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Effects of Oral Administration of Non-genotoxic Hepato-hypertrophic Compounds on Metabolic Potency of Rat Liver

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It remains uncertain why non-genotoxic compounds that result in liver hypertrophy cause liver tumors. In an effort to resolve this issue, we examined whether liver post-mitochondrial fraction (S9) prepared from rats treated with non-genotoxic compounds affected the genotoxicity of pro-mutagens. Known hepatotoxic compounds, such as piperonyl butoxide (PBO), decabromodiphenyl ether (DBDE), beta-naphthoflavone (BNF), indole-3-carbinol (I3C) and acetaminophen (AA), were orally administered to male and female F344 rats at doses sufficient to cause liver hypertrophy. Rats received diets containing each test compound for 3 days, 4 weeks or 13 weeks, and were then kept for 4 weeks without the test chemical. S9 prepared from the livers of each group was used for the Ames test with 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), benzo[a]pyrene (BaP) and N-nitrosodimethylamine (NDMA). In both sexes, liver hypertrophy was observed following administration of all test compounds, and was then reversed to the control state when administration ceased. The mutagenicity of MeIQx, BaP and NDMA increased with the use of S9 derived from rats treated with non-genotoxic compounds other than AA. DBDE administration had a marked effect on the mutagenicity of BaP (over a 30-fold increase in females) and NDMA (about a 20-fold increase in males). To estimate the involvement of metabolic enzymes in the alteration of mutagenicity, we measured the activity of ethoxyresorufin- O-deethylase (EROD) and methoxyresorufin- O-demethylase (MROD) (phase I enzymes), and UDP-glucuronosyltransferase (UGT) and glutathione S-transferase (GST) (phase II enzymes) in each S9 sample. The activity of phase I enzymes increased, even at the 3rd day following administration, and then decreased gradually, except in the case of AA, while the activity of phase II enzymes increased slightly. These results suggest that non-genotoxic hepatohypertrophic compounds may be partly involved in carcinogenesis by modulating the metabolism of pre-carcinogens incorporated from the environment, in a manner that is dependent on sex and pre-incorporated chemicals.

Key words: liver hypertrophic compound, metabolism, mutation, Ames test

Introduction

We are surrounded by numerous environmental risk factors that not only include natural products, but also anthropogenic sources such as environmental pollutants, industrial chemicals and food additives. Large amounts of chemical products are produced every day. As a result, our health is affected in a manner corresponding to the combined impact of these factors, which includes the effects of genotoxic and non-genotoxic factors. The carcinogenic process might thus be affected by environmental stress, and this poses a serious problem for analyses involving environmental assessments. It has been reported that non-genotoxic hepatotoxic compounds induce liver tumors accompanied by hypertrophy. Numerous investigations have been conducted in an effort to elucidate the mechanisms involved with tumor formation by non-genotoxic compounds. Hepatotoxic compounds also frequently induce cytochrome P450 (CYP) gene expression in rodents (1–3), and the combined effects of environmental chemicals might lead to the modulation of carcinogen
metabolism, such as through changes in CYPs-activity (4) or the inhibition of detoxification. Although there are substantial reports detailing the involvement of CYP-gene or protein induction or suppression, few reports have dealt with investigations concerning changes observed in genotoxicity in the presence of liver fraction as a result of the effects caused by treatment with non-genotoxic hepatocarcinogens.

This study represents a unique trial designed to observe temporal changes in the liver during the administration of hypertrophic compounds. In this study, we used the Ames test to examine the liver S9 fraction. S9 was prepared from F344 rat liver following treatment with five hypertrophic compounds; piperonyl butoxide (PBO), decabromodiphenyl (DBDE), beta-naphthoflavone (BNF), indole-3-carbinol (I3C) and acetaminophen (AA). These compounds are known as inducers of both CYP, and phase II detoxification enzymes. PBO, a domestic insecticide and a pesticide synergist that is widely used along with pyrethroids as a grain protector, is hepatocarcinogenic in rats (5) and mice (6), and induces some CYP family members (7). DBDE is a widely used brominated flame retardant, and humans are exposed to this compound through the ingestion of food, the living environment and occupational settings. The Environmental Protection Agency (EPA) performed a toxicological review of DBDE and reported that CYP is induced (8). Bruchazier et al. showed that DBDE administration induces CYP1A/2B (9). BNF, a synthetic derivative of naturally occurring flavonoids, is a well-known tumor promoter and acts through the induction of CYP1A enzymes via aryl hydrocarbon receptor activation (10,11). I3C, an autolysis product of the abundant glucobrassicin in cruciferous vegetables, has been widely investigated (12). Investigations have shown that I3C induces phase I and phase II metabolic enzymes in various species (13,14), and in neonatal rats exposed transplacentally (15). Furthermore, chemopreventive effects on tumor incidence have been shown in animal models, due either to the modulation of xenobiotic-metabolizing enzymes, such as phase I and phase II enzymes, or electron-scavenging activity (16). AA is widely used as an inexpensive, effective and over-the-counter nonsteroidal analgesic-antipyretic drug. It is relatively safe at therapeutic doses, but produces fatal hepatotoxicity when taken in excess doses (17).

Hepatic phase I and phase II enzymes play an important role in the metabolism of xenobiotics in mammals. Phase I enzymes, typically CYP1A, 2A and 3A, catalyze alterations to the structure and physiochemical properties of xenobiotics. These transformed compounds often retain bioactivity and may, in some cases, manifest increased reactivity with cellular components such as DNA. In contrast, phase II enzymes, including UDP-glucuronosyltransferase (UGT), sulphotransferase, acetyltransferase and glutathione S-transferase (GST), act through conjugation and subsequent alterations to the structure and physiochemical properties of metabolites produced by phase I enzymes, generally resulting in reduced bioactivity. In this study, we measured the activity of ethoxyresorufin-O-deethylase (EROD), methoxyresorufin-O-demethylase (MROD), UGT and GST in liver S9 used in the Ames test, as the level of mutagenic activity is thought to reflect the intricate metabolism of xenobiotics.

Mutation profiles of 2-amino-3,8-dimethylimidazo [4,5- f]quinoline (MeIQx), benzo[a]pyrene (BaP) and N-nitrosodimethylamine (NDMA) varied depending on sex and the nature of the administered compound. In most cases, the mutagenicity increased in the presence of S9 from treated rats and phase I enzyme activity was also enhanced. Changes in mutagenicity and enzyme activity were observed according to the profile of the hypertrophic state following administration of test compound. Changes in potency in terms of increased mutation, which may lead to cancer, appear to be caused by the modulation of metabolic enzyme activity.

Materials and Methods

Chemicals: Piperonyl butoxide (PBO) [51–03–6] and decabromodiphenyl ether (DBDE) [1163–19–5] were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Beta-naphthoflavone (BNF) [6051–87–2] was obtained from Tokyo Kasei Kogyo (Tokyo, Japan), and indole-3-carbinol (I3C) [700–06–1] and acetaminophen (AA) [103–90–2] were purchased from Tokyo Kasei Kogyo Ltd. (Osaka, Japan). Beta-naphthoflavone (BNF) was prepared from F344 rat liver following treatment with five hypertrophic compounds. PBO, a synthetic derivative of naturally occurring flavonoids, is a well-known tumor promoter and acts through the induction of CYP1A enzymes via aryl hydrocarbon receptor activation (10,11). I3C, an autolysis product of the abundant glucobrassicin in cruciferous vegetables, has been widely investigated (12). Investigations have shown that I3C induces phase I and phase II metabolic enzymes in various species (13,14), and in neonatal rats exposed transplacentally (15). Furthermore, chemopreventive effects on tumor incidence have been shown in animal models, due either to the modulation of xenobiotic-metabolizing enzymes, such as phase I and phase II enzymes, or electron-scavenging activity (16). AA is widely used as an inexpensive, effective and over-the-counter nonsteroidal analgesic-antipyretic drug. It is relatively safe at therapeutic doses, but produces fatal hepatotoxicity when taken in excess doses (17).

Animal treatment and S9 preparation: Male and female specific-pathogen-free Fischer 344/DuCrj (F344) rats aged 4 weeks were purchased from Charles River Japan Inc. (Tokyo, Japan). In this study, two independent experiments were performed using different compounds. In each experiment, rats were randomly allocated to 3 or 4 groups, including a group that did not receive chemicals during the course of the experiments, each consisting of 5 males and 5 females. Animal protocols were reviewed and approved by the Animal Care and Use Committee of the National Institute of Health.
Effects of Rat-S9 Treated with hHypertrophic Compounds

Sciences, Japan. Five-week-old rats were fed a pelleted diet containing a test compound at a dose capable of inducing liver hypertrophy for 13 weeks, and were then maintained for 4 weeks under a diet without the compound. We used PBO at 20,000 ppm and DBDE at 50,000 ppm in the first experiment, and BNF at 5,000 ppm, I3C at 2,000 ppm and AA at 12,500 ppm in the second experiment. Livers were dissected at 3 days, 4 weeks and 13 weeks following the commencement of chemical administration, and at 4 weeks after chemical administration had ceased. Hepato-hypertrophy was estimated by calculating the relative liver weight (g/100 g body weight). Part of the dissected liver was pooled within the same group, and pooled sections were homogenized in 3 volumes of ice-cold 0.15 M KCl and centrifuged at 9000 \( \times \) g for 10 min at 4°C. This supernatant fraction (S9) was immediately frozen and kept at -80°C until subsequent use.

**Ames test:** The ability of S9 to transform indirect mutagens to reactive direct mutagens showing mutagenicity was examined using the Ames test with a pre-incubation procedure, as previously described (18). A tester strain of *Salmonella typhimurium* TA98 was used for the assay with MeIQx and BaP, and TA100 was used for NDMA. Bacteria were incubated for 30 min at 37°C with the mutagen and S9 mix containing 10% S9 in 0.15 M sodium phosphate buffer (pH 7.4), 15 mM MgCl\(_2\), 8 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADPH, 4 mM NADH and 5 mM ATP, and poured onto a minimal glucose agar plate (in triplicate). After plates were kept at 37°C for 48 h, the number of revertant colonies on each plate was counted.

**Measurement of enzyme activity:** CYP 1A activity in S9 was determined by measuring the generation of resorufin from ethoxy- and methoxyresorufin, as previously described (19). UGT activity was determined by measuring the reduction in absorbance at 405 nm of p-nitrophenol released by the enzyme reaction, and GST activity was determined by measuring the increase in absorbance of glutathione-conjugate with CDNB substrate, as previously reported (20). Protein concentration was determined using the Bradford method (21).

**Results**

**Toxicity and hypertrophy:** Temporal changes in body-weight and relative liver weight of rats during the experiment are shown in Fig. 1. Based on changes in body weight, almost all compounds were toxic, whereas BNF and I3C were not toxic in male rats. On the other hand, hypertrophy was observed with all compounds as
relative liver weights increased significantly following treatment with every compound, and the effects of PBO were particularly marked. The observed hypertrophic effects were similar in both males and females, and were reversed when administration ceased, except in the case of PBO and DBDE.

**Mutagenicity of MeIQx, BaP and NDMA in the presence of S9 from rats treated with hypertrophic compounds:** The mutagenicity increased in a dose-dependent manner in most experiments. Mutagenic activity is shown as the number of revertants per pmole of MeIQx, per nmole of BaP, and per μmole of NDMA, as calculated from the slope of the dose-response curve (Table 1). S9 fractions from non-treated rats exhibited similar mutagenicity in the case of MeIQx and NDMA in both males and females. In contrast, the mutagenicity of BaP was 5 to 10 times higher when male S9 from non-treated rats was used, compared with female S9. Additionally, the ability of S9 from non-treated male rats to show BaP mutagenicity changed during the experimental period, unexpectedly increasing until 13 weeks and then decreasing, while the ability of female S9 remained at low levels throughout the experiment. In an effort to compare the mutagenicity displayed by the prepared S9 fractions, the relative mutagenicity is shown as a ratio of the mutagenicity displayed by treated S9 vs. the mutagenicity displayed by non-treated S9 (Fig. 2). We confirmed that enhancing effects are present when the ratio is greater than two.

The mutagenicity of MeIQx was 2 to 4 times higher following treatment with test compound when male S9 from PBO, DBDE, BNF or I3C-treated rats was used. The elevated mutagenicity was largely maintained during administration, and then declined to levels seen in untreated rat S9 when administration had ceased, except in the case of DBDE. In contrast, female S9 from compound-treated rats showed little effect on the mutagenicity of MeIQx.

The effects of S9 on the mutagenicity of BaP differed markedly between males and females. As shown in Fig. 2, S9 activity in BaP-induced mutations in male rats was seldom elevated by treatment with compounds, while the activity was slightly elevated by treatment with DBDE. On the other hand, S9 from female rats treated with DBDE and PBO strongly enhanced BaP mutagenicity, while treatment with BNF or I3C resulted in slight enhancement. The potency for elevating mutations in S9 decreased when administration had ceased, while that from DBDE-treated rats continued to increase during the entire course of the experiments.

The mutagenicity of NDMA was negligible using S9 from non-treated rats. As S9 from non-treated female
rats did not have the potency to show mutations in experiment 1, the relative activities with PBO and DBDE could not be calculated, although the mutation rates were elevated. Generally, the mutation induced by NDMA with PBO-, DBDE- and 3IC-treated S9 exhibited similar increases in both males and females (Table 1 and Fig. 2). Elevated activities decreased markedly to untreated levels when administration had ceased, even
in the case of DBDE, while the effects of which continued in the case of MeIQx and BaP. BNF and AA showed no effect on S9 activity with NDMA.

**Effects of oral administration of hypertrophic compounds on phase I and II enzyme activity in liver S9:**
In an effort to estimate factors responsible for the changes in S9 activity, we measured the activity of EROD and MROD (phase I enzymes) (Table 2), and UGT and GST (phase II enzymes) (Table 3). The CYP1A family comprises inducible enzymes that play a role in the metabolism of xenobiotics. The results shown in Table 2 reveal that administration of almost every test compound significantly enhanced MROD and EROD activity in a similar manner in males and females, although administration of AA suppressed this activity. In general, enzyme activities were highest in S9 from rats treated for 3 days to 4 weeks, and then decreased gradually to control levels, except for the case of DBDE, where activity was maintained even after administration had ceased. The phase II enzyme UGT was generally enhanced in a similar manner in males and females. DBDE-treated rat S9 showed the highest activities after 4 and 13 weeks. GST activity increased slightly but significantly in all S9 samples.

**Discussion**
It remains uncertain why non-genotoxic liver hypertrophic compounds cause liver tumors in rodents. It is widely thought that environmental stress leads to an elevated cancer risk in humans. Environmental stress includes both genotoxic factors and non-genotoxic factors associated with foods, drugs, and environmental components such as water, soil and air. Humans are unavoidably exposed to the combined action of these factors. We believe that non-genotoxic compounds induce carcinogenesis due to combined effects consisting of large amounts of non-genotoxic compounds and small amounts of genotoxic compounds incorporated as contaminants. To investigate the combined effects in relation to liver tumors, we examined whether the potential of a liver fraction to activate carcinogens/mutagens is affected by treatment with a hypertrophic compound. To this end, the Ames test was employed in the presence of S9 from non-genotoxic hypertrophic compound-treated rats; i.e., we scored the number of revertants induced by indirect mutagens and observed changes in the mutagenicity with administration time. F344 rats were fed PBO, DBDE, BNF, 13C or AA at high doses. S9 was prepared from dissected liver after 3 days, 4 weeks and 13 weeks following administration of the compound, and the genotoxicity of indirect mutagens, such as MeIQx, BaP and NDMA, was determined by the Ames test using the S9 fractions prepared above. Additionally, we measured changes in enzyme activity in the

<table>
<thead>
<tr>
<th>Compound</th>
<th>Period of treatment</th>
<th>UGT (pmol/min/mg protein)</th>
<th>GST (nmol/min/mg protein)</th>
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<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>None</td>
<td>3 days</td>
<td>36 ± 10</td>
<td>39 ± 17</td>
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<td>4 weeks</td>
<td>34 ± 7</td>
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<tr>
<td></td>
<td>17 weeks</td>
<td>34 ± 5</td>
<td>36 ± 8</td>
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<tr>
<td>PBO</td>
<td>3 days</td>
<td>50 ± 11*</td>
<td>33 ± 10</td>
</tr>
<tr>
<td></td>
<td>4 weeks</td>
<td>85 ± 8**</td>
<td>64 ± 12**</td>
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<td></td>
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<td>77 ± 2**</td>
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<tr>
<td></td>
<td>17 weeks</td>
<td>91 ± 5**</td>
<td>66 ± 7**</td>
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<tr>
<td>DBDE</td>
<td>3 days</td>
<td>39 ± 10*</td>
<td>63 ± 7**</td>
</tr>
<tr>
<td></td>
<td>4 weeks</td>
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<td>115 ± 10**</td>
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<td></td>
<td>13 weeks</td>
<td>117 ± 3**</td>
<td>107 ± 9**</td>
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<tr>
<td></td>
<td>17 weeks</td>
<td>50 ± 9**</td>
<td>69 ± 5**</td>
</tr>
<tr>
<td>BNF</td>
<td>3 days</td>
<td>87 ± 2**</td>
<td>81 ± 10**</td>
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<tr>
<td></td>
<td>4 weeks</td>
<td>38 ± 5**</td>
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<tr>
<td></td>
<td>13 weeks</td>
<td>28 ± 4*</td>
<td>89 ± 14**</td>
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<td>17 weeks</td>
<td>16 ± 8</td>
<td>48 ± 5</td>
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<tr>
<td>13C</td>
<td>3 days</td>
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<tr>
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<td>17 weeks</td>
<td>45 ± 14</td>
<td>37 ± 12</td>
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</table>

Significance of differences relative to activity exhibited by no treatment (None) group was evaluated by Student’s t test. *P < 0.05 and **P < 0.01 for increase, and †P < 0.05 for decrease.
S9 fraction, namely that of EROD and MROD (phase I enzymes), and UGT and GST (phase II enzymes).

Results showed that the potency to activate mutagens was elevated by treatment of rats with the hypertrophic compounds tested, except for AA, and that the elevated potency continued during administration of the hypertrophic compounds and decreased when administration had ceased, except for DBDE.

AA treatment had no effect on S9 activity, in that the mutagenicity of the mutagens remained unchanged during the course of the experiments and enzyme activity remained constant or decreased. The NTP program confirmed that the carcinogenicity of AA was inconclusive in female F344 rats, and that no carcinogenic effects were observed in male rats (17). Our finding that the mutagenicity of indirect mutagen was not affected supports the notion that involvement of AA in carcinogenesis is either undetermined or negligible. Kim et al. reported that a subtoxic dose of AA induces CYP2E1 (22). Although CYP2E1 is known to be involved in the metabolism of NDMA (23), the mutagenicity of NDMA in our study was not affected by AA-S9. It is possible that the dose or administration-pathway employed in this study may have been responsible for the observed lack of CYP2E1 induction.

BNF has been widely investigated and is known to be a potent tumor promoter and inducer of CYPs (10). In our study, the activity of EROD and MROD was enhanced during the administration period (Table 2), in agreement with expression of CYP genes and proteins (to be published elsewhere). The mutagenicity profile could be accounted for by considering the enzyme activity, in that the mutagenicity of MeIQx and BaP increased with BNF-induced S9, but NDMA was not activated by CYP1A1/2 induced by BNF treatment.

PBO, DBDE and I3C treatment increased S9 activity with every mutagen used in this study. Although PBO was the most hypertrophic among the compounds examined in this study, DBDE had the greatest effect on mutagenicity and metabolic enzymes. Watanabe et al. reported that PBO induces a wide range of molecules of the CYP family, such as CYP1A1, CYP2B1/2, CYP3A and CYP4A, in F344 male rats, thus suggesting that PBO is a tumor promoter (24), and enhances the activity of UGT and GST. We observed elevated enzyme activities (Table 2), in addition to CYP gene and protein expression, and is in agreement with their report (to be published by Gamou et al.). On the other hand, Muguruma et al. showed that PBO enhances the expression of oxidative and metabolic stress-related genes such as CYP1A1, CYP2A5, CYP2B9, CYP2B10 and P450 oxidoreductase in mice, suggesting that PBO induces liver tumors via oxidative stress due to the production of ROS (7). Tasaki et al. also reported that PBO treatment induces oxidative stress and oxidative DNA damage in mice (25). The mutagenic profile presented in this study is in agreement with the profile of enzyme induction, in that the increased mutagenicity of MeIQx, BaP and NDMA decreased when administration of PBO had ceased. It is possible that PBO incorporated as an environmental contaminant enhances the risk of liver tumors through a combination of effects associated with the activation of enzymes and oxidative damage through enzymatic reactions. A synergistic effect between PBO and BaP has been reported in grass shrimp larvae (26). DBDE appeared to be the most effective at elevating BaP mutagenicity, and the increased mutagenicity was observed even after administration had ceased. MROD and EROD activities were enhanced by DBDE treatment even at 3 days, and remained at high levels after administration had ceased. In contrast, although enzyme activities were also enhanced by BNF, the activities decreased to control levels when administration had ceased. Elevated UGT activity, which is related to the metabolism of BaP (27), decreased when DBDE administration had ceased, although phase I enzyme activity was maintained after administration had ceased. This change in BaP metabolic enzymes appears to be related to the continuity of mutagenicity at high levels during the course of the experiment. The effect of DBDE on the mutagenesis of MeIQx and BaP was observed regardless of sex, while differences in the effect on the mutagenesis of NDMA were observed based on sex.

Manhães-Rocha et al. reported that Schistosoma infection modulated the expression of CYPs in mice in a strain- and gender-specific manner (28). Since the only CYP enzymes examined were MROD and EROD, the activities of which did not differ between males and females, the aforementioned gender-specific difference observed may be caused by an alteration of enzymes other than those examined in this study.

I3C is found in various cruciferous plants and has been widely investigated as a chemopreventive component through the activation of phase I and phase II enzymes (29). On the other hand, there have been reports suggesting that long exposure to I3C promotes carcinogenesis in rats (30). We observed that MROD, EROD and UGT enzyme activity was enhanced by I3C treatment and that mutagenicity was also elevated, except in the case of BaP with male S9. As the rate of elevated phase I enzyme activities was higher than that of UGT activity, I3C treatment might provide promoting effects on rats in this experiment.

As shown in Table 1, the mutagenicity of BaP was higher with male S9 than with female S9 from rats not treated with hypertrophic compounds. The higher levels of BaP mutagenicity may be related to sex-dependent CYP expression, similar to a report showing that expression of CYP2B and CYP2C is higher in male rat liver compared with female rat liver (31). Although we...
did not measure the activity of these CYP2 enzymes, it is considered that BaP might be constitutively metabolized by CYP2, in addition to being metabolized by CYP1A following induction. However, we were unable to determine the manner by which DBDE effectively elevated the potency by activating the premutagenic form of BaP to the corresponding mutagenic form, in spite of the similar activity of CYP1A induced by DBDE and PBO.

It appears that phase II enzymes play a much smaller role in the changes in mutagenicity observed in this study, as their effects were slight in comparison with phase I enzymes, and were similar for each compound tested. We previously reported that these compounds regulate the expression of various genes involved in DNA damage and hormone receptors other than metabolic enzymes (32–35). However, we have yet to identify the genes linking hepatic potency and elevated mutations, and more specifically, we have yet to account for the fact that the most hypertrophic compound (PBO) was not the most effective compound (DBDE) in enhancing mutations.

Taken together, the present study suggests that non-genotoxic hypertrophic compounds have the potential to elevate the risk of carcinogenesis induced by environmental genotoxic compounds through various changes in metabolic pathways, such as phase I and phase II enzymes, endocrinal effects and the oxido-redox status of cells. These changes are effected when non-genotoxic compounds are incorporated with small amounts of contaminating genotoxic compounds.

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Conflicts of Interest: None of the authors have any conflicts of interest with this study.

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