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Cytotoxic and xenoestrogenic effects via biotransformation of *trans*-anethole on isolated rat hepatocytes and cultured MCF-7 human breast cancer cells

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Abstract

The metabolism and action of *trans*-anethole (anethole) and the estrogen-like activity of the compound and its metabolites were studied in freshly isolated rat hepatocytes and cultured MCF-7 human breast cancer cells, respectively. The incubation of hepatocytes with anethole (0.25–2.0 mM) caused a concentration- and time-dependent cell death accompanied by losses of cellular ATP and adenine nucleotide pools. Anethole at a weakly toxic level (0.5 mM) was metabolized to 4-methoxycinnamic acid (4MCA), 4-hydroxy-1-propenylbenzene (4OHPB), and the monosulfate conjugate of 4OHPB; the levels of 4OHPB sulfate and 4MCA reached approximately 20 and 200 μM within 2 hr, respectively, whereas that of free unconjugated 4OHPB was less than approximately 0.5 μM. At a moderately toxic concentration (1.0 mM), unconjugated 4OHPB reached approximately 10 μM, followed by abrupt loss of 3′-phosphoadenosine 5′-phosphosulphate (PAPS). Based on cell viability and adenine nucleotide levels, 4OHPB was more toxic than anethole and 4MCA. The addition of 2,6-dichloro-4-nitrophenol (50 μM), an inhibitor of sulfotransferase, enhanced the anethole-induced cytotoxicity associated with losses of ATP, PAPS, and 4OHPB sulfate, and symmetrically increased the unconjugated 4OHPB concentration. 4OHPB as well as diethylstilbestrol (DES) and bisphenol A (BPA), which are known xenoestrogenic compounds, competitively displaced 17β-estradiol bound to the estrogen receptor α in a concentration-dependent manner; IC_{50} values of these compounds were approximately 1×10^{-5}, 1×10^{-8} and 5×10^{-5} M, respectively. 4OHPB also caused a concentration (10^{-8} to 10^{-6} M)-dependent proliferation of MCF-7 cells, whereas neither anethole nor 4MCA (10^{-9} to 10^{-5} M) affected cell proliferation. However, at higher concentrations (>10^{-4} M), 4OHPB rather than anethole and 4MCA was cytotoxic. These results suggest that the biotransformation of anethole induces a cytotoxic effect at higher concentrations in rat hepatocytes and an estrogenic effect at lower concentrations in MCF-7 cells based on the concentrations of the hydroxylated intermediate, 4OHPB.

Keywords: Anethole; Metabolism; Xenoestrogen; Cytotoxicity; Hepatocytes; MCF-7 cells

1. Introduction

trans-Anethole (anethole; 1-methoxy-4-(1*E*)-1-propenylbenzene) is a benzene ring with a single methoxy group *para* to the double-bonded propenyl group, occurs naturally as a major component of the essential oils in fennel and star anise, and is also present in numerous plants such as dill, basil, and tarragon. The *trans* isomer is by far more abundant (>99%) than the *cis* isomer in natural oils [1]. It is widely used as a popular aniseed flavoring agent in a variety of confectioneries and in alcoholic and non-alcoholic beverages and has been used as an important ingredient of herbal medicines for thousands of years. Because its usage is so widespread, even if consumption in the diet is low, the potential toxicity of anethole has been studied *in vivo* and *in vitro* to assess its various toxicologic properties [2]. Anethole was not potently toxic in genotoxic, mutagenic, and immunotoxic studies and in some short- and/or long-term dietary studies. The liver was identified as a

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Abbreviations: BPA, bisphenol A; DCNP, 2,6-dichloro-4-nitrophenol; DES, diethylstilbestrol; DMSO, dimethyl sulfoxide; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*-(2-ethanesulfonic acid); 4MCA, 4-methoxycinnamic acid; 4OHPB, 4-hydroxy-propenylbenzene; PAPS, 3′-phosphoadenosine 5′-phosphosulphate.

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target of anethole-induced toxicity in rats and mice; oral administration of anethole (30–900 mg/kg/day) to rats and mice caused dose-related increases in liver weights accompanied by hepatocellular hypertrophy or hydropic hepatocytes [2]. In addition, it has been reported that anethole is well absorbed when administered via the oral route in rats and humans, undergoes extensive metabolism, and is excreted in the urine and that the metabolism of anethole proceeds via *O*-demethylation, ω-side-chain oxidation, and side-chain epoxidation [3–5].

Although many natural and man-made compounds are ubiquitous in the environment, little is known about the potential risks to humans of exposure to known xenoestrogens. Consequently, considerable attention has focused on dietary phytoestrogens such as flavonoids as well as synthetic aromatic compounds bearing hydroxyl groups as endocrine-disrupting chemicals exhibiting weak estrogenic activity in *in vivo* [6,7] and *in vitro* [8] bioassays. Recently, we have reported that the estrogenic activity is induced by biotransformation, either via the direct hydroxylation of aromatic compounds or via dealkylation subsequent to hydroxylation of the *para*-substituted group in aromatic compounds such as benzophenones, which are used as UV-absorbers and ingredients of pharmaceutical and agricultural chemicals [9,10]. The metabolic pathway and toxic effects of anethole have been studied *in vivo* [2–5] and/or *in vitro* [11], although the relationship between metabolism and estrogenic activity has not been clarified. The freshly isolated rat hepatocyte system abounds in various drug-metabolizing enzymes and their cofactors associated with phase I and phase II and is useful for the study intracellular target sites and temporal sequences leading to cell injury by chemicals and their metabolites [12]. In the present study, we first investigated the metabolism and action of anethole in isolated rat hepatocytes and then assessed the potential estrogenic activities of the compound and its intermediates using a competitive binding assay for recombinant human estrogen receptor α (ERα) and a proliferative assay of MCF-7 cells which are estrogen-responsive human breast cancer cells.

2. Materials and methods

2.1. Materials

The chemical compounds used were obtained from the following companies: *trans*-anethole, 4MCA and BPA (purities of >98%) from Tokyo Kasei Kogyo Co., Ltd.; 17β-estradiol (E_2), DES, PAPS, β-glucuronidase, sulfatase (type VI, β-glucuronidase-free *Aerobacter aerogenes*), HEPES and bovine serum albumin from Sigma Chemical Co.; 4OHPB (purity of >99%), DCNP and collagenase from Wako Pure Chemical Industries Ltd. All other chemicals were of the highest purity commercially available.

2.2. Isolation and incubation of hepatocytes

Male F344/DuCrj (240–260 g) rats were obtained from Charles River Japan Inc. and were housed in wire-bottom cages. The rats were allowed food (CE-2, Clea Japan Inc.) and water *ad libitum* before hepatocytes were prepared. Hepatocytes (10^6 cells/mL) were isolated by collagenase perfusion of the liver and suspended in Krebs–Henseleit buffer, pH 7.4, containing 12.5 mM HEPES and 0.1% albumin, as described previously [12]. All incubations were performed in rotating, round-bottomed flasks at 37°, under a constant flow of humidified carbogen (95% O_2/5% CO_2). Reactions were started by the addition of anethole and its metabolites dissolved in DMSO (final concentration, <1%). Corresponding control groups received an equivalent volume of DMSO. Aliquots of incubation mixture were taken at intervals to monitor cell death and the concentrations of intracellular adenine nucleotides, PAPS, and anethole and its metabolites.

In other experiments using DCNP as an inhibitor of sulfate conjugation [13,14], the inhibitor at a concentration of 50 μM was added to the hepatocyte suspension and preincubated for 15 min prior to the addition of anethole. Aliquots of incubation mixtures were taken after definite period (90 min) to determine these parameters described above.

2.3. Biochemical and morphological assays

Adenine nucleotides (ATP, ADP and AMP) and PAPS in hepatocytes were determined by a modification of HPLC, as described by Jones [15] and Uesugi *et al.* [16], respectively. Briefly, aliquots of cell suspension (0.5 mL) were deproteinized with perchloric acid (final concentration, 3.8%). The perchloric acid extracts were neutralized with 10 M KOH and centrifuged to remove insoluble potassium perchlorate. An aliquot (50 μL) of the neutralized supernatant was introduced into a HPLC system equipped with a reverse-phase TSK-gel ODS-120T column (4.6 mm i.d. × 250 mm, 5-μm particle size; Tosoh Co.) and a UV absorbance detector (260 nm). The mobile phase was 0.1 M potassium phosphate buffer (pH 6.0) and the flow rate was 1.5 mL/min. Adenine nucleotides were quantified by injection of known amounts of authentic ATP, ADP and AMP. For the determination of PAPS, an aliquot (20 μL) of the neutralized supernatant was injected into an ion-pair reversed-phase HPLC system equipped with an analytical Capcell pak C18 column (4.6 mm i.d. × 250 mm, 5-μm particle size; Shiseido Co.) equipped with a UV absorbance detector (260 nm). The mobile phase was 0.1 M triethylamine phosphate buffer (pH 7.5)/methanol (95:5, by volume), and the flow rate was 1.0 mL/min. Hepatocyte viability was assessed by Trypan blue (0.16%, w/v) exclusion under a light microscope, and initial cell viability in each experiment was more than 85%. The number of blebbed hepatocytes was counted by light microscopy and expressed as the percentage of

Trypan blue excluding cells which exhibited multiple surface protrusions.

2.4. Determination of anethole metabolites by HPLC

An equal volume of cold methanol was added to the cell suspension and the mixture was filtered through a membrane cartridge (pore size of cellulose membrane, 0.45 μm; 13 mm i.d. × 20 mm; Millex-SLHV, Millipore Co.). The eluate (20 μL) was injected onto the analytical Capcell pak C18 column equipped with a UV absorbance detector (260 nm). The mobile phase was methanol/0.1 M ammonium dihydrogen phosphate (60:40, by volume, pH 5.3), and the flow rate was 1.0 mL/min. Anethole, 4MCA and 4OHPB were identified by co-chromatography with these authentic compounds or by LC-ESI/MS. The recoveries for these compounds were checked by the addition of known amounts of authentic compounds to hepatocyte suspensions, and were more than 85%.

Conjugates derived from anethole in the cell suspensions were enzymatically hydrolyzed with β-glucuronidase or β-glucuronidase-free sulfatase and the hydrolytic products were identified and/or determined by HPLC. Briefly, aliquots of cell suspensions were treated with a cell disrupter (Sonifier Branson Sonic Power Co.) in ice-water for 20 s and then filtered through an ultrafiltration cartridge (limit of elution, molecular weight <5000; Ultrafree-MC centrifugal filter unit, Millipore Co.). The eluate adjusted to pH 4.5 with sodium acetate buffer was incubated with β-glucuronidase (about 500 U) or sulfatase (about 2 U) for 3 hr at 37° and then the mixture (40 μL) was injected into the HPLC system.

2.5. Identification of metabolites derived from anethole by mass spectroscopy coupled with HPLC

An equal volume of chilled methanol was added to the cell suspension and the mixture was centrifuged for 5 min at 200 *g*. The supernatant fraction was concentrated by solid-phase extraction using C_{18} Sep-Pak cartridges (Waters Co.), and was also subjected to negative electrospray ionization/mass spectroscopy (HPLC-ESI/MS). The mass spectra of anethole metabolites were obtained using a LC/MS system, consisting of a Waters mass spectrometer ZMD and a Waters Alliance 2690 pump (Waters Co.). The column was eluted with a linear gradient of acetonitrile–water (solvents modified with 1% (v/v) acetic acid); the gradient started at 30% acetonitrile and changed to 80% during 30 min and the flow rate was 0.2 mL/min. The MS spectrum was scanned from 100 to 500 Da in 1.0 s and produced with a corn voltage of 30 V.

2.6. Competitive binding assay

Competitive binding between 17β-estradiol and various compounds was determined using an estrogen-R(α) competitor screening kit (Wako Pure Chemical Industries Ltd.); the kit consists of recombinant human ERα coated on the bottom of 96-well multiwell plates and fluorescein-labeled 17β-estradiol as the competitor for the assay. DES and other compounds dissolved in DMSO were added to a reaction solution containing fluorescein-labeled 17β-estradiol, and the mixture (100 μL) was added into each well. The concentrations of the various compounds used in this study ranged from 5×10^{-10} to 5×10^{-4} M. After 2 hr of incubation at room temperature, the mixture, which contained free compounds or the 17β-estradiol unbound to ERα, in wells was aspirated and exchanged with the assay solution (100 μL). The concentration of fluorescein-labeled 17β-estradiol bound to ERα on the bottom was measured in a CytoFluor 4000 fluorescence plate reader (PerSeptive Biosystems Inc.) with filters set for 485-nm excitation and 535-nm emission. The results are expressed as percentages of the fluorescence values for the reaction solution without samples.

2.7. MCF-7 cell proliferation assay

MCF-7 cells, cultured human breast cancer cells, were purchased from American Type Culture Collection. Cells were cultured in phenol red free-RPMI-1640 medium, supplemented with 5% fetal calf serum (FCS), 15 mM HEPES, 50 U/mL penicillin, 50 μg/mL streptomycin and 10 ng/mL insulin at 37° with 5% CO_2 in air at saturating humidity, and were routinely passed at approximately 80% confluence. Prior to initiating experiments, cells were seeded in and attached to 96-well multiwell plates at 4×10^3 per well in 0.3 mL of RPMI-1640 medium, supplemented with 5% estrogen-free FCS. After 24 hr, the medium was replaced with the same volume of the above medium containing 17β-estradiol (1 nM) as a positive control, anethole or its metabolites: the concentrations of anethole and its metabolites used in this study ranged from 1 nM to 100 μM. Estrogen-free FCS was prepared using the dextran–charcoal procedure [17]. The cells were cultured for 6 days and cell numbers in each well were determined using a cell proliferation assay kit (cck-8; Dojindo Laboratories Co.); 20 μL of WST-8 solution, 5 mM 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt and 0.2 mM 1-methoxy phenazinium methylsulfate dissolved in 150 mM KCl, were added to wells containing 0.3 mL of medium with cells. After incubation for 2 hr at 37° in a humidified 5% CO_2 atmosphere, the absorbance of the well was read at 450 nm (reference wavelength, 650 nm) by a microculture plate reader (model 450; Bio-Rad Laboratories). The assay, a modified tetrazolium colorimetric assay, is based on metabolic reduction of WST-8 to its corresponding formazan and the coloration obtained is directly proportional to the number of viable cells. The coloration was found to be linear for up to 120 min after addition of the dye-containing medium, with cell numbers exceeding 3×10^4.

2.8. Statistical analysis

Statistical analysis was performed by one-way analysis of variance, followed by Dunnett's multiple comparison test. Statistical significance was assumed at $P < 0.05$.

3. Results

3.1. Toxic effects of anethole on rat hepatocytes

The incubation of rat hepatocytes with anethole (0.25–2.0 mM) caused a concentration- and time-dependent cell death accompanied by a depletion of intracellular ATP and total adenine nucleotides, and the formation of surface blebs, indicating cellular morphological damage (Fig. 1). Although the cells that were blebbing were not dead, assessed by Trypan blue uptake, they may be beginning to die. Anethole at >1.0 mM elicited a rapid depletion of total nucleotide pools, whereas the loss of ATP was reflected by concomitant increases in the levels of ADP and AMP (data not shown).

3.2. Metabolism of anethole in isolated rat hepatocytes

As shown by the HPLC elution profiles, anethole (retention time; RT, approximately 31 min) at concentration of 1.0 mM (Fig. 2) was converted to several metabolites during a 1.5-hr incubation. The metabolites eluted in peaks (a) and (c) were identified as 4MCA (RT, 3.3 min) and 4OHPB (RT, 9.5 min), which are known as metabolites of anethole in rat hepatocytes [11], by co-chromatography with both authentic compounds or by LC-ESI/MS. The metabolite eluted in peak (b) was enzymatically hydrolyzed with sulfatase, not with β-glucuronidase, to yield free 4OHPB, indicating that the metabolite was a sulfate of 4OHPB. Further, the conjugate was identified as a monosulfate by LC-ESI/MS (Fig. 3); the ESI/MS spectrum showed a base peak at *m/z* 212, corresponding to $M - H^-$. The MS spectrum of peak (b) also showed a fragment ion at *m/z* 132 (4OHPB $- H^-$), which corresponds to a loss of 81 (sulfate moiety; 81 amu), $M - SO_3H^-$. From these results, the metabolite of peak (b) was tentatively identified as a monosulfate conjugate of 4OHPB. The materials (RT < 3.2 min) eluted prior to 4MCA resisted hydrolysis by sulfatase and/or β-glucuronidase, because treatment of hepatocyte-extract with both enzymes produced neither new apparent peaks in HPLC elution profiles nor marked increases in the amounts of materials eluted subsequent to 4MCA excluding 4OHPB. In addition, metabolic intermediates derived from the side-chain epoxidation did not identify in the HPLC elution profiles.

To understand the metabolism of anethole, time courses for the change in levels of the compound and its metabolites in hepatocytes were investigated at a non (0.25 mM)-, a low (0.5 mM)- or a moderate (1.0 mM)-toxic concentration of anethole (Fig. 4). At the concentration of 0.5 mM, the levels of 4MCA and 4OHPB sulfate increased with time followed

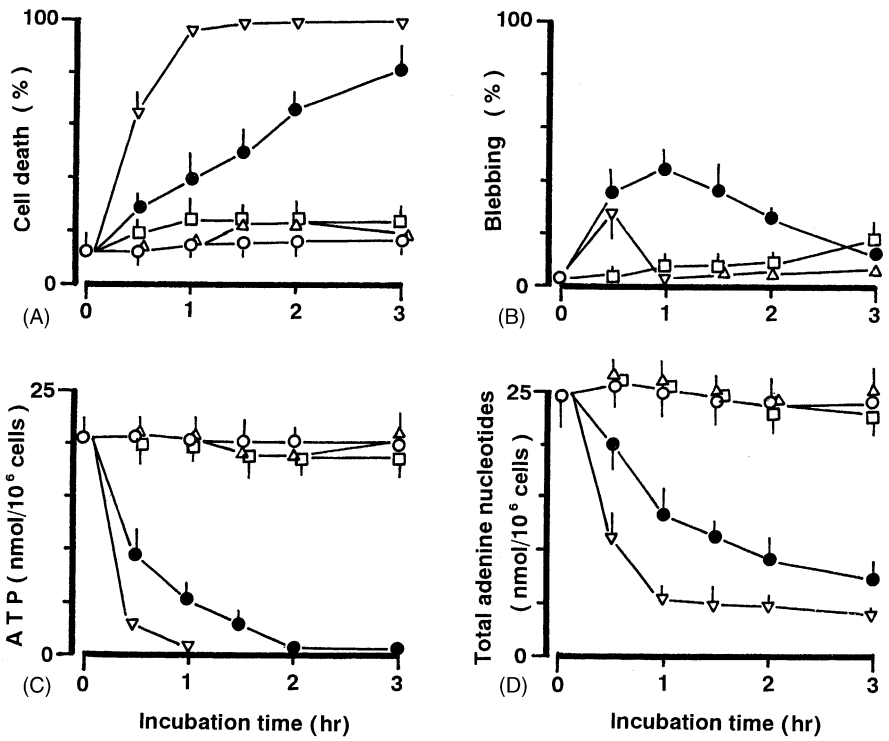

Fig. 1. Effects of anethole on cell death (A), blebbing (B), and intracellular levels of ATP (C) and total adenine nucleotides (D) in isolated rat hepatocytes. Hepatocytes were incubated at 10^6 cells/mL in Krebs–Henseleit buffer, pH 7.4, with no addition (○), 0.25 mM (Δ), 0.5 mM (□), 1.0 mM (●) and 2.0 mM (▽) as described in Section 2. Results are expressed as the means ± SE of three experiments.

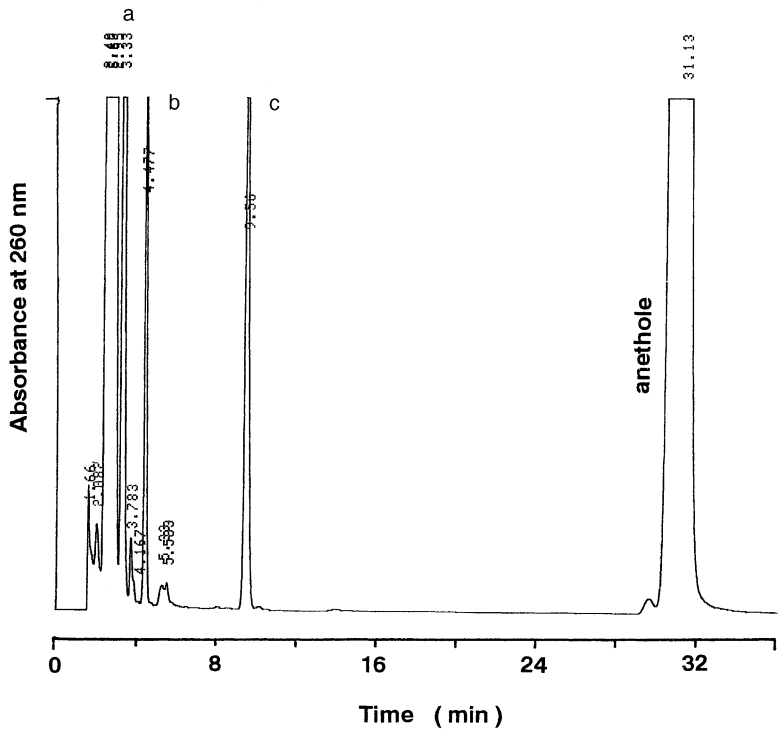

Fig. 2. Typical HPLC elution profiles of metabolites in hepatocyte (10^6 cells/mL) suspensions treated with 1.0 mM of anethole for 90 min. Peaks are 4MCA (a; retention time (RT), 3.3 min), sulfate of 4OHPB (b; RT, 4.4 min), 4OHPB (c; RT, 9.5 min) and anethole (RT, 31.1 min).

by a loss of anethole and reached approximately 200 and 20 μM within 2 hr, respectively, whereas free unconjugated 4OHPB was maintained at less than approximately 0.5 μM during the incubation. The time courses of changes in these metabolites were similar between 0.25 and 0.5 mM of anethole, the amount of each metabolite was dose-dependent. On the other hand, at 1.0 mM of anethole, the level of unconjugated 4OHPB reached an apparent steady-state at approximately 9 μM within 2 hr accompanied by an abrupt loss of activated sulfate PAPS, which is a essential cosubstrate in the sulfoconjugation reaction. The maximum concentrations of 4OHPB sulfate and 4MCA in hepatocytes treated with 1.0 mM anethole were less than those from 0.5 mM anethole. In a supplementary experiment, 4OHPB was sulfated by the rat liver cytosol fraction in the presence of PAPS (0.9 mM) and that the rates of sulfation derived from 4OHPB 0.5 and 1.0 mM after 30-min incubation at 37° were 54.2 and 53.4 pmol/min/mg protein, respectively. This indicates that the activity of sulfotransferase was not affected by the high concentration of 4OHPB.

3.3. Comparative cytotoxicity of anethole, 4OHPB and 4MCA in rat hepatocytes

The relative toxicities of anethole, 4OHPB and 4MCA at a concentration of 0.5 mM were compared by measuring

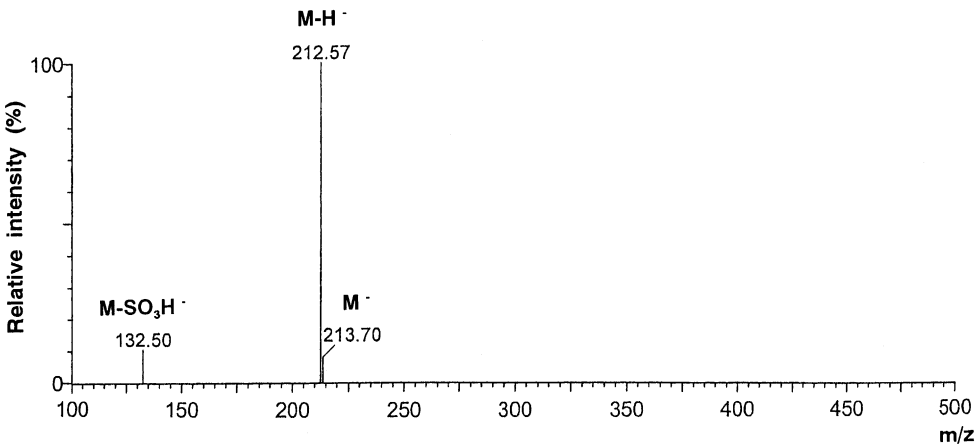

Fig. 3. Typical-ESI mass spectrum of peak (b) in Fig. 2.

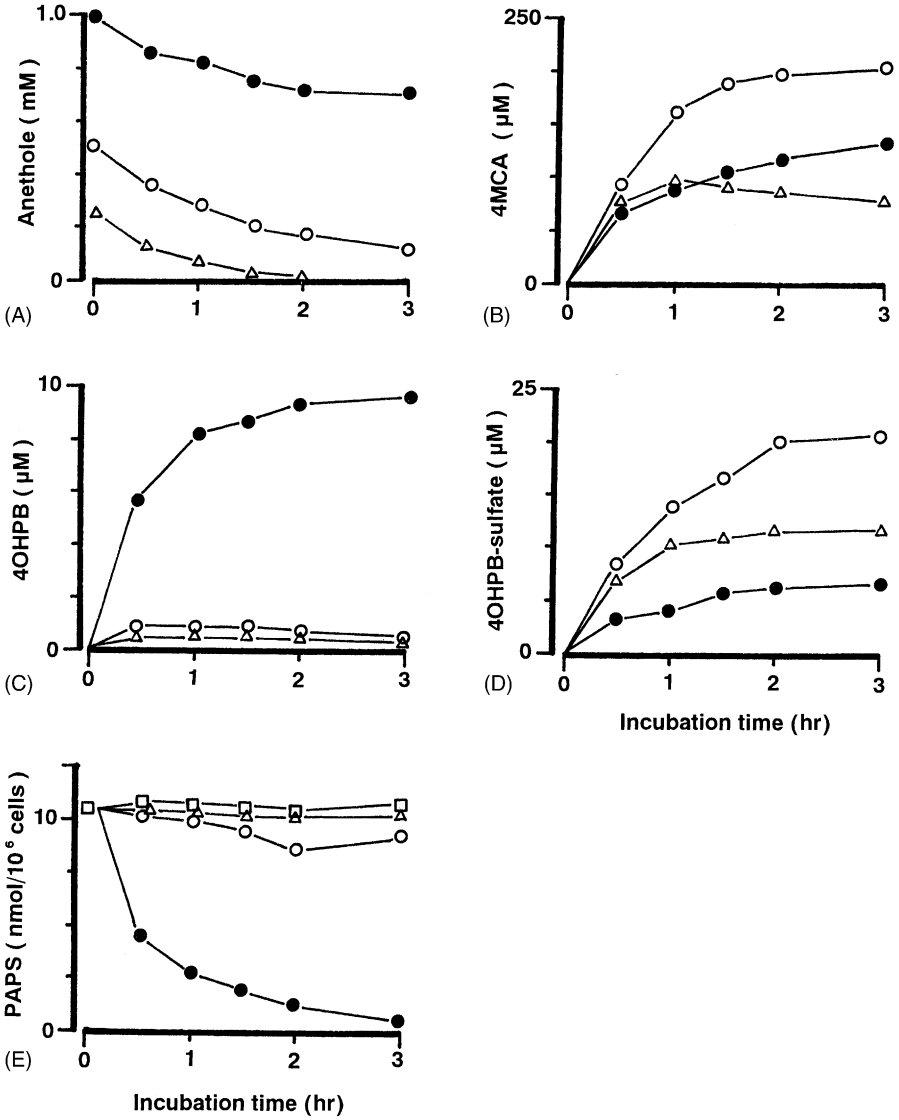

Fig. 4. Changes in the levels of anethole (A) and its metabolites; 4MCA (B), 4OHPB (C) and sulfate of 4OHPB (D) in rat hepatocyte suspensions. Hepatocytes (10^6 cells/mL) were incubated with 0.25 mM (Δ), 0.5 mM (○) and 1.0 mM (●) of anethole for 3 hr. (E) Effect of anethole on intracellular levels of PAPS; no addition (○), 0.25 mM (Δ), 0.5 mM (□), 1.0 mM (●). Results are the averages of two or three experiments.

their effects on cell killing and the levels of ATP and total adenine nucleotides in isolated rat hepatocytes (Table 1). After a 90-min incubation, 4OHPB caused a decrease in cell viability accompanied by losses of intracellular ATP and adenine nucleotide pools, whereas there were no significant differences in toxicity among untreated, anethole- and 4MCA-treated hepatocytes.

Table 1
Comparative cytotoxicity of anethole, 4OHPB and 4MCA in isolated rat hepatocytes

Treatments (0.5 mM)	Cell death (%)	ATP (nmol/10^6 cells)	Total adenine nucleotides (nmol/10^6 cells)
None (control)	19.7 ± 3.2	18.7 ± 3.0	23.3 ± 3.0
Anethole	20.1 ± 2.9	19.9 ± 1.9	20.9 ± 2.7
4OHPB	56.1 ± 5.5*	5.2 ± 0.8*	13.1 ± 3.0*
4MCA	18.9 ± 2.0	18.0 ± 2.2	22.8 ± 1.7

Hepatocytes were incubated with each compound (0.5 mM) in Krebs–Henseleit buffer at 37° for 90 min. The values of cell death, ATP and total adenine nucleotides are expressed as the means ± SE of three determinations.
* Significant difference from values for untreated (DMSO) hepatocytes ($P < 0.05$).

3.4. *Effect of a sulfotransferase inhibitor on anethole-induced cytotoxicity*

The effects of DCNP, an inhibitor of sulfotransferase, on metabolism and cytotoxicity induced by anethole were examined (Table 2). The pretreatment of hepatocytes with DCNP (50 μM) enhanced and accelerated anethole (0.5 or 1.0 mM)-induced cell killing as well as the loss of cellular ATP. The biotransformation of 4OHPB sulfate was inhibited by DCNP, whereas the amount of free unconjugated 4OHPB was increased. In addition, the cotreatment caused a dose-dependent loss of PAPS levels as well as the

Table 2
Effects of a sulfotransferase inhibitor (2,6-dichloro-4-nitrophenol; DCNP) on anethole-induced cytotoxicity in hepatocytes

Treatments		Cell death (%)	ATP (nmol/10^6 cells)	PAPS (nmol/10^6 cells)	4MCA (μM)	4OHPB (μM)	4OHPB-sulfate (μM)	Anethole (μM)
Anethole (mM)	DCNP (μM)							
–	–	19.6 ± 3.6	19.8 ± 3.0	10.3 ± 2.9				
–	50	18.5 ± 4.3	20.1 ± 2.8	11.3 ± 3.0				
0.5	–	20.8 ± 5.3	18.6 ± 2.9	7.7 ± 2.6	170	0.4	15.6	204
0.5	50	45.9 ± 4.0*,†	12.3 ± 2.0*,†	5.6 ± 2.1*	174	3.8	3.3	190
1.0	–	53.6 ± 7.6*	5.3 ± 2.8*	1.7 ± 1.6*	114	11.7	6.4	713
1.0	50	82.6 ± 4.3*,‡	0.4 ± 1.0*,‡	0.3 ± 0.2*,‡	128	19.9	0.4	726

Hepatocytes (10^6 cells/mL) were incubated in Krebs–Henseleit buffer at 37° for 90 min. DCNP (50 μM) was added to the hepatocyte suspensions 15 min prior to exposure of anethole (0.5 or 1.0 mM). Results of cell death, ATP and PAPS are expressed as the means ± SE of three experiments and those of anethole and its metabolites are expressed as the means of two experiments.

* Significant differences between untreated group and treated groups ($P < 0.05$).

† Significant differences between anethole (0.5 mM) group and anethole (0.5 mM) plus DCNP group ($P < 0.05$).

‡ Significant differences between anethole (1.0 mM) group and anethole (1.0 mM) plus DCNP group ($P < 0.05$).

depletion of ATP levels. Neither the levels of 4MCA and remaining anethole in hepatocyte suspension were affected by DCNP, nor the inhibitor itself affected any of the parameters: the cell viability and cellular levels of ATP and PAPS.

3.5. Competitive binding assay of anethole and its metabolites for estrogen receptor α (ERα)

To compare affinities among anethole, 4OHPB, 4MCA and known xenoestrogenic compounds, DES and BPA, to the estrogen receptor, the ability of these compounds to bind to ERα was assayed with that of fluorescein-labeled 17β-estradiol (Fig. 5). DES displaced with very high affinity fluorescein-labeled 17β-estradiol bound to ERα, while 4OHPB and BPA displaced in a competitive manner the 17β-estradiol bound to the receptor. Fifty percent inhibitory values (IC_{50}) of DES, 4OHPB and BPA were approximately 1×10^{-8}, 1×10^{-5} and 5×10^{-5} M, respectively. However, both anethole and 4MCA at concentrations from 10^{-9} to 10^{-4} M did not impair the binding of 17β-estradiol to ERα.

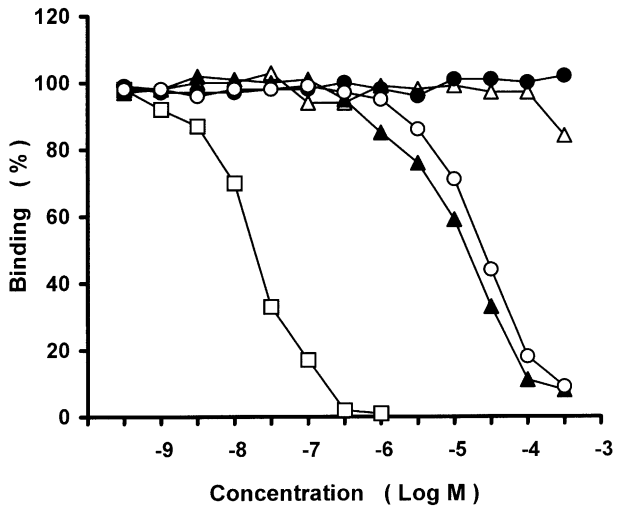

Fig. 5. Competitive binding assay of anethole and its metabolites, bisphenol A (BPA) and diethylstilbestrol (DES) for human recombinant estrogen receptor α (ERα). Ability of these compounds for binding to ERα was via competition with that of fluorescein-labeled 17β-estradiol. Results are expressed as the means from three determinations. Keys: anethole (●), 4MCA (Δ), 4OHPB (▲), BPA (○), DES (□).

3.6. Proliferative effects of anethole and its metabolites on MCF-7 cells

Fig. 6 shows the effects of anethole and its metabolites, 4OHPB and 4MCA, on MCF-7 cells derived from estrogen-responsive human breast cancer cells. The proliferative values were compared with those for untreated control cells. Relative to control (approximately 12.1×10^3 cells per well) on day 6, 4OHPB increased cell numbers in a concentration-dependent manner from 10^{-8} to 10^{-6} M and at 10^{-6} M, the maximum concentration, the compound increased cell numbers approximately 1.9-fold. Despite this, neither anethole nor 4MCA at concentrations from 10^{-9} to 10^{-5} M affected the cell proliferation. The concentration of 17β-estradiol is typical of a positive control in assays evaluating the estrogenicity of the above compounds, and the proliferation induced by estradiol in this study is consistent with previous studies [18,19]. On the other hand, all compounds at a concentration of 10^{-4} M elicited a considerable decrease in cell numbers relative to the control; viable percentages of MCF-7 cells exposed to anethole, 4OHPB and 4MCA were approximately 80, 25 and 85% of untreated cells, respectively. Thus, this indicates that these compounds at a higher concentration caused toxic and/or growth inhibitory effect to MCF-7 cells; the ranking in terms of cytotoxicity being 4OHPB > anethole or 4MCA.

4. Discussion

The results obtained in the present study show that in isolated rat hepatocytes, anethole induced a concentration- and time-dependent loss of cell viability, followed by

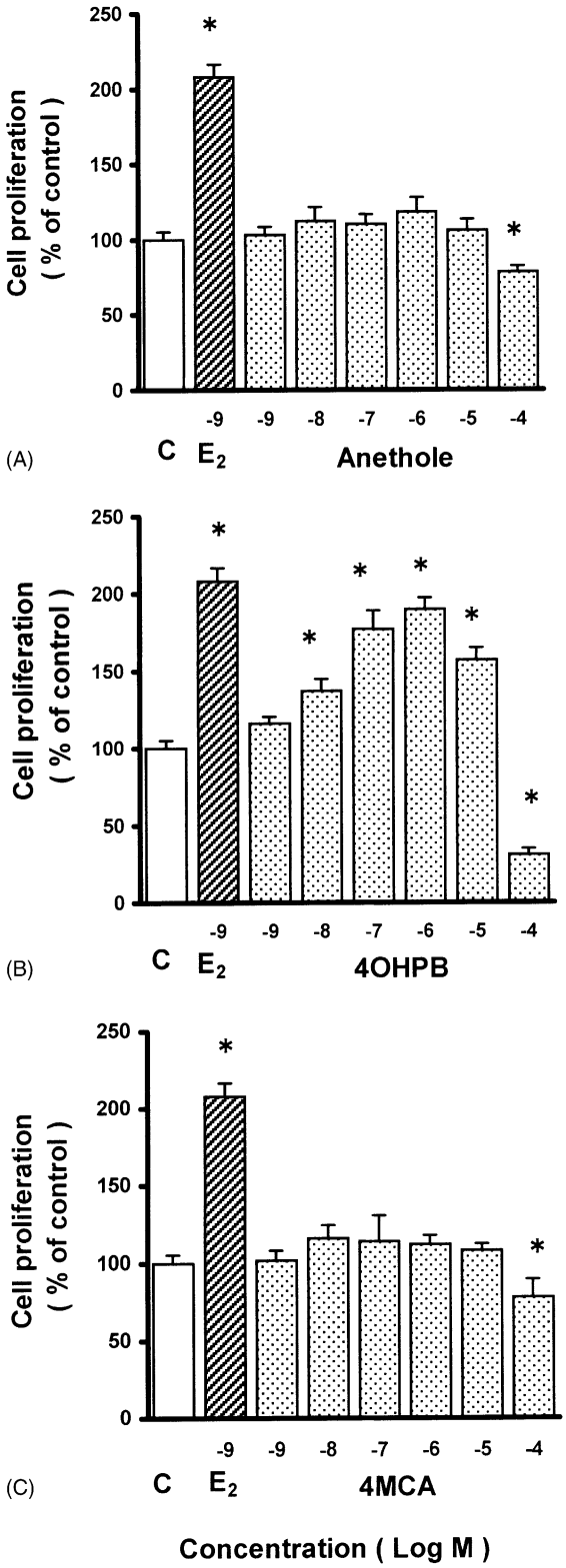

Fig. 6. Effects of anethole (A) and its metabolites, 4OHPB (B) and 4MCA (C), on the growth of MCF-7 cells. These compounds were tested at concentrations ranging from 10^{-9} M (−9) to 10^{-4} M (−4) for 6 days. Results are the means ± SE of three or four determinations. C and E_2 indicate untreated control and 17β-estradiol (10^{-9} M)-treated cells, respectively. (∗) Significant differences between the control group and treated groups ($P < 0.05$).

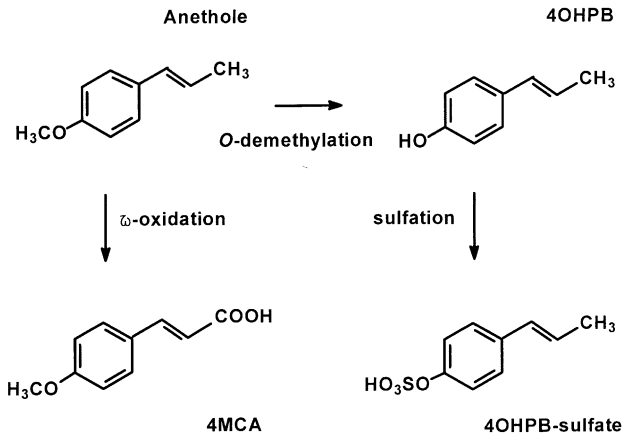

Fig. 7. Proposed metabolism of anethole in rat hepatocytes: anethole; 4MCA, 4-methoxycinnamic acid; 4OHPB, 4-hydroxy-propenylbenzene, and sulfate of 4OHPB.

decreases in intracellular levels of ATP and total adenine nucleotide pools. These results confirm, at least in part, that the liver is the target organ of anethole in rats [2]. Although at high concentrations anethole itself was toxic, at a weakly toxic level (<0.5 mM) the compound is rapidly converted to at least three metabolites: 4MCA via ω-side-chain oxidation and 4OHPB via *O*-demethylation in phase I, and a conjugate of 4OHPB via sulfation in phase II (Fig. 7). In connection with this, it was reported that the metabolism of anethole proceeds via *O*-demethylation and side-chain ω-oxidation, which are major pathways in rats and humans receiving doses of 200–300 mg/kg and 15 μg/kg body weight, respectively, and that the metabolic pathway shifted from *O*-demethylation to side-chain oxidation with increasing dose in rats [2,4,5,20].

Sulfate conjugation is a major metabolic pathway in the biotransformation and detoxification of xenobiotics such as phenols and related compounds, and endobiotics in mammals [21]. It is known that the extent of sulfate conjugation in rat hepatocytes is limited by the intracellular concentration of the activated sulfate donor, PAPS. The biosynthesis of PAPS is dependent on intracellular ATP levels and mitochondrial function as a site of ATP production, and PAPS acts as a rate-limiting component in sulfate conjugation [21,22]. In addition, it has been reported that the rate of PAPS production decreases with decreasing ratios of ATP/ADP or ATP/AMP [23]. We found that at a moderately toxic concentration (1.0 mM), anethole caused an abrupt increase in free unconjugated 4OHPB accompanied by a decrease in cellular levels of ATP, PAPS and 4OHPB sulfate (Figs. 1 and 4) and that the activity of sulfotransferase was not affected by 4OHPB at 0.5 and 1.0 mM in a supplementary experiment. These results suggest that the decrease of 4OHPB sulfate may be associated with the inhibition of synthesis of PAPS via an abrupt loss of ATP caused by the high dose of anethole. Even if hepatocytes have the ability to maintain a stable PAPS pool [24], the decrease in the level of PAPS may be

related to the simultaneous reduction in ATP, followed by loss of cell viability, as well as the consumption by sulfate conjugation.

In the presence of an inhibitor of sulfotransferase, anethole-induced cytotoxicity was enhanced in a concentration-dependent manner (Table 2). DCNP (50 μM) alone had no effect on cell viability, ATP and PAPS levels, and the formation of 4MCA during a 90-min incubation, whereas free unconjugated 4OHPB accumulated in hepatocyte suspensions, followed by the inhibition of 4OHPB sulfate formation and the loss of cell viability. A comparative analysis of anethole and its metabolites showed that 4OHPB was more toxic than the parent compound and 4MCA when rat hepatocytes were incubated with each compound (0.5 mM) for 90 min (Table 1) or MCF-7 cells were cultured with 10^{-4} M of each compound for 6 days (Fig. 6). In previous studies, we reported that phenolic compounds such as 4-hydroxybenzoate esters (parabens) [25,26], bisphenol A [27], DES [27], and propyl gallate [28] induced acute cytotoxicity in rat hepatocytes, accompanied by ATP depletion via impairment of mitochondrial function related to oxidative phosphorylation and/or membrane potential ($\Delta\Psi$). The toxic effect of bisphenols on hepatocytes is related to the concentration of unconjugated and/or unmetabolized parent compounds remaining in hepatocyte suspensions [9,27,28]. The relationship between the structure of chavicol and its analogues and their nematocidal potency has been studied in *C. elegans* and it has been reported that the hydroxyphenyl group is necessary for the nematocidal activity and that the activity of 4-propenylphenol (4OHPB) is approximately 7-fold that of anethole [29]. In a preliminary experiment, the addition of 4OHPB (0.25–1.0 mM) to Krebs–Henseleit buffer (pH 7.4) did not induce an increase in oxygen consumption (data not shown), which indicates that autoxidation via formation of superoxide anion radicals does not occur readily [30]. Although the mechanism of anethole-induced cell death is unclear, these results suggest that the accumulation of 4OHPB is an important factor in the onset of cell death and that the formation of 4OHPB sulfate depends on cell viability and intracellular ATP level. However, we are unable to exclude the possibility that an intermediate derived from epoxidation of the side-chain acts as a trigger for cytotoxicity induced by anethole in rat hepatocytes, since an inhibitor of epoxide hydrolase also enhances toxicity [11].

Anethole is a main constituent of the essential oils derived from fennel and anise, which are known to have estrogen-like effects as well as pharmacological effects in herbal medicines. Recently, the xenoestrogenicity of BPA, alkylphenols, and other phenolic compounds has been demonstrated in a number of *in vitro* and *in vivo* assays [7,8]. In this study, anethole itself did not exhibit a proliferative effect on MCF-7 human breast cancer cells with estrogen receptor, whereas 4OHPB caused a proliferation of MCF-7 cells at concentrations ranging from 10^{-8} to 10^{-6} M (Fig. 6). The reason why the parent compound was not estrogenic in MCF-7 cells may be that these cells are unable to transform anethole to the estrogenic 4OHBP. In addition, the estrogenic potency of 4OHPB can be reproduced in a cell-free assay; 4OHPB as well as BPA elicits a concentration-dependent displacement of 17β-estradiol bound to ERα (Fig. 5). Based on the relative IC_{50} values, binding potency for ERα is DES $\gg$ 4OHPB $>$ BPA $>$ anethole and 4MCA. It is well established that hydrophobicity, as demonstrated by its close correlation with the partition coefficient of xenobiotics, is often associated with biological action, which is expressed as a structure–toxicity or –activity relationship [31]. The *n*-octanol/water partition coefficient (log *p*), a physical property used extensively to describe the lipophilicity or hydrophobicity of chemicals, of DES, BPA, and 4OHPB is approximately 5.07, 3.64, and 2.83, respectively [32–34]. All of these compounds have a common structure with units of a 4-mono- or 4,4′-dihydroxy group in the aromatic rings. The log *p* of anethole and 4MCA is 3.39 and 2.68, respectively, both compounds have very weak or negligible estrogenic activity (Figs. 5 and 6), and neither has a 4-hydroxy group. Thus, there is no good strong correlation between estrogenic potency and log *p* in anethole and its metabolites, while it is apparent that the existence of a hydroxy group in the aromatic ring is necessary for the onset of estrogenic effects.

It is known that phenolic compounds with an alkyl group in the *para* (or 4)-position on the aromatic ring stimulate the proliferation of MCF-7 cells [35,36]. For example, acetamidophenol (acetaminophen) significantly increased the proliferation of MCF-7 cells, and the relative potency of its isomers in stimulating the proliferation of cells was *p*- $>$ *m*- $>$ *o*-acetamidophenol, indicating that the position of the hydroxy group on the aromatic ring influences the estrogenic effect [37]. In addition, Routledge *et al.* [38] reported that alkyl esters of 4-hydroxybenzoic acid, known as parabens, are weakly estrogenic *in vitro*. On the other hand, the onset of estrogenic effects of biphenyls is associated with hydroxylated intermediates produced by microsomal cytochrome P-450 monooxygenase. In previous studies [9,10], we reported that unsubstituted benzophenone was enzymatically converted to at least three metabolites, benzhydrol, 4-hydroxybenzophenone, and a sulfate, in rat hepatocytes and that the effects of 4-hydroxybenzophenone on the proliferation of MCF-7 cells *in vitro* and uterotrophic response in immature female rats were estrogenic, whereas neither the parent compound nor benzhydrol was essentially active. The estrogenic activity of 2-hydroxy-4-methoxybenzophenone (benzophenone-3) is attributed to the action of a 4-hydroxylated intermediate through biotransformation by oxidative *O*-dealkylation [10]. Based on the cumulative findings, it appears that there are at least two mechanisms for the onset of estrogenic activity produced by aromatic compounds: one is direct interaction between an intrinsic 4-hydroxylated compound and the estrogen receptor; and the other is indirect action by 4-hydroxylated intermediates via biotransformation through either hydroxylation or

O-dealkylation. While experimental data on the estrogenic potential of 4OHPB sulfate are not obtained in this study, it can be assumed that the conjugate would not have the capability to interact with the estrogen receptor, because neither glucuronide nor sulfate derived from xenoestrogenic compounds, bisphenol A and alkylphenols, interacts with the estrogen receptor and affects estrogenic activity in MCF-7 cells [39,40]. Since anethole may become estrogenic via biotransformation, it will be necessary to investigate the estrogenic potency of the compound *in vivo*.

In conclusion, the results of present study show that the incubation of hepatocytes with anethole causes a concentration- and time-dependent decrease in cell viability, accompanied by losses of intracellular ATP, adenine nucleotide pools, and PAPS, and that anethole is converted enzymatically to its metabolites: 4OHPB and its sulfate and 4MCA. The cytotoxicity of anethole is enhanced by a sulfotransferase inhibitor, which increases free unconjugated 4OHBP levels, and 4OHPB is more toxic to hepatocytes than the parent molecule or 4MCA. In *in vitro* estrogenic assays, 4OHPB competitively displaces 17β-estradiol bound to ERα in a concentration-dependent manner and enhances the proliferation of MCF-7 cells, whereas neither the parent compound nor 4MCA at the concentrations used affects cell proliferation and competitive binding. These results suggest that the hydroxylated intermediate 4OHPB rather than the parent molecule induces cytotoxic and/or estrogen-like effects through biotransformation.

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