated gradient controller. Absorbance was monitored at 254 nm with a Waters Model 440 multiwavelength detector. Chiral stationary phase (CSP) HPLC was performed with a Shimadzu LC-10AD VP (Shimadzu Scientific Instruments, Columbia, MD) system equipped with Hitachi D-2500 chromatographic Integrator.

Resolution of (±)-1,2-DHD-6-NC by non-chiral stationary phase column—(±)-1, 2-DHD-6-NC was converted to bis(–)-menthyloxy esters with (–)-menthoxycetyl chloride and the pair of diastereomers was then separated by HPLC. The procedure for preparation of bis((–)-menthyloxy esters has been described previously (21). Briefly, to a solution of (±)-1, 2-DHD-6-NC (1 μmol) in anhydrous pyridine (300 μL) was added dropwise 1.5 equivalent (–)-menthoxycetyl chloride under ice bath temperature. The reaction mixture was stirred at 0–5° C for 12 h. The solvent was concentrated *in vacuo* and passed through a short silica gel column to produce essentially quantitative yield. The diastereomeric bisester was resolved by HPLC on a Lichrosorb silica 60 Å normal phase column (5 μm, 250 × 4.6 mm) using 10% ether in cyclohexane isocratic gradient.

Resolution of (±)-1,2-DHD-6-NC by chiral stationary phase column—(±)-1, 2-DHD-6-NC was resolved on chiral stationary phase column [(*S,S*)Whelk-O1, 5 μm, 250 × 4.6 mm, Regis Technologies Inc. Morton Grove, IL] which is based on 1-(3, 5-dinitrobenzamido)-1, 2, 3, 4-tetrahydrophenanthrene. The mobile phase is 4% isopropyl alcohol, 2% CH₂Cl₂ and 1% acetic acid in hexane at flow rate of 3 mL/min, isocratic gradient.

Measurement of UV circular dichroism (UV-CD) and optical rotatory dispersion (ORD)

UV-CD spectra of each enantiomer dissolved in methanol were obtained using a Jasco Model J-710 spectropolarimeter (Jasco Inc, UK) with a quartz cell of 1 cm path length at ambient temperature. The CD analyses were performed using a measurement range from 350 to 210 nm, a 2 nm bandwidth, 3 accumulations, and a 50 nm/min scanning speed. Before UV-CD measurement, UV spectrum was recorded on a spectrophotometer (Beckman Coulter, DU®640, Fullerton, CA). The spectrum was scanned in the wavelength range of 210–350 nm. The concentration of the enantiomer solution was adjusted to ensure the best quality of the UV-CD spectrum. CD spectra are expressed by ellipticity for a solution of 1.0 absorbance unit at a specific wavelength (238 nm) per ml of methanol. The measurement of ORD was performed as described previously (22).

Incubation of 6-NC with rat liver microsomes—6-NC (40 μmole in 5 mL DMSO) was incubated at 37°C for 1h in a 200 mL incubation medium. The incubation mixture consisted of 100 mM potassium phosphate buffer (pH 7.4), 3 mM of MgCl₂, 200 units of glucose-6-

phosphate dehydrogenase, 1 mM of NADP⁺, 4 mM of glucose-6-phosphate and 1 mg/mL protein equivalent of liver microsomes from Aroclor 1254-treated Sprague-Dawley rats (In Vitro Technology, Inc, MD). At the end of incubation, 6-NC and its metabolites were extracted with sequential additions of acetone (200 mL) and ethyl acetate (400 mL) and the organic phase was dried with anhydrous Na₂SO₄, filtered and concentrated *in vacuo*. The residue was dissolved in 5 mL of THF/methanol (1:1, v/v) and the major metabolite 1, 2-DHD-6NC (17) was isolated by HPLC using a reverse-phase Vydac C₁₈ semi-preparative column (Separations Group, Hesperia, CA). The elution solvent system is as follows: a linear gradient from 40% MeOH in H₂O to 55% MeOH in H₂O over 30 min and to 60% MeOH in H₂O over 15 min at a flow rate of 2 mL/min; the flow rate was then increased to 3.75 mL/min over 5 min with isocratic 60% MeOH in H₂O followed by a linear gradient from 60% MeOH to 100 % MeOH in 30 min at a flow rate of 3.75 mL/min. The retention time of 1, 2-DHD-6-NC was 32.2 min. The collected fractions containing 1, 2-DHD-6-NC were concentrated and characterized by UV (17). The collected 1, 2-DHD-6-NC metabolite was further resolved by either non-chiral or chiral stationary phase HPLC methods as described above.

Cell culture and treatment—The mammary epithelial cell (MEC) line derived from a *lacI* (BigBlue™) Fisher 344 rat was grown in DMEM/F12 (1:1) with 5% FBS. Cells were treated twice with each enantiomer, with a 3-day period between treatments.

Mutagenesis assay—After treatment, cells were processed according to previously published procedures (13). Briefly, DNA was extracted using a Recoverase kit (Stratagene, LaJolla, CA). Phage packaging was carried out using a phage packaging mix prepared from bacterial strains *E. coli* NM759 and BHB2688. The *cII* mutagenesis assay was then employed. In *E. coli* 1250, under specified conditions (25 °C) only mutants give rise to phage plaques, whereas at 37 °C all infected cells give rise to plaques, providing a phage titer. The ratio of mutant to non-mutant plaques is the mutant fraction (MF). For mutational spectra, mutants were cored from petri dishes and the agar plugs were mixed with 100 mL phage buffer and spread on selective plate to confirm mutant phenotype and purify mutant phages. The *cII* genes in the mutants were subjected to amplification by PCR and sequencing of the *cII* gene was performed as described previously (13) by Roderick Haesevoets, University of Victoria, B.C., Canada.

Results and Discussion

Resolution of (±)-1, 2-DHD-6-NC

The enantioselective HPLC separation of racemic (±)-1, 2-DHD-6-NC was achieved by both non-chiral stationary phase and chiral stationary phase columns. Figure 1A and 1B (Top Panels) show representative HPLC traces of enantiomeric separation of the synthetic racemic (±)-1,2-DHD-6-NC by non-chiral and chiral stationary phase HPLC methods, respectively. Figure 1A and 1B (Bottom Panels) show representative HPLC traces of enantiomeric 1, 2-DHD-6-NC metabolites catalyzed by S-9 (details are described below). The retention times (min) and absolute configurations are also indicated in the figures. For non-chiral stationary phase HPLC method, the racemic (±)-1,2-DHD-6-NC was converted quantitatively to a mixture of bis-(–)-menthyloxyacetyl ester (21) which were then subjected to HPLC separation.

Enantiomers of oxygenated derivatives of a large number of PAH have been resolved by HPLC using Pirkle's chiral stationary phase columns (23). The resolution of enantiomers by chiral stationary phase column depends on a number of parameters such as column stationary phase, mobile phase composition, mobile phase pH, flow rate, and injection volume, which need to be carefully optimized. Based on a test of a total of 8 columns, the Pirkle-Concept Whelk-O1 chiral column was chosen in this study. The chiral stationary phase of this column is based on

1-(3, 5-dinitrobenzamido)-1, 2, 3, 4-tetrahydrophenanthrene and belongs to π -electron acceptor/ π -electron donor class. In the earlier studies, the majority of elution order-absolute configuration relationships of resolved enantiomers have been established. Consistent with the results obtained from chrysene *trans*-1,2-dihydrodiol and other dihydrodiol enantiomers (24), the [*S,S*]-enantiomer is less retained by the chiral column than the [*R,R*]-enantiomer. Our results also showed that the elution order of dihydrodiol enantiomers on chiral column were opposite to that obtained from bis-($-$)-menthyloxyacetyl ester derivatives on non-chiral column; these results are in line with those reported for other dihydrodiols derived from numerous PAH (25).

Absolute configurations of enantiomeric 1, 2-DHD-6-NC

Determination of absolute configuration of enantiomeric 1, 2-DHD-6-NC is based on the assumption that compounds of similar structure exhibit similar optical characteristics. Thus, the absolute structures of the separated enantiomers can be estimated by comparing experimental data obtained by chiral spectral methods with those spectra obtained from compounds possessing similar structures with known configuration. The UV-CD method is suitable for chiral compounds containing at least one chromophoric group, which is the case here. Therefore, the UV-CD method was used in this study for the estimation of the absolute structure.

The absolute configurations of chrysene *trans*-1, 2-dihydrodiol have been identified previously by Yang et al (24). Both 1,2-DHD-6-NC and chrysene *trans*-1, 2-dihydrodiol share the same chrysene backbone where their chiral center resides; the only difference in their structures is the residual nitro functional group which is far removed from the chiral center and does not contribute to their overall chirality. Assuming that the nitro group does not affect the overall UV-CD behavior of the whole compounds, their UV-CD spectra should look very similar. According to this hypothesis, we compared the UV-CD of these two analogs and assigned their absolute configurations as ($-$)-[*R,R*]- and ($+$)-[*S,S*]-1, 2-DHD-6NC. The ORD spectra of the two enantiomers were also obtained to confirm the assignment (data not shown). The ($-$)-[*R,R*]- and ($+$)-[*S,S*]-1, 2-DHD-6-NC showed a pair of nearly symmetric CD spectra (Figure 2).

Metabolism of 6-NC by rat liver microsomes and the enantiomeric composition of its metabolite ($\pm$)-1, 2-DHD-6-NC

The metabolism of 6-NC by liver microsomes from Aroclor 1254-treated Sprague-Dawley rats was examined under conditions optimal with respect to protein concentration, substrate concentration and incubation time as described earlier (17). The major metabolite obtained under the experimental conditions was ($\pm$)-1, 2-DHD-6-NC which accounted for nearly 60% of the total organic extractable metabolites (17). The incubation mixture was separated by HPLC using a reverse-phase Vydac C₁₈ semi-preparative column (Separations Group, Hesperia, CA). The fractions containing ($\pm$)-1, 2-DHD-6-NC were collected (retention time = 32.2 min) and the enantiomeric composition of ($\pm$)-1, 2-DHD-6-NC was determined using both non-chiral and chiral stationary phase HPLC methods as described above. Figure 1 (A and B, Bottom Panels) show the representative HPLC traces of ($\pm$)-1, 2-DHD-6-NC metabolite resolved by non-chiral and chiral stationary phase, respectively. The area under the curve corresponding to each enantiomer was determined. Our results showed that the two enantiomers generated by the metabolism of 6-NC by liver microsomes from Aroclor 1254-treated male rats of Sprague-Dawley, yield 88% ($-$)-[*R,R*] and 12% ($+$)-[*S,S*] enantiomers.

The stereochemical course of diol formation from a parent PAH is likely to be determined by several factors such as stereoselectivity of microsomal cytochrome P450 in the metabolic epoxidation of the double bond to produce arene oxides, the stereoselectivity of the epoxide

hydrolase-mediated hydration of arene oxide to *trans*-dihydrodiol and spontaneous hydration and/or racemization of the arene oxides. Several studies have shown that the degree of enantiomeric purity of the dihydrodiol metabolites formed depended on the source of the metabolizing system and the structure of the parent PAH compound (16). In contrast to other chrysene dihydrodiols, the stereoselective epoxidation reactions at the 1, 2-double bond of chrysene have been shown to depend on the types of inducers used to treat the rats (24,26). Studies have also suggested that the differences in the optical purity of metabolically formed chrysene *trans*-1, 2-dihydrodiol are more likely due to different contents of cytochrome P450 isozymes present in various liver microsomal preparations. In contrast, epoxide hydrolases contained in liver microsomes from various preparations have identical catalytic function with respect to the hydration of an enantiomeric epoxide to dihydrodiol with a certain degree of optical purity (27,28). Aroclor 1254-induced rat liver homogenate supernatant (liver S-9) has been routinely used as an exogenous metabolic activation system for the evaluation of mutagenicity of xenobiotics. Aroclor-1254 is known to induce the activity of various cytochrome P450 isozymes including CYP1A2 which appears to play a major role in the metabolism of 6-NC to 1, 2-DHD-6NC by human liver microsomes (29,30). However, the effect of different types of cytochrome P450 inducers from various species on the stereoselective metabolism of 6-NC to 1, 2-DHD-6-NC require additional studies.

Mutagenesis assay

Since one of our goals of this study was to evaluate the effect of stereochemistry on the mutagenicity of 1, 2-DHD-6-NC, we attempted to determine which of the enantiomers had a mutational profile most similar to that of 6-NC. The mutation fractions (MF) and mutational profiles of [*R,R*] and [*S,S*]-enantiomers were examined in the *cII* gene of *lacI* mammary epithelial cells (MEC) *in vitro*. Mutation was divided into seven classes consisting of the six possible base pair substitutions and insertions/deletions. Our results showed that [*R,R*]-isomer is a significantly ($p < 0.01$) more potent mutagen compared to the [*S,S*]-isomer; the MF level in [*R,R*]-enantiomer increased ~2.7-fold than [*S,S*]-enantiomer at dose 0.25 μ M, 3 fold at dose 0.5 μ M and 2.8 fold at dose 0.75 μ M (Figure 3). Although the mutagenic potency of the [*R,R*]-isomer was several fold higher than that of [*S,S*]-isomer, the mutational spectra produced by these two enantiomers were quite similar (Figure 4). The major types of mutation induced by the [*R,R*]- enantiomer are AT > GC, AT > TA and GC > TA substitutions; the mutation spectra of the [*S,S*]- isomer was similar to the [*R,R*]-isomer, but a lower percentage of AT > GC mutations was observed. The mutation spectra of [*R,R*]- and [*S,S*]-enantiomers are similar to those obtained from the mammary gland of rats treated with 6-NC (Figure 4).

The structures/conformations of PAH-DNA adducts have been thought to influence the mutagenic patterns of these so-called “bulky” carcinogens, in addition to host biological factors (31,32). In this study, we presumed that upon using the same biological systems (such as *lacI* mammary epithelial cells used here) the varied mutagenic patterns induced by these two enantiomers will result from the different stereochemistry between them. We predicted that the (–)-[*R,R*]- and (+)-[*S,S*]-1,2-DHD-6NC would undergo further nitroreduction to their corresponding (–)-[*R,R*]- and (+)-[*S,S*]-1,2-DHD-6-NHOH-C which can react with DNA in a stereoselective manner or produce different DNA adduct conformations accountable for their varied mutagenic patterns. Like certain stereoisomeric PAH metabolites (33,34), our results also showed that the (–)-[*R,R*]- and (+)-[*S,S*]-1,2-DHD-6-NC exhibited similar mutational spectra. In fact we predicted that the enantiomers possess similar mutational profiles which may result from the fact that the binding of the isomers to the necessary metabolic enzymes occurs at the chiral centers, but the reaction of the proximate mutagen with DNA occurs at the nitrogen, which is further removed from the chiral center of 1, 2-DHD-6-NC. Nevertheless, the significant higher mutation fraction of (–)-[*R,R*]- than (+)-[*S,S*]-enantiomer indicated an important role of stereochemistry in the mutagenicity of this compound.

Based on the results of the present study that *[R,R]*-1,2-DHD-6-NC was the major isomer formed in Aroclor-1254-induced rat liver microsomes as well as the assumptions that the more mutagenic isomer would contribute more to the overall mutagenicity of 6-NC, we hypothesize that *[R,R]*-1,2-DHD-6-NC is the proximate carcinogen of 6-NC in the rat mammary gland *in vivo*. Ongoing studies in our laboratory are testing this hypothesis. Whether the stereoselectivity will be retained when the optically active (–)-*[R,R]*- and (+)-*[S,S]*-1,2-DHD-6-NC are further metabolized to the corresponding putative ultimate carcinogenic hydroxylamino metabolites will be assessed in future studies.

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Abbreviations

NO ₂ -PAH	nitropolynuclear aromatic hydrocarbons
6-NC	6-nitrochrysene
6-AC	6-aminochrysene
N-OH-6-AC	N-hydroxy-6-aminochrysene
1	2-DHD-6-NHOH-C
<i>trans</i> -1	2-dihydroxy-1,2-dihydro-N-hydroxy-6-aminochrysene
1	2-DHD-6-NC, <i>trans</i> -1, 2-dihydroxy-1, 2-dihydro-6nitrochryse
1	2-DHD-6-AC, 1,2-dihydroxy-6-aminochrysene

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Fig 1.

Representative HPLC traces of (±)-1, 2-DHD-6-NC resolved by (A) non-chiral stationary and (B) chiral stationary phase HPLC methods. **A:** (±)-1, 2-DHD-6-NC was converted to bis(-) menthyloxy esters with (-)-menthoxyacetyl chloride followed by HPLC separation on a Lichrosorb silica 60 Å normal phase column (5 µm, 250 × 4.6 mm) using 10% ether in cyclohexane isocratic gradient mobile phase. The top panel represents the synthetic (±)-1, 2-DHD-6-NC and the bottom panel represents the (±)-1, 2-DHD-6-NC metabolite obtained from 6-NC using rat liver S-9 fraction. **B:** 1, 2-DHD-6-NC was resolved by chiral stationary phase [(S,S)Whelk-O1, 5 µm, 250 × 4.6 mm, Regis Technologies Inc. Morton Grove, IL] using 4% isopropyl alcohol, 2% CH₂Cl₂ and 1% acetic acid in hexane at flow rate of 3 mL/min, isocratic gradient. The top panel represents the synthetic (±)-1, 2-DHD-6-NC and the bottom panel represents the (±)-1, 2-DHD-6-NC metabolite obtained from 6-NC using rat liver S-9 fraction.

Fig 2.
CD spectra of (-)-[R,R]- (—) and (+)-[S,S]- (----)-1,2-DHD-6-NC in methanol. The vertical axis is expressed in ellipticity.

Fig 3.

Comparative mutation fraction (MF) of *[R,R]*- and *[S,S]*-1,2-DHD-6-NC in mammary epithelial cells *in vitro*. MF is expressed in units of mutants/ 10^5 plaque forming units (mean $\pm$ S.D., $n=3$). * $p < 0.01$.

Fig 4. Comparative mutagenic profiles of *[R,R]*- and *[S,S]*-1,2-DHD-6-NC in mammary epithelial cells (MEC) *in vitro* and 6-NC *in vivo*.

Scheme 1.
Metabolic activation pathway of 6-NC.