

Structural toxicity relationship of 4-alkoxyphenols' cytotoxicity towards murine B16-F0 melanoma cell line.

Majid Y. Moridani¹, Mike Moore², Richard A. Bartsch³, Yanfei Yang³, Souzan Heibati-Sadati³

¹Department of Pharmaceutical Sciences, School of Pharmacy; Department of Pediatrics, School of Medicine, Texas Tech University HSC, Amarillo, Texas, USA

²School of Pharmacy, Texas Tech University HSC, Amarillo, Texas, USA

³Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas, USA

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ABSTRACT. PURPOSE. The aim of this study was to identify phenolic agents that could form quinone reactive intermediate metabolites in melanocytes in order to be effective as anti-melanoma agents; but were not metabolized by liver P450 metabolizing enzymes in order to have minimal toxicity towards the liver. **METHODS.** Tyrosinase, an enzyme present abundantly in melanocytes was selected as a molecular target for the treatment of malignant melanoma. Ten alkoxyphenols were investigated for their metabolism by tyrosinase/O₂, rat liver P450 microsomal/NADPH/O₂ metabolizing systems and for their toxicity towards B16-F0 melanoma cells. **RESULTS.** All the alkoxyphenols showed a dose- and time-dependent toxicity towards B16-F0 cells except 2-iso-propoxyphenol. 4-n-Hexyloxyphenol demonstrated the greatest toxicity towards B16-F0 cells while minimally depleting glutathione in microsomal preparations at its calculated LC₁₀ and LC₅₀ lethal concentrations for B16-F0. At 100 μM concentrations, 4-t-butoxyphenol showed the lowest amount of glutathione depletion by microsomal P450 system. Alkoxyphenols with at least two alkyl groups derivatized at alpha carbon of alkoxy group showed minimal rates of metabolism by tyrosinase/O₂ metabolizing system. A quantitative structural toxicity relationship equation was also derived, $\text{LogLC}_{50}(\mu\text{M}) = -0.265(\pm 0.064)\text{LogP} + 2.482(\pm 0.179)$. **CONCLUSIONS.** 4-n-hexyloxyphenol was identified as a potential lead anti-melanoma agent against B16-F0 melanoma cells with minimal metabolism by rat liver P450 microsomal preparation.

INTRODUCTION

Malignant melanoma is one of the deadliest cancers known to man. It is estimated that 55,100 new invasive melanoma cases are diagnosed in the USA every year of which 7,910 will be expected to die from the disease (1). The estimated lifetime risk for melanoma among Americans is 1 in 74. Currently, the therapy for melanoma includes surgical intervention, which has a high rate of treatment failure in highly metastatic and advanced cases that are usually fatal. Systemic chemotherapy is often the only resource, but the results to date have been very disappointing and the lack of selective cytotoxicity often leads to intolerable side effects (2). With increasing occurrence of this disease, there is a clear and urgent need for an improved treatment regimen with enhanced specificity.

Tyrosinase, an enzyme found abundantly only in melanocytes, was selected as a molecular target for 4-hydroxyanisole (4-HA) in the past. 4-HA is a simple phenolic agent which was first shown by Riley (3) to be a melanocytotoxic agent. Tyrosinase was shown to catalyze the oxidation of 4-HA to 4-methoxycatechol and its o-quinone, which reacted readily with nucleophiles (4–6). In addition, melanoma toxicity may result from the covalent binding of the o-quinone to protein thiols and/or glutathione (GSH) depletion (7) and inhibition of mitochondrial electron transport (8). This ultimately leads to desirable melanoma cell death. 4-HA was the only compound from this class that was tested in clinical trials as an anti-melanoma agent (3, 9). Depigmentation and tumor shrinkage resulted from both the topical application of 4-HA (3) and intra-arterial infusions of 4-HA into patients' legs (9). Unfortunately, 4-HA clinical trials were terminated because serious liver damage occurred (10) but there were no insights into the mechanisms resulting in induced liver toxicity. It was recently shown that 4-HA was also metabolized by liver P450s via arene epoxidation route to p-quinone (Figure 1), a reactive metabolite, which was highly toxic to isolated rat hepatocytes (6).

Corresponding author: Majid Moridani, Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, 1300 Coulter Drive, Amarillo, Texas, 79106, USA. majid.moridani@ttuhsc.edu

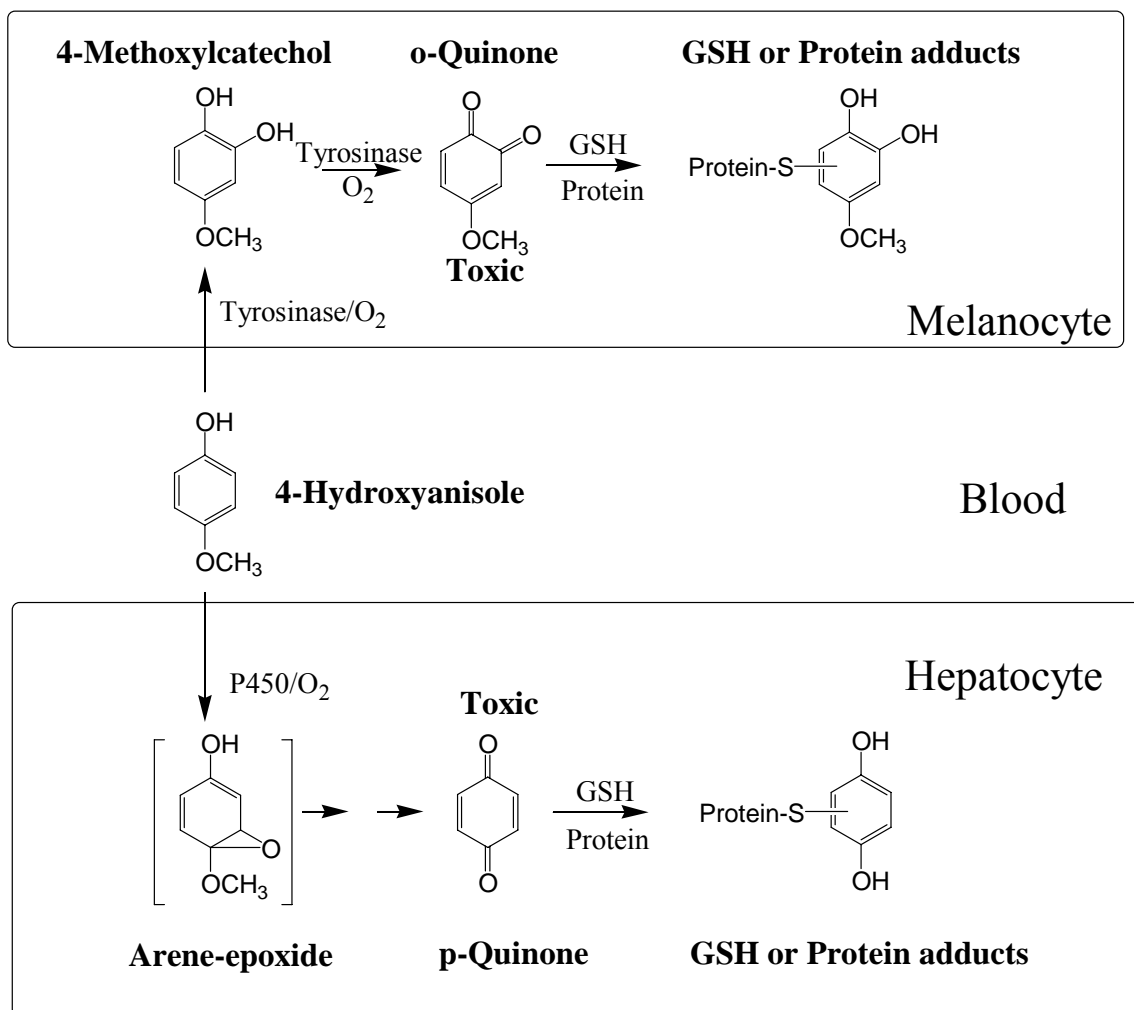


Figure 1: Metabolism pathway for 4-HA in melanocyte and hepatocyte. 4-HA was metabolized by liver P450s via arene epoxidation route to p-quinone, a reactive metabolite, which can deplete GSH by conjugate formation. P-Quinone was shown to be highly toxic to isolated rat hepatocytes (6). 4-HA was also metabolized by melanocyte tyrosinase to form 4-methoxycatechol and then an o-quinone which can react with intracellular GSH and is toxic towards melanoma cells.

In the current work, we sought to identify a phenolic compound with minimum toxicity towards the liver but yet efficacious against melanoma. We thus investigated ten alkoxyphenol compounds with various linear aliphatic side chains and their branched analogues (Figure 2) for their metabolism by tyrosinase/O₂, rat liver P450 microsomal preparation/NADPH/O₂ metabolizing systems, and for their toxicity towards the B16-F0 mouse

melanoma cell line. Our data indicates that all alkoxyphenols tested in this work demonstrated toxicity towards murine B16-F0 melanoma cell line. It was postulated that only 4-nHP (4-n-hexyloxyphenol) demonstrated a significant advantage over other alkoxyphenols with respect to GSH depletion by rat liver microsomal P450s and therefore its toxicity towards the liver.

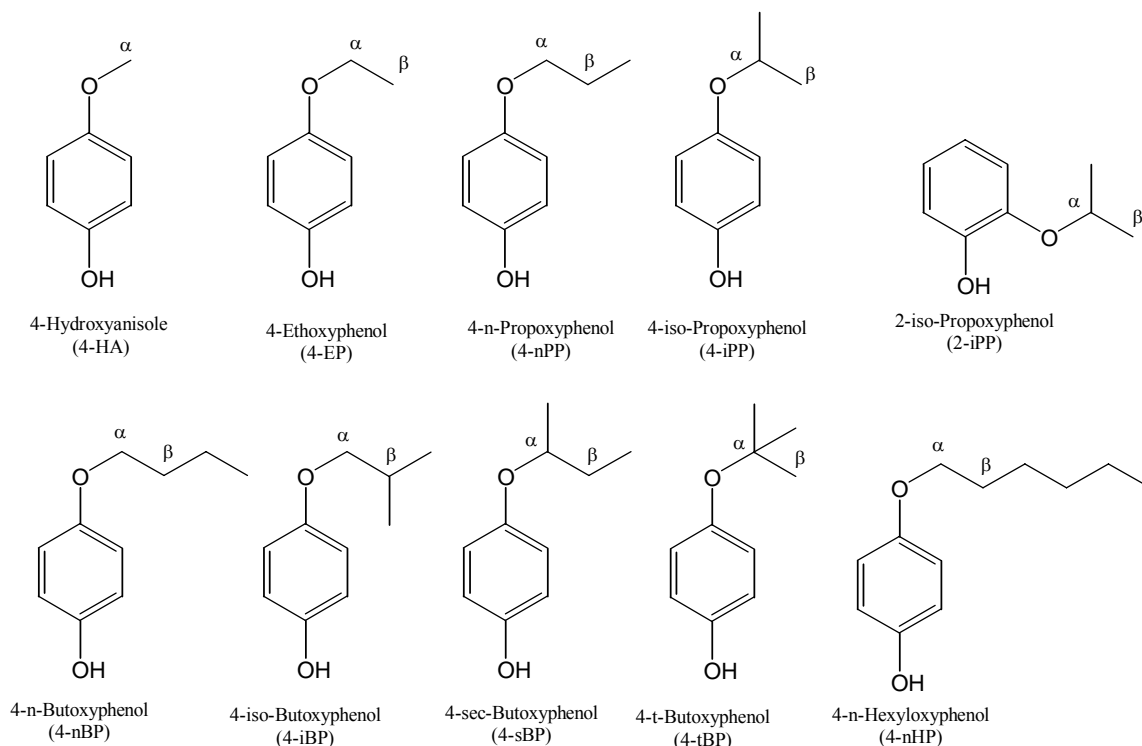


Figure 2: Chemical structure of alkoxyphenols. The positions of hydrogen and the side chains are marked as α and β on the aliphatic side chain of alkoxyphenols.

EXPERIMENTAL SECTION

Materials

All materials, solvents and reagents were purchased from either Sigma-Aldrich, USA, or Fisher-Scientific, USA. DETAPAC (diethylenetriaminopentaacetic acid) was purchased from Sigma-Aldrich, USA. Mushroom tyrosinase was used throughout this study. Phenolic agents that were investigated in this study included: 4-HA (4-hydroxyanisole); 4-EP (4-ethoxyphenol); 4-nPP (4-n-propoxyphenol); 4-iPP (4-iso-propoxyphenol); 2-iPP (2-iso-propoxyphenol); 4-nBP (4-n-butoxyphenol); 4-iBP (4-iso-butoxyphenol); 4-sBP (4-sec-butoxyphenol); 4-tBP (4-t-butoxyphenol); and 4-nHP (4-n-hexyloxyphenol). 4-t-Butoxyphenol was purchased from Matrix Scientific, USA. 4-iPP, 4-iBP and 4-sBP were synthesized (11). Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. 11965-092), fetal bovine serum (Cat. No. 10082-139), Penicillin-Streptomycin; (10,000 units/mL, Cat. No. 15140-122) and Versene (1:5000 Cat. No. 15040-066) were purchased from Invitrogen, USA. The mouse melanoma B16-F0 cell line was obtained from American Type Culture Collection (ATCC), USA.

METHODS

Syntheses of 4-iso-propoxyphenol, 4-iso-butoxyphenol and 4-sec-butoxyphenol

All alkoxyphenols were commercially available except 4-iso-butoxyphenol, 4-sec-butoxyphenol and 4-iso-propoxyphenol, which were prepared by adaptation of a method published by Naish-Byfield et al (11). Briefly, KOH (0.020 mol) dissolved in ethanol (80 mL) was added dropwise over a 1 h period to a stirred solution of hydroquinone (0.10 mol) and the appropriate alkyl bromide (0.020 mol) in ethanol (400 mL) at reflux under nitrogen. After overnight refluxing and cooling to room temperature, excess base was neutralized with acetic acid. The inorganic salts were filtered and the solvent was evaporated *in vacuo* to give a mixture of hydroquinone and the 4-alkoxyphenol. Dichloromethane was added and the mixture was filtered to remove most of the excess hydroquinone. The filtrate was dried over magnesium sulfate and evaporated *in vacuo*. The crude product was purified by recrystallization or column chromatography. 4-iso-Butoxyphenol was obtained in 50% yield after recrystallization from dichloromethane-hexane (mp 53-64°C). IR (deposit on a NaCl plate from

dichloromethane solution): 3359 (OH), 1235 (CO), 1039 (CO) cm^{-1} . ^1H NMR (300 MHz, CDCl_3): δ = 1.01 (d, J = 6.6 Hz, 6H, CH_3), 1.99-2.07 (m, 1H, CH), 3.66 (d, J = 6.6 Hz, 2H, CH_2), 4.37 (s, 1H, OH), 6.76-6.77 (m, 4H, ArH).

4-sec-Butoxyphenol was isolated in 30% yield after chromatography on silica gel. IR (film): 3359 (OH), 1230 (CO), 1100 (CO) cm^{-1} . ^1H NMR (300 MHz, CDCl_3): δ = 0.98 (t, J = 7.2 Hz, 3H, CH_3CH_2), 1.25 (d, J = 6.0 Hz, 3H, CH_3 CH), 1.47-1.82 (m, 2H, CH_2), 4.08-4.12 (m, 1H, CH), 4.59 (s, 1H, OH), 6.75-6.77 (m, 4H, ArH). Analysis, Calculated for $\text{C}_{10}\text{H}_{14}\text{O}_2$: C, 72.26; H, 8.44. Found: C, 71.86; H, 8.45.

4-iso-Propoxyphenol was isolated in 50% yield after chromatography on silica gel. IR (film): 3359 (OH), 1226 (CO), 1120 (CO) cm^{-1} . δ = 1.29 (d, J = 6.0 Hz, 6H, CH_3), 4.38-4.42 (m, 1H, CH), 4.69 (s, 1H, OH), 6.75-6.77 (m, 4H, ArH). Analysis, Calculated for $\text{C}_9\text{H}_{12}\text{O}_2$: C, 71.03; H, 7.95. Found: C, 71.29; H, 7.90.

UV-VIS spectroscopy of tyrosinase mediated metabolism of alkoxyphenols

The spectra of a solution containing alkoxyphenol (100 μM) and tyrosinase (20 U/mL) were recorded in the absence and presence of GSH (200 μM) using a GBC UV-Visible spectral spectrophotometer (GC Scientific, Australia). The spectra of the mixture were obtained when GSH was added to the solution either before or after the addition of tyrosinase. The control spectrum was that of the respective alkoxyphenol solution (100 μM) in phosphate buffer [0.1 M (pH 7.4) containing DETAPAC (1 mM)].

Tyrosinase mediated GSH depletion assay

Tyrosinase (10 μL ; 2500 U/mL) was added to a mixture of alkoxyphenol (100 μM) and GSH (200 μM) in a final volume of 1 mL phosphate buffer (0.1 M, pH 7.4, DETAPAC 1mM). The mixture was pre-incubated for 30, 90, and 180 min at 37°C. A 250 μL aliquot was added to trichloroacetic acid (25 μL ; 30% w/v), vortexed and left at room temperature for 5 min. A 100 μL aliquot of the supernatant was then added to a mixture of Ellman's reagent 5,5'-dithiobis-(2-nitrobenzoic acid) DTNB (25 μL ; 2 mg/mL) and Tris/HCl buffer (875 μL ; 0.1 M, pH 8.94), and then vortexed. The absorbance of the solution was observed at 412 nm (12, 13). The standard curve for GSH measurement gave a regression coefficient of greater than 0.99 over the

range of 5-200 μM GSH concentrations (data not shown).

Animal housing and protocol

Adult male Sprague-Dawley rats, 250–300g, were obtained from Charles River Laboratories, USA, fed ad libitum, were allowed to acclimatize for 1 week on clay chip bedding in a room with a 12 h light photocycle, an environmental temperature of 21-23 °C and 50-60% relative humidity. The animal protocols used in current investigation for rat liver microsomal preparation were reviewed and approved by Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center, Amarillo, TX.

Rat liver microsomal preparation

The rats were anesthetized by sodium pentobarbital (60 mg/kg) before surgery in order to prepare the animal before liver removal. Hepatic microsomes were prepared by differential centrifugation as described previously (14). Briefly, the liver was removed and weighed in a beaker on ice. The liver was cut into pieces and washed by cold KCl (154 mM): Tris/HCl (50 mM, pH 7.4) buffer solution then suspended into 4 volumes of KCl:Tris/HCl buffer. The tissue was gently homogenized using an electrical homogenizer and subsequently by a handheld glass tissue grinder before centrifuging at 1935 g (Beckman Avanti J-25I, Beckman Rotor- JA-25.5) at 4 °C for 15 min to remove tissue and cell debris. The supernatant was centrifuged at 12,100 g at 4 °C for 15 min to remove subcellular organelles followed by centrifugation at 100,000 g (Beckman Optima LE-80K, Beckman Rotor- 45 Ti) at 4 °C for 1 h. The supernatant was discarded and microsomes were separated and suspended in 5 mL Tris/HCl buffer (100 mM, pH 7.4) containing 1 mM DETAPAC. The mixture was homogenized using a handheld glass tissue grinder after which an additional 15 mL Tris/HCl buffer was added and the solution was aliquoted in 750 μL and stored at -70°C for subsequent use. Microsomal protein content was determined by a modified Lowry method (15).

CYP2E1 induced rat liver microsomal preparation

CYP2E1 induced microsomes were prepared from rats treated (i.p.) with inducing agent pyrazole (200 mg/kg/day) (16) for 2 consecutive days before sacrificing the rats on the 3rd day.

Microsomal mediated GSH depletion assay

The amount of GSH conjugates formed was determined colorimetrically using Ellman's reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (13). Incubation mixtures contained in a final volume of 1 mL phosphate buffer (0.1 M, pH 7.4, DETAPAC 1mM), 2 mg/mL rat liver microsomes, 200 μ M GSH, 1 mM NADPH, and 100 μ M alkoxyphenols. The mixtures were gently mixed at 37 °C from which 250 μ L aliquots were taken at 30, 60, and 90 min time-interval points into Eppendorf tubes containing 25 μ L trichloroacetic acid (30% w/v). Following protein precipitation and centrifugation for 5 min, the GSH levels of a 100 μ L aliquot of the supernatant was determined by the addition of 0.1 M Tris/HCl buffer, pH 8.94 (875 μ L), and 2 mg/mL DTNB (25 μ L). The reduced DTNB formed was determined at 412 nm on a GBC spectral spectrophotometer.

Cell Culture

The mouse B16-F0 cell line was obtained from American Type Culture Collection, USA. A frozen B16-F0 cell vial was washed twice with DMEM media before culturing in 8 mL DMEM supplemented with fetal bovine serum (FBS) (10%) and streptomycin/penicillin (100 U/mL) in a T-25 flask (17). All the cell culture processes were carried out in a type II vertical laminar air flow chamber. The B16-F0 cultures were kept at 37 °C under a 5% CO₂ atmosphere in a TS Autoflow CO₂ Water-Jacketed Incubator (NUAIRE, USA). The culture medium was changed when acidification was indicated by the pH indicator and when needed. To detach the cells from the flask, the media was first removed from the flask. The remaining media was then washed out using sterile phosphate buffered saline (PBS) (2-3 mL). Subsequently, Versene (2-5 mL) was added to the flask and the sample was incubated for 2-3 min in the incubator to provide time for cells to detach. The detached cells were rinsed with ~10 mL of pre-warmed sterile PBS to dilute Versene. The mixture was transferred into a 50 mL tube. The flask was additionally rinsed with sterile PBS and the content was added to the rest of the cells collected. The cells were then spun down at 800 RPM (Beckman GPR Centrifuge, USA) for 3-5 min. The PBS-Versene mixture was aspirated off. Pelletted cells were then re-suspended in DMEM media (supplemented by FBS 10% and antibiotics 100 U/mL) followed by splitting the mixture into one T-75 flask containing 30 mL media (25% of the

media was supplemented from the previous culture step as conditioning media).

Cell viability

To determine cell viability, the cells obtained from each flask were suspended in 4 mL of DMEM media supplemented by FBS 10% and antibiotics 100 U/mL (contained 25% media from the previous culture step as conditioning media). The cells were counted using trypan blue exclusion method (18) for determining the viability.

MTT assay in 96 well plates

To evaluate cytotoxicity, cells were obtained from exponentially growing 90-95% confluent cultures and seeded at 12,500 cells/well in 96-well plates. The cells were kept in 100 μ L fresh DMEM media (supplemented by FCS 10% and antibiotics 100 U/mL) for 24 h to allow cell adhesion and environmental adaptation. Subsequently, the cells were treated with additional 150 μ L DMEM (supplemented with FCS 10% and antibiotics 100 U/mL) containing various concentrations of alkoxyphenols for 1-4 days. At 24 h interval, the medium was removed and the wells were washed three times using DMEM media alone before adding 40 μ L of 2 mg/mL yellow tetrazolium dye (3 - (4, 5-dimethylthiazolyl - 2) - 2, 5 - diphenyltetrazolium bromide) (MTT) (17). The plates were returned to the incubator for a period of 4 h. The residual MTT solutions were removed from wells and then 200 μ L of DMSO was added to each well. The plates were stored at room temperature in a dark place for an additional 2 h before reading them at 570 nm using XFluor Plate Reader (Tecan US, Inc, USA). All experiments were performed in triplicate. An analysis of variance (ANOVA) of repeated means was carried out to compare the percentage of surviving cells in the cultures for different concentrations of each compound. t-test was used to compare the results of toxicity of the alkoxyphenols with 4-HA.

LC₁₀ and LC₅₀ calculation

Lethal concentrations (LC) which can cause 10% and 50% of the cell death were calculated from the linear regression equation derived from graphing the viability of the cells at day 2 (on x axis) versus the concentration of the drug (on y axis).

Partition coefficient

Partition coefficient values were estimated using the LogP software available at www.LogP.com.

Table 1: Metabolism mediated percentage GSH depletion of alkoxyphenols by tyrosinase and rat liver microsomal preparations

Alkoxyphenols	Eq. constructed from data (day 2) ^a	B16-F0 toxicity (μM) (day 2)					LogP ^d	Metabolism mediated %GSH depletion						
		Observed LC ^b		Estimated ^c LC ₅₀ (Eq.2)		Tyrosinase/O ₂ ^e		Normal liver microsomes/NADPH/O ₂ ^f		2E1 induced liver microsomes/NADPH/O ₂ ^f				
		LC ₁₀	LC ₅₀	Log LC ₅₀	LC ₅₀			Log LC ₅₀	30 min	90 min	180 min	90 min	30 min	60 min
4-hydroxyanisole	LC= - 1.2 viability (%) + 118	10	58**	1.76	117	2.07	1.56	87±3†	100	100**	10±3	30±3	42±3	58±2**
4-ethoxyphenol	LC= - 1.4 viability (%) + 145	18	74	1.87	90	1.95	2.00	7±1	43±2	87±3	11±3	27±3	43±2	52±5
4-n-propoxyphenol	LC= - 1.5 viability (%) + 132	<1	58	1.76	68	1.83	2.45	4±1	54±5	87±4	23±4	39±4	52±3	61±3
4-iso-propoxyphenol	LC= - 1.9 viability (%) + 181	14	88	1.94	72	1.86	2.35	2±1	9±2	13±2*	24±3	42±2	57±3	67±3
2-iso-propoxyphenol	LC= - 4.6 viability (%) + 484	70	254	2.40	75	1.88	2.29	1±1	9±2	17±2*	18±4	41±2	51±4	56±4
4-n-butoxyphenol	LC= - 1.0 viability (%) + 96	6	46	1.66	52	1.71	2.90	21±2	88±4	100	27±3	45±3	58±2	69±3
4-iso-butoxyphenol	LC= - 1.2 viability (%) + 134	24	74	1.87	55	1.74	2.81	49±3	87±4	100	32±4	47±3	73±3	75±3
4-sec-butoxyphenol	LC= - 1.1 viability (%) + 118	19	62	1.79	56	1.75	2.78	1±1	7±2	17±3*	25±3	42±4	60±4	66±3
4-t-butoxyphenol	LC= - 4.3 viability (%) + 414	27	200*	2.30	55	1.74	2.80	1±1	5±1	19±2*	33±5	8±3	19±1	25±3*
4-n-hexyloxyphenol	LC= - 1.8 viability (%) + 116	<1	26*	1.41	29	1.46	3.87	94±2†	100	100	38±3	42±4	60±4	66±3

^a LC (lethal concentration) equation was derived from regression analysis of viability data on the day 2 versus concentration.

^b LC₁₀ and LC₅₀ were calculated from equations presented in the first column (see above).

^c The values were calculated from $\text{LogLC}_{50} (\mu\text{M}) = -0.265\text{LogP} + 2.482$ (QSTR Eq.2).

^d LogP was calculated from LogP software available from www.logP.com.

^e The mixture contains tyrosinase (25 units/mL), alkoxyphenol (100 μM) and GSH (200 μM) in a final volume of 1 mL phosphate buffer (0.1 M, pH 7.4, DETAPAC 1mM).

^f The reaction mixture contains microsomes (2 mg/mL), alkoxyphenol (100 μM) and GSH (200 μM) in a final volume of 1 mL phosphate buffer (0.1 M, pH 7.4, DETAPAC 1mM).

* Significantly different (t-test, $P < 0.05$) from 4-HA (marked as **) for selected data.

† As effective as 4-HA for being metabolized by mushroom tyrosinase/O₂ metabolizing system.

RESULTS

UV-VIS spectroscopy of tyrosinase mediated metabolism of alkoxyphenols

The progression of alkoxyphenol's oxidation was monitored by tyrosinase/O₂ oxidizing system using a UV-VIS spectroscopy method, which showed a distinct peak at 420-470 nm with a characteristic indicative of o-quinone formation. Addition of glutathione at the beginning of the metabolism reaction resulted in the significant loss in the absorbance of the 420-470 nm peaks. The UV-VIS spectra of this peak for all the alkoxyphenols were developed over 1 min except for 2-iPP and 4-tBP that were found to have a 20-fold lower rate of oxidation by tyrosinase/O₂ metabolizing system.

Tyrosinase mediated GSH depletion assay

As shown in Table 1, alkoxyphenols depleted GSH in the tyrosinase/O₂ metabolizing system in the following decreasing order: 4-HA >> 4-nBP, 4-iBP, and 4-nHP >> 4-EP and 4-nPP >> 4-iPP, 2-iPP, 4-sBP and 4-tBP. 4-HA depleted 1.8 molecular equivalent of GSH per mole at 30 min incubation. 4-nBP, 4-iBP, and 4-nHP depleted 1.8 molecular equivalent of GSH per mole whereas 4-EP and 4-nPP depleted GSH on mole per mole basis after 90 min incubation. 4-iPP, 2-iPP, 4-sBP and 4-tBP depleted GSH only to an average equivalent of 0.15 molar per mole with tyrosinase/O₂ metabolizing system during the 90 min incubation. It was found that additional substitution/s on the alpha carbon of alkoxy group prevented the metabolism of the molecule by tyrosinase/O₂. For instance, the rate of metabolism for 4-butoxyphenol series (4-nBP, 4-iBP, 4-sBP, and 4-tBP) by tyrosinase was diminished in the following decreasing order 4-tBP < 4-sBP < 4-nBP and 4-iBP < 4-HA which suggests that the presence of a non linear side chain on the alpha carbon atom of the alkoxy group may interfere with the molecular fit into the tyrosinase enzyme active site. Negligible GSH depletion occurred in the absence of the enzyme.

Glutathione conjugate formation by rat hepatocyte microsomes

The amount of GSH depleted as a result of alkoxyphenol metabolism catalyzed by rat liver microsomes/NADPH/O₂ was determined to be between 1.2 -1.5 equivalents of GSH per mole except for 4-tBP which was 0.5 equivalent per mole of GSH after 90 min of incubation. At 100 μM

concentration (Table 1), 4-tBP showed the lowest rate of GSH depletion by microsomal P450/NADPH/O₂ metabolizing system, which corresponded to 2.3 fold less than 4-HA. This suggests that 100% of the alkoxyphenols in the reaction mixture underwent glutathione conjugation except 4-tBP which could be due to hindrance imposed by the presence of the bulky t-butoxy group in its molecular structure. It was noted that the alkoxyphenols were metabolized by CYP 2E1 induced rat liver microsomes more readily than when non-induced/standard rat microsomal P450 system was used. Negligible GSH depletion occurred in the absence of the enzyme.

Cell viability

Cell viability of the cultured murine B16-F0 melanoma cell line was measured using trypan blue exclusion test (18) and was always greater than 95% before seeding the cells into the 96 well plates for MTT assay.

Cytotoxicity in B16-F0 melanoma cell line

The LC₅₀ (2 day) concentrations were determined by MTT assay (17) as a measure of melanoma cell viability (Figure 3). The required concentrations of compounds that can cause 50% decrease in melanoma cell viability (LC₅₀ μM) on the second day are given in Table 1. ANOVA and regression analysis of the toxicity of each of the alkoxyphenols at various doses showed the cytotoxicity to be dose- and time-dependent with a ranking order of 4-nHP>>4-nPP, 4-HA, 4-sBP, 4-iBP, 4-EP>4-iPP>>4-tBP>2-iPP except for 2-iPP.

Partition coefficient

The partition coefficients of the alkoxyphenols were estimated using the LogP software (www.logp.com) and were in a decreasing order of 4-nHP>>4-nBP, 4-iBP, 4-sBP, 4-tBP>>4-nPP>4-iPP and 2-iPP>>4-EP>>4-HA, thus indicating that the lipid solubility of the alkoxyphenol increases as the size of the aliphatic group and/or the number of carbon atoms on the aliphatic side chain group increases (Table 1).

One parameter quantitative structure toxicity relationship

The data in Table 1 was used to derive Eq. 1 as a one-parameter model for quantitative structure toxicity relationship (QSTR) for describing the toxicity of the alkoxyphenols towards B16-F0 melanoma cell line.

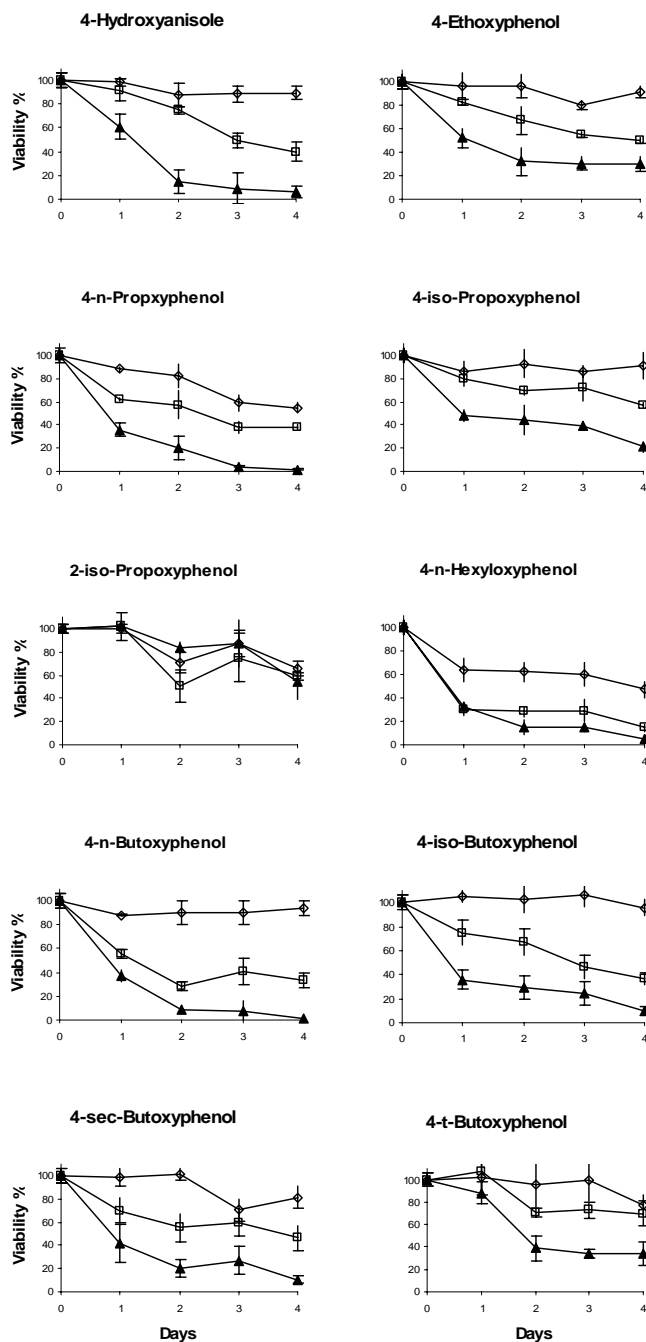


Figure 3: The cytotoxicity of alkoxyphenols towards B16-F0 mouse melanoma cell line. All assays were carried out in triplicate. The concentrations of the reagents tested were 10 μM , 50 μM and 100 μM except for 2-iso-propoxyphenol and 4-t-butoxyphenol that were 10 μM , 100 μM and 250 μM . All the alkoxyphenols showed a dose- and time-dependent toxicity towards B16-F0 cells except 2-iso-propoxyphenol. 4-n-Hexyloxyphenol demonstrated the greatest toxicity towards B16-F0 cells.

$$\text{Log}LC_{50} (\mu\text{M}) = -0.182 (\pm 0.153) \text{Log}P + 2.345 (\pm 0.405)$$

($n=10$, $R^2=0.150$, P value for $\text{Log}P$ term = 0.268; P value for intercept term < 0.001)

Eq. 1

As shown in Eq. 2 (Figure 4) the exclusion of outliers (4-HA, 2-iPP and 4-tBP; shown as ● symbol) from the rest of the data points (shown as ◇

symbol) greatly improved the QSTR equation between the alkoxyphenols LogLC_{50} (μM) and their LogP values. The calculated LogLC_{50} values from Eq. 2 were similar to the experimental data (Table 1). The outlier 4-HA (Table 1) was 1.7 fold more toxic than the toxic value calculated from Eq. 2 whereas both 2-iPP and 4-tBP were 3.6 fold less toxic.

However, the toxicity of the seven alkoxyphenols was well predicted by Eq. 2 (Table 1).

$$\text{LogLC}_{50} (\mu\text{M}) = -0.265 (\pm 0.064) \text{LogP} + 2.482 (\pm 0.179)$$

($n=7$, $R^2=0.773$, P value for LogP term = 0.009; P value for intercept term < 0.0001)

Eq. 2

Outliers: 4-HA, 2-iPP, and 4-tBP

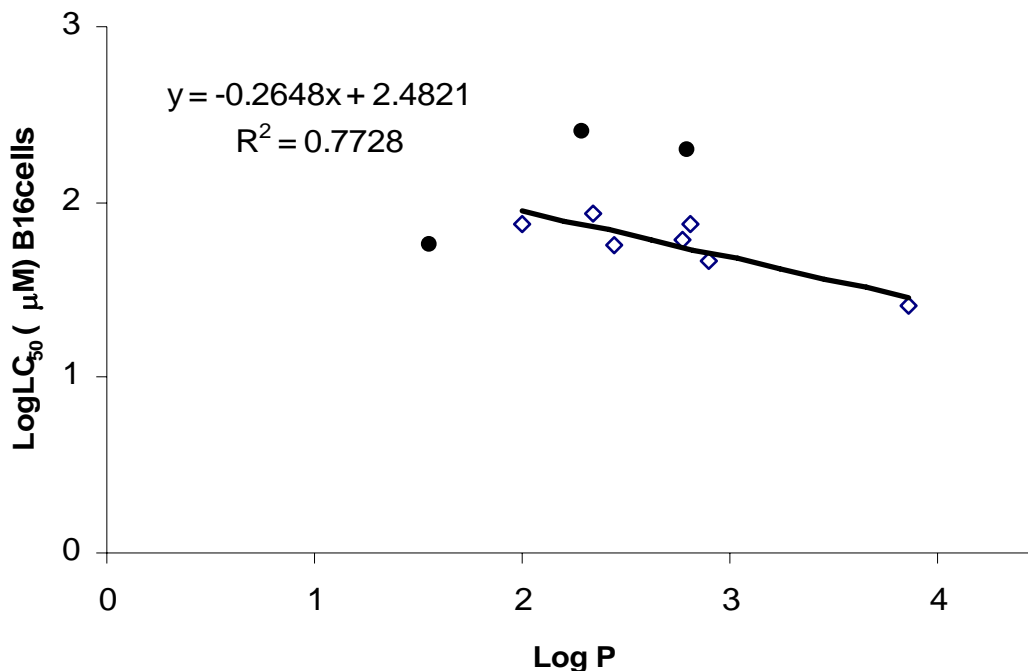


Figure 4: Graphical presentation of quantitative structure toxicity relationship (QSTR) for alkoxyphenols in B16-F0 mouse melanoma cell line. 4-HA (4-hydroxyanisole); 4-EP (4-ethoxyphenol); 4-nPP (4-n-propoxyphenol); 4-iPP (4-iso-propoxyphenol); 2-iPP (2-iso-propoxyphenol); 4-nBP (4-n-butoxyphenol); 4-iBP (4-iso-butoxyphenol); 4-sBP (4-sec-butoxyphenol); 4-tBP (4-t-butoxyphenol); and 4-nHP (4-n-hexyloxyphenol). The toxicity of alkoxyphenols increases with an increase in its LogP value and lipid solubility. 4-n-Hexyloxyphenol demonstrated the greatest toxicity towards B16-F0 cells. 4-HA, 2-iPP and 4-tBP are outliers as (●).

DISCUSSION

The aim of this investigation was to identify alkoxyphenolic agents that were metabolized at a lower rate by rat liver microsomes/NADPH/ O_2 but could still be metabolized by the tyrosinase/ O_2 enzyme and therefore be relatively toxic towards B16-F0 melanoma cells when compared to 4-HA.

Riley group had previously investigated the structure activity relationship of tyrosinase dependent cytotoxicity of a series of substituted linear alkoxyphenols (11). However, this study did not investigate the metabolism of this group of phenols

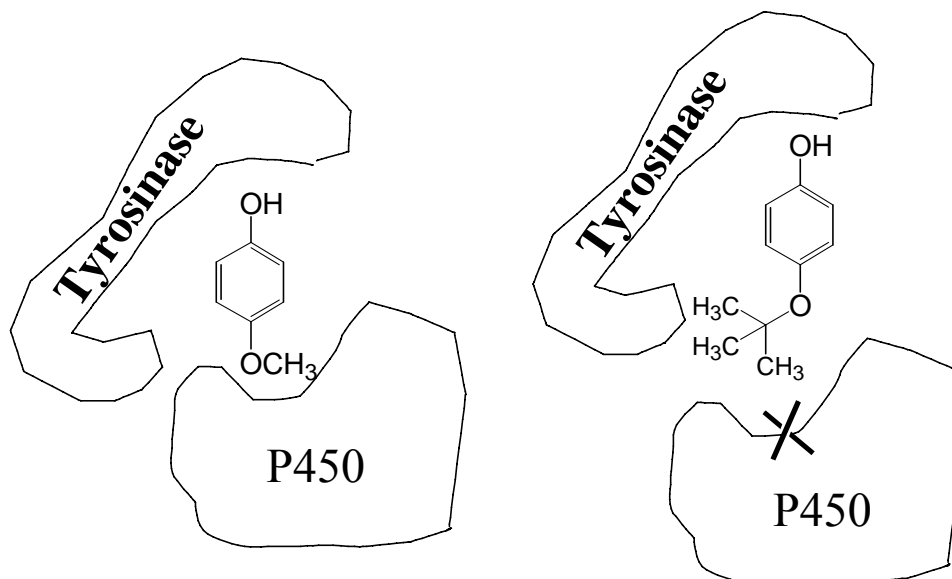
by liver P450 enzymes. Moreover, the study did not look into the effect of substitution on the alpha and beta carbons of the alkoxy group on the metabolism by tyrosinase/ O_2 and P450/NADPH/ O_2 metabolizing systems. Such information is invaluable in drug design of a safer anti-melanoma phenolic agent.

Therefore, ten alkoxyphenol compounds (Figure 2) with various linear and branched alkoxy side chains were selected to investigate the effect of substitution on the alpha and beta positions of the alkoxy side chain groups on the metabolism by tyrosinase/ O_2 , rat liver microsomes/NADPH/ O_2 systems and the toxicity towards murine melanoma

B16-F0 cell line. 4-HA (4-hydroxyanisole); 4-EP (4-ethoxyphenol); 4-nPP (4-n-propoxyphenol); 2-iPP (2-iso-propoxyphenol); 4-nBP (4-n-butoxyphenol); 4-tBP (4-t-butoxyphenol); and 4-nHP (4-n-hexyloxyphenol) were available commercially. 2-iPP was selected because it possessed a bulky group at the ortho position to the phenolic functional group. We hypothesized that the presence of a bulky group such as isopropoxy at ortho position of 2-iPP would prevent the phenol group from undergoing metabolism by tyrosinase/ O_2 . Three additional alkoxyphenols 4-iPP (4-iso-propoxyphenol), 4-iBP (4-iso-butoxyphenol) and 4-sBP (4-sec-butoxyphenol) were synthesized (11). These three compounds were selected to test if the substitution on the alpha carbon group (e.g. 4-iPP, 4-sBP and 4-tBP) could substantially reduce the metabolism by the P450 metabolizing system without any significant change in their metabolism by the tyrosinase/ O_2 metabolizing system. It was hypothesized that the presence of a bulky groups such as t-butoxy as substitutes to the methyl group of 4-HA in a phenolic compound such as 4-tBP should

lower the rate and extent of its metabolism by rat liver P450 enzyme, largely due to restrict hindrance imposed by the size of the group on the aromatic ring leading to a reduced rate of arene epoxide formation (Figure 5). Such molecular optimization may also increase toxicity towards the melanoma cell line due to the greater lipophilicity of the t-butoxy group in comparison to the methoxy group of 4-HA.

It was noted that the replacement of each hydrogen atom on the methoxy group ($O-CH_3$) of 4-HA substantially reduced the molecule's ability to undergo metabolism by tyrosinase monooxygenase system. For instance, 4-EP ($O-CH_2CH_3$) and 4-nPP ($O-CH_2CH_2CH_3$) with only one substitution on the alpha carbon (Figure 2) showed at least a 5-fold decrease in their ability to undergo metabolism by tyrosinase/ O_2 . This decrease was compensated as the length of the aliphatic chain was increased in compounds such as 4-nBP ($O-CH_2CH_2CH_2CH_3$, 4-iBP, and 4-nHP ($O-CH_2CH_2CH_2CH_2CH_2CH_3$) which could be due to an increase in the lipophilic properties of the corresponding molecules.



4-Hydroxyanisole (4-HA):
both P450 and tyrosinase can access the ring to metabolize 4-HA to toxic metabolites

4-t-Butoxyphenol (4-tBP): introduction of a bulky group such as t-butoxy should prevent the metabolism by P450s to toxic metabolite whereas tyrosinase still can metabolize it to catechol.

Figure 5: The proposed effect of restrict hindrance of t-butoxy on metabolism of 4-t-butoxyphenol by liver P450 enzyme and melanoma tyrosinase

The decrease in the rate of metabolism and the extent of GSH depletion was even more significant for the alkoxyphenols having two or three alkyl substitutes on the alpha carbon atom such as for 4-iPP (O-CH(CH₃)₂), 2-iPP (O-CH(CH₃)₂), 4-sBP (O-CH(CH₃)CH₂CH₃) and 4-tBP (O-C(CH₃)₃) which showed a 30-fold decrease in the extent of GSH depletion by the tyrosinase/O₂ metabolizing system. For 4-tBP, this could be because it possessed a bulkier group than other alkoxy groups. 2-iPP also possesses a bulky group [iso-propoxy (O-CH(CH₃)₂)] at the ortho position to the phenolic functional group which prevents the phenol group undergoing metabolism by tyrosinase/O₂ system.

Unlike alpha substitution, the derivatization on the beta carbon atom of aliphatic side chain did not significantly alter the rate of the metabolism of these compounds by tyrosinase/O₂ metabolizing system (Figure 2; Table 1).

The mechanism of the 4-HA metabolism by isolated rat hepatocytes and rat liver microsomes was previously investigated (6). It was found that P450 plays a major role in the metabolism of 4-HA to p-quinone and its induced cytotoxicity towards isolated rat hepatocytes. In addition, it was shown that P450 inhibitors could significantly abolish P450 mediated 4-HA induced cytotoxicity metabolism. Three mechanistic pathways were proposed for 4-HA metabolism by the P450 system (6) which included: ipso attack, O-demethylation, and arene epoxidation pathways. Because of the absence of any electronic withdrawing group in the molecular structure of 4-HA the ipso attack mechanism was not considered as a viable route for 4-HA metabolism. The investigators were unable to identify formaldehyde, a metabolic product of 4-HA metabolism if it was metabolized via the O-demethylation pathway. Therefore, by exclusion it was concluded that arene epoxidation was the correct mechanistic metabolism route for p-quinone formation by the P450/NADPH/O₂ metabolizing system (6). Other investigators were also suggested a similar mechanism of metabolism for arene epoxide formation (19). These findings led us to conclude that the introduction of a bulky group such as t-butoxy into the chemical structure of alkoxyphenol may prevent and/or limit the metabolism of 4-t-butoxyphenol by P450 system. Besides, t-butoxy lacks a hydrogen atom on the alpha carbon immediately next to oxygen atom (-O-C(CH₃)₃). This makes it unlikely for the molecule to undergo

O-dealkylation and consequently may lead to a minimal metabolism by P450/NADPH/O₂ and toxicity towards liver.

It was found that all the alkoxyphenols tested in this work showed toxicity towards murine B16-F0 melanoma cell line. The cytotoxicity of these alkoxyphenols were shown to be dose- and time-dependent with a ranking order of 4-nHP>>4-nPP, 4-HA, 4-sBP, 4-iBP, 4-EP>4-iPP>>4-tBP>2-iPP except for 2-iPP. Our data also showed that 2-iPP and 4-tBP were poor substrates for tyrosinase and thereby the least toxic substances against melanoma cells (Table 1). It was postulated that the observed enhanced toxicity could be due to their higher degree of lipid solubility, which provides these molecules with an ability to cross the cell membrane more readily. Such increase in lipid solubility could ultimately compensate for the restrict hindrance imposed by the additional derivatization on the alpha position on alkoxy group as a preventive factor for metabolism by tyrosinase/O₂ as discussed above. Based on the data presented in the Tables 1 and 2, it is expected that 4-nHP at LC₁₀ and LC₅₀ concentrations leads to a lower amount of GSH depletion by microsomal/NADPH/O₂ than 4-HA and, therefore, 4-nHP was identified as a potential lead anti-melanoma agent in this study.

Furthermore, our data demonstrate a direct relationship between toxicity toward B16-F0 cells and the degree of lipophilicity via one parameter QSTR Eq. 2, which can be considered an invaluable tool for estimating the toxicity of untested alkoxyphenols in future. There is no clear reason for the anomalous behavior of 4-HA, 2-iPP and 4-tBP except that the alkoxy group of the latter two phenols may hinder tyrosinase to hydroxylate the aromatic ring effectively to the corresponding catechol analogues.

The presence of 4-HA as an outlier with more toxicity implies that other factors or specific mechanisms other than hydroxylation and o-quinone formation mediated by tyrosinase/O₂ are involved in the toxicity towards B16 melanoma cells. Previously Passi et al (8) reported that melanoma toxicity might also result from inhibition of mitochondrial electron transport. It was recently shown that polyphenols induced hepatocyte cytotoxicity correlated with mitochondrial membrane potential (20) and a collapse of hepatocyte mitochondrial membrane preceded the cytotoxicity of most phenols towards rat liver hepatocytes.

Table 2: The calculated percentage GSH depletion by microsomal metabolizing system calculated for LC₁₀ and LC₅₀ (μM) of the alkoxyphenols

Alkoxyphenol	Lethal concentration		%GSH depleted by P450/NADPH/O ₂ metabolizing system		
	Calculated LC ₁₀ (μM) ^a	Calculated LC ₅₀ (μM) ^a	Calculated for LC ₁₀ (μM) concentration ^b	Calculated for LC ₅₀ (μM) concentration ^b	Measured at 100 (μM) concentration
4-hydroxyanisole*	10**	58**	6%**	33%**	58±2**
4-n-propoxyphenol	1	58	<1%	35%	61±5
4-n-butoxyphenol	6	46	4%	32%	69±6
4-t-butoxyphenol	27	200	7%	50%	25±3*
4-n-hexyloxyphenol*	<1*	26*	<1%*	17%*	66±3

* Significantly different (*t*-test, $P < 0.05$) from 4-HA (marked as **) for selected data.

^a LC₁₀ and LC₅₀ concentrations were calculated from equations presented in Table 1.

^b GSH% depletions at LC₁₀ and LC₅₀ were calculated by dividing the LC concentration by 100 followed by multiplying the product by GSH% depletion at 100 (μM).

In addition, we investigated the QSTR for phenols (21), catechols (22) and polyphenols (23) in isolated rat hepatocytes in which it was found that the phenols with higher lipophilicity, bond dissociation or σ^+ values or with lower pKa and redox potential were more toxic towards hepatocytes (21-23). However, one should note that all the phenols studied in this work differ only significantly in their lipid solubility properties but not other physico-chemical properties. Our previous studies in hepatocyte (21-23) indicate that one or a combination of mechanisms; i.e. mitochondrial uncoupling, phenoxy radical, or phenol metabolism to quinone methides and quinones, contribute to phenol cytotoxicity towards hepatocytes depending on the phenol chemical structure. Therefore, a similar cytotoxic mechanism may contribute to the cytotoxicity of alkoxyphenols towards melanoma B16-F0 cell line.

In summary, 4-nHP was identified as a potential lead anti-melanoma compound. However, before a conclusion can be made on how effective 4-nHP might be in the treatment of melanoma, further investigations into its mechanism of toxicity, in vivo metabolism and pharmacokinetic profiles are required and these are currently under investigation in our laboratory.

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