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Structure-toxicity relationship of phenolic analogs as anti-melanoma agents: An enzyme directed prodrug approach

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Abstract

The aim of this study was to identify a phenolic prodrug compound that is minimally metabolized by rat liver microsomes, but yet could form quinone reactive intermediates in melanoma cells as a result of its bioactivation by tyrosinase. In current work, we investigated 24 phenolic compounds for their metabolism by tyrosinase, rat liver microsomes and their toxicity towards murine B16-F0 and human SK-MEL-28 melanoma cells. A linear correlation was found between toxicities of phenolic analogs towards SK-MEL-28 and B16-F0 melanoma cells, suggesting similar mechanisms of toxicity in both cell lines. 4-HEB was identified as the lead compound. 4-HEB (IC₅₀ 48h, 75 μ M) showed selective toxicity towards five melanocytic melanoma cell lines SK-MEL-28, SK-MEL-5, MeWo, B16-F0 and B16-F10, which express functional tyrosinase, compared to four non-melanoma cells lines SW-620, Saos-2, PC3 and BJ cells and two amelanotic SK-MEL-24, C32 cells, which do not express functional tyrosinase. 4-HEB caused significant intracellular GSH depletion, ROS formation, and showed significantly less toxicity to tyrosinase specific shRNA transfected SK-MEL-28 cells. Our findings suggest that presence of a phenolic group in 4-HEB is critical for its selective toxicity towards melanoma cells.

Keywords

4-hydroxyanisole; tyrosinase; melanoma; B16-F0; SK-MEL-28; ethyl 4-hydroxybenzoate

1. Introduction

Skin cancer is one of the most common types of cancer in the United States [1]. Although rare in comparison to basal and squamous cell carcinoma of the skin, melanoma is the most lethal cancer of the skin. Human epidemiological studies have revealed a significant increase in the incidence of melanoma in Western populations; the number of cases worldwide has doubled in the past twenty years [2]. In its early stages malignant melanoma can be cured by surgical resection, but once it has progressed to the metastatic stage it is refractory to treatment and

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does not respond to the currently available therapies [3]. Novel chemotherapeutic agents for the treatment of patients with disseminated malignant melanoma are urgently needed. Because the majority of chemotherapy leads to severe systemic toxicity [4], strategies that can enhance selective drug delivery to melanoma are highly desirable. Because tyrosinase enzyme is expressed abundantly and upregulated in melanoma cells [5], it is considered to be a suitable molecular target for an enzyme directed prodrug bioactivation approach for selective drug delivery to melanoma [6]. It has also been reported that tyrosinase is present in relatively high concentrations in human melanoma tissue [7]. In a study of tyrosinase prepared from normal skin and metastatic melanoma from the same patient, 46 to 95 units per mg were found in the skin and 17,000 to 19,500 units per mg in the melanoma [7]. In this approach, phenolic based prodrugs are bioactivated to catechols and then to quinones in melanoma cells as a result of their oxidation by tyrosinase (Fig. 1). Using this approach, 4-hydroxyanisole (4-HA) was previously investigated in melanoma clinical trials [8-10]. However these clinical trials were terminated later because 4-HA caused serious liver toxicity [11]. Previously, we reported that 4-HA undergoes metabolism by liver P450 enzymes to form p-quinone which is cytotoxic to isolated rat hepatocytes [10]. Based on these findings, we hypothesized that P450 mediated bioactivation of 4-HA in hepatocytes contributed in 4-HA induced liver toxicity. Recently, using the tyrosinase directed prodrug bioactivation approach; we investigated ethyl 4-hydroxybenzoate (4-HEB), acetylsalicylic acid (ASA), N-acetyl-para-aminophenol (APAP) as anti-melanoma agents and investigated their mechanism of toxicity in human SK-MEL-28 melanoma cells [12-14]. Our findings suggested that the mechanism of 4-HEB, ASA, APAP toxicity in SK-MEL-28 included o-quinone formation, intracellular GSH depletion, ROS formation, and mitochondrial toxicity [12-14].

In the current study, our aims were: 1) to identify a lead phenolic agent that was minimally metabolized by rat liver microsomal preparations but yet could form quinone reactive intermediates in melanoma as a result of its bioactivation by tyrosinase [15]. Twenty four phenolic analogs of 4-HA and 4-HEB (Fig. 2) were investigated for their metabolism by tyrosinase, rat liver microsomal preparation and for their toxicity towards melanoma cell lines; 2) to demonstrate the selective toxicity of the lead compound towards melanoma cell lines compared to non melanoma cells lines; 3) to investigate the role of phenolic group as an essential functional group in the chemical structure of the lead prodrug compound in drug induced toxicity in SK-MEL-28 melanoma cells. A tyrosinase specific shRNA was also used to investigate the role of tyrosinase in phenolic based prodrug bioactivation.

2. Materials and methods

2.1 Materials

All materials, solvents and reagents used in this work were analytical grade with the highest degree of purity and were purchased either from Sigma-Aldrich, St. Louis, MO or Fisher-Scientific, Pittsburgh, PA. Mushroom tyrosinase was used throughout this study because purified human tyrosinase is not available commercially. DMSO was used to dissolve the phenolic agents for addition to the cell culture. Because the compounds were dissolved in DMSO, the final concentration of DMSO was 0.1% and 1% v/v in cell culture media of the cells treated with drugs. Therefore, the media for control cells contained 0.1% and 1% v/v DMSO in the experiment. DETAPAC (diethylenetriaminepentaacetic acid) was purchased from Sigma-Aldrich; St. Louis, MO. Phenolic agents that were investigated in this study were split into four groups (Fig. 2) and included: (group A prodrugs) 4-hydroxy derivatives: 4-hydroxyanisole (4-HA, **I**); 4-hydroxytoluene (4-HT, **II**), 4-hydroxybenzyl alcohol (4-HBAL, **III**), 4-hydroxyacetophenone (4-HAP, **IV**), 4-hydroxybenzoic acid (4-HBA, **V**), ethyl 4-hydroxybenzoate (4-HEB, **VI**); (group B prodrugs) 3-hydroxy derivatives: 3-hydroxyanisole (3-HA, **VII**), 3-hydroxytoluene (3-HT, **VIII**), 3-hydroxybenzyl alcohol (3-HBAL, **IX**), 3-

hydroxyacetophenone (3-HAP, **X**), 3-hydroxybenzoic acid (3-HBA, **XI**), ethyl 3-hydroxybenzoate (3-HEB, **XII**); (group C prodrugs) 2-hydroxy derivatives: 2-hydroxyanisole (2-HA, **XIII**), 2-hydroxytoluene (2-HT, **XIV**), 2-hydroxybenzyl alcohol (2-HBAL, **XV**), 2-hydroxyacetophenone (2-HAP, **XVI**), 2-hydroxybenzoic acid (2-HBA, **XVII**), and ethyl 2-hydroxybenzoate (2-HEB, **XVIII**); and (group D prodrugs) control derivatives that lack a hydroxy functional group on the aromatic ring: anisole (**XIX**), toluene (**XX**), benzyl alcohol (**XXI**), acetophenone (**XXII**), benzoic acid (**XXIII**), and ethyl benzoate (**XXIV**). In addition, 4-methoxyanisole and ethyl 4-methoxybenzoate (E-4MB) which are the methoxy analogs of 4-HA and 4-HEB, respectively, were also included in this study to investigate the role of the 4-hydroxy functional group of 4-HA and 4-HEB in toxicity. All phenolic analogs used in this study were of analytical grade ($\geq 99\%$ purity) and available commercially.

Ribonuclease A (Cat. No. R6513) and propidium iodide (Cat. No. P4170) were obtained from Sigma-Alrich, St. Louis, MO. The SureSilencing shRNA plasmid (Cat. No. KH01771N for the Neomycin resistance) for human tyrosinase was obtained from SuperArray Bioscience Corporation, Frederick, MD. The Anti-Tyrosinase monoclonal antibody (232 $\mu\text{g}/\mu\text{l}$; Cat. No. 05-647) was obtained from Upstate Innovative Cell Signalling Solutions, Lake Placid, NY.

2.2 Cell Lines and Culture Conditions

Modified Eagle Medium Alpha (MEM) (1 $\times$) (Cat. no. 32571-036), fetal bovine serum (FBS) (Cat. No. 10082-139), and penicillin-streptomycin (10,000 units/mL; Cat. No. 15140-122) were purchased from American Type Culture Collection (ATCC[®]), Manassas, VA. Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. 11965-092) and Versene (1 $\times$, 0.2g EDTA 4Na/L in phosphate-buffered saline) (1:5000 Cat. No. 15040-066) were purchased from Invitrogen, Grand Island, NY. RPMI medium 1640 (1 $\times$) (Cat. no. 11875-119) was obtained from Invitrogen Corporation, Grand Island, NY. Cell lines were obtained from ATCC[®], Manassas, VA.

2.3 Tyrosinase mediated GSH depletion assay

A previously described method [12,13,16] using Ellman's reagent (DTNB) [17] was employed to measure the extent of GSH depletion as a result of the enzymatic oxidation of the phenolic agent by tyrosinase/ O_2 . The experiment was performed in three replicates.

2.4 Rate of AA and NADH oxidation mediated by metabolic bioactivation of the phenolic compound in the presence of tyrosinase/ O_2

A previously described method [18] was used to measure the rate of ascorbic acid (AA) and NADH oxidation mediated by metabolism of the phenolic prodrug compound by tyrosinase/ O_2 [12,13,16]. The experiment was performed in three replicates.

2.5 Animal housing and protocol

Adult male Sprague-Dawley rats, 250–300g, were obtained from Charles River Laboratories, Wilmington, MA, fed *ad libitum*, were allowed to acclimatize for 1 week on clay chip bedding in a room with a 12 h light photo cycle, an environmental temperature of 21–23°C and 50–60% relative humidity. The rats were housed under specific pathogen free conditions in accordance with the animal protocols used in current investigation. The animal protocols 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.

2.6 Rat liver microsomal preparation

The CYP2E1 induced microsomes were prepared from rats treated (*i.p.*) with inducing agent pyrazole (200 mg/kg/day) by differential centrifugation as described previously [16]. CYP2E1

induced microsomes were used because our previous study showed that 4-HA was more cytotoxic towards CYP2E1 induced isolated rat hepatocytes in comparison with normal rat hepatocytes [10]. The experiment was performed in three replicates.

2.7 Enzymatic oxidation of phenolic agent by rat liver microsomal preparation

A previously described method using Ellman's reagent (DTNB) [17] was employed to measure the extent of GSH depletion as a result of the enzymatic oxidation of the phenolic agent by rat liver P450 microsomal preparation/NADPH/O₂ system [13,16]. The experiment was performed in three replicates.

2.9 Cell viability

SK-MEL-28, Me Wo, B16-F0, B16-F10, SK-MEL-24, C32, SW-620, Saos-2, PC-3, and BJ cells were grown as described previously [13,14,19]. All the cells were seeded at 40,000 cells/well except that SK-MEL-5 cells which were seeded at 4,000 cells/well in 24-well plates. The cell viability assay was performed using yellow tetrazolium dye (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide) (MTT) [13,14,19]. Results from three independent experiments in triplicate were averaged and presented.

2.10 IC₅₀ calculation

The required concentration (IC₅₀ in μM) of the phenolic agent that can lead to a 50% decrease in cell viability 48 h after incubation was calculated from the logarithmic regression equation derived from graphing the viability of the cells at 48 h versus the concentration of the phenolic agent. Results from three independent experiments in triplicate were averaged and presented.

2.11 Tyrosinase shRNA transfection

Tyrosinase-proficient SK-MEL-28 cells (2×10^6) were transfected with the 1 μg plasmid DNAs (of all shRNA clones) for 48 h using the FuGENE6 reagent (Roche Diagnostics Corporation, Indianapolis, IN) as previously described [21,30].

shRNA3, which was most efficient in curtailing tyrosinase expression (Fig. 9A) was chosen for drug sensitivity experiments. Briefly, SK-MEL-28 cells were seeded at 160,000 cells/well in 6-well plates. 24 h after seeding, the cells were transfected with the shRNA clone as described in the previous section. 24 h later, the untreated controls and the transfected cells along with the non specific shRNA (negative control) transfected cells were treated with 4-HEB (75 μM) and incubated further for 48 h. Cell viability was assayed by MTT assay [19] by the procedure detailed above. Results from three experiments were averaged and presented.

2.12 Intracellular GSH measurement

Exponentially growing cells were seeded at 1×10^6 cells/mL in DMEM Alpha media supplemented with (10%) FBS in 24-well plates. 3 h after incubation at 37°C, the cells were treated with an additional 1 mL of media with varying concentrations of 4-HEB and E4MB for 1, 2 and 3 h, respectively. Intracellular GSH was determined as described previously [12, 13,22] in melanocytic SK-MEL-28 and amelanotic C32 melanoma cells [23]. Results from three experiments were averaged and presented.

2.13 Reactive oxygen species (H₂O₂) formation

Melanocytic SK-MEL-28 and amelanotic C 32 cells were seeded at 50,000 cells per well in 96-well black microplates (Cat. No. 3603, Corning Incorporated, Corning NY). Reactive oxygen species (ROS) formation was determined using 2',7'-dichlorofluorescein diacetate as described previously [12,24]. Results from six replicates were averaged and presented.

2.14 Cell cycle analyses

0.3×10^6 melanocytic SK-MEL-28 melanoma cells were plated in a T-75 flask in 16 ml MEM media and allowed to attach overnight. 24 h after seeding the cells, the desired concentrations (75-500 μ M) of phenolic prodrug compound were added to the T-75 flask in 4 ml media. Equal volume of DMSO was added to control. 48 h after incubation, cells were detached using trypsin-EDTA solution (2-5 mL). Cell cycle distribution was assessed by flow cytometry after staining the cells with propidium iodide as described previously [25] in melanocytic SK-MEL-28 melanoma cells. Results from three independent experiments were averaged and presented.

2.15 Partition coefficient

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

2.16 Statistical Analysis

Results from three to six replicate experiments have been reported as mean $\pm$ SD. An analysis of variance (ANOVA) was carried out to compare the percentage of surviving cells for each compound at various concentrations followed by Bonferoni's post t-test.

3. Results

3.1 Tyrosinase mediated GSH depletion assay

Most of the 4-hydroxy derivatives tested in this study underwent metabolic oxidation by tyrosinase/ O_2 metabolizing system more than their corresponding 2-hydroxy and 3-hydroxy derivatives (Table 1). As an example, the order of metabolism for 4-hydroxy derivatives by tyrosinase/ O_2 was 4-hydroxyanisole $\gg$ 4-hydroxytoluene $>$ 4-hydroxybenzyl alcohol $>$ 4-hydroxyacetophenone $>$ 4-hydroxybenzoic acid and ethyl 4-hydroxybenzoate (4-HEB).

In order to investigate the role of phenolic functional group in the oxidation of these compounds, ethyl 4-methoxybenzoate (E4MB), in which the 4-hydroxy functional group is masked by a methoxy group was also investigated for its metabolism by tyrosinase. When metabolized by tyrosinase at 2 h incubation time, 4-HEB depleted 24% of GSH, which translates to 0.48 mol GSH per mole of 4-HEB oxidation whereas E4MB underwent negligible metabolism by tyrosinase/ O_2 at 2 h incubation (Table 3). Negligible GSH depletion occurred in the absence of the enzyme. Our findings suggests that the presence of the phenolic functional group in 4-HEB played a central role in 4-HEB metabolic bioactivation to a catechol and then a quinone by tyrosinase, which could then react with GSH in the solution (Fig. 1).

3.2 The rate of AA and NADH oxidation

4-HEB and E4MB were investigated for their ability to oxidize ascorbic acid (AA) and NADH in the presence of tyrosinase. The rate of AA and NADH oxidation as a result of 4-HEB metabolism by tyrosinase were 143 nM of AA and 389 nM of NADH per 4-HEB (μ M) per tyrosinase (unit/ml) per min, respectively (Fig. 3). However, the rate of AA and NADH oxidation mediated by tyrosinase in the presence of E4MB, which lacks the phenol functional group, were 8 nM of AA and 50 nM of NADH per E4MB (μ M) per tyrosinase (unit/ml) per min, respectively (Fig. 3, Table 3), which were found to be significantly lower than 4-HEB. These findings indicate that because 4-HEB possesses a phenolic functional group it can readily undergo bioactivation by tyrosinase to a quinone, which can in turn oxidize AA and NADH, rapidly.

3.3 Metabolism by rat liver P450 microsomal preparation/NADPH/O₂

Previously, we showed that CYP2E1 induced isolated rat hepatocytes were more susceptible to the toxic effects of phenolic based compounds [26]. CYP2E1 induced rat liver microsomes was used to investigate the metabolism of these phenol. There was no distinctive order of metabolism observed for the 2-, 3- and 4-hydroxy derivatives when the compounds were incubated by the CYP2E1 induced rat liver microsomal preparations (Table 1). 4-HEB and 4-hydroxybenzoic acid were found to be metabolized the least by rat liver microsomal preparation. Negligible GSH depletion occurred in the absence of the enzyme.

3.4 Cell viability

The cell viability was measured using trypan blue exclusion test [20] and was always greater than 90% before seeding the cells.

3.5 Anti-proliferative effects in cell lines

The required concentrations of the compound that can cause 50% decrease in cell viability 48 h after incubation were reported as (IC₅₀ μM or mM, 48 h) and are given in Table 1. Our findings indicated that the toxicity of the compounds with phenolic functional group were dose- and time-dependent. The compounds that lack the hydroxy functional group were used as negative controls. The negative controls showed no dose- and time-dependency at concentrations between 1-5 mM.

The order of toxicity (IC₅₀ at 48 h) for 4-hydroxy derivatives in both human SK-MEL-28 and murine B16-F0 melanoma cell lines was identical. For instance, the order of toxicity in human SK-MEL-28 melanoma cells was: 4-hydroxyanisole and ethyl 4-hydroxybenzoate >>> 4-hydroxytoluene, 4-hydroxybenzyl alcohol >> 4-hydroxyacetophenone > 4-hydroxybenzoic acid. A linear correlation $IC_{50} (SK-ME-28) = 0.98 \times IC_{50} (B16-F0) + 0.17$; $n=20$, $R^2=0.94$ was found between the toxicity of phenolic analogs towards human SK-MEL-28 and murine B16-F0 melanoma cells, suggesting similar mechanisms of toxicity may exist in both cell lines (Fig 3). 2-Hydroxyanisole, 3-hydroxyanisole, 4-hydroxybenzoic acid, and 4-hydroxyacetophenone were not included in the correlation as they were considered as outliers because they significantly showed more toxicity towards murine B16-F0 melanoma cell line than in human SK-MEL-28, suggesting that their mechanism of toxicity may differ in these two cell lines.

The IC₅₀ (48 h) for E4MB in human SK-MEL-28 cells melanoma cells was found to be 750 μM, demonstrating a 10-fold decrease in E4MB toxicity when compared to 4-HEB. These findings indicate that the presence of 4-hydroxy functional group in chemical structure of 4-HEB is essential for its enhanced toxicity towards melanoma cells.

When tested at 75 μM concentration, 4-HEB showed considerable toxicity in five melanocytic human SK-MEL-28, SK-MEL-5, MeWo cells and melanocytic murine B16-F0 and B16-F10 melanoma cell lines resulting in 52±4%, 57±5%, 55±8%, 62±11%, and 60±8% anti-proliferative effects, respectively (Fig. 7). However, 4-HEB (75 μM) showed 24±3% and 28±4% anti-proliferative effects towards amelanotic SK-MEL-24 and C32 cells, which do not express functional tyrosinase [23], respectively, whereas it showed 52±4% anti-proliferative effect in human melanocytic SK-MEL-28 melanoma cells (Fig. 8), which expresses functional tyrosinase [23]. In addition, 4-HEB (75 μM) demonstrated significantly lower toxicity when tested against non-melanoma cell lines such as PC-3 prostate cells (viability 85±3%), BJ skin fibroblast cells (viability 89±7%), Saos-2 osteosarcoma cells (viability 86±1%) and non melanoma SW-620 colorectal adenocarcinoma cells (viability 89±4%), which do not express tyrosinase (Fig. 7).

3.6 Drug sensitivity assay using tyrosinase shRNA transfected cells

4-HA (50 μ M) and 4-HEB (75 μ M) toxicity in SK-MEL-28 cells was investigated in tyrosinase shRNA transfected cells. A non specific shRNA (NS-shRNA) plasmid was used as control (Fig. 9A). Transfection with the shRNA clone 3 which curtailed tyrosinase expression by 50% prevented the toxicity of 4-HEB (75 μ M) for $24 \pm 7\%$ in SK-MEL-28 melanoma cells (Fig. 9B). Cells transfected with non specific shRNA plasmid (NS-shRNA) did not offer any protection against 4-HEB toxicity in SK-MEL-28 melanoma cells.

3.7 Intracellular GSH measurement

Our findings indicate that 4-HEB (75, 150, and 300 μ M) depleted 37%, 41%, and 45% of intracellular GSH, respectively, at 2 h incubation with human melanocytic SK-MEL-28 melanoma cells (Fig. 5A), whereas 4-HEB (75, 150, and 300 μ M) depleted only 8%, 13%, and 16% of intracellular GSH, respectively, at 2 h incubation with human amelanotic C32 melanoma cells (Fig. 5B). Additionally, E4MB were investigated for its effect on intracellular GSH depletion. Our findings indicate that E4MB (0.75, 1, and 1.5 mM) depleted 10%, 13%, and 15% of intracellular GSH, respectively, at 2 h incubation with human melanocytic SK-MEL-28 melanoma cells (Fig. 5C), and depleted 6%, 7%, and 10% of intracellular GSH, respectively, at 2 h incubation with human amelanotic C32 melanoma cells (Fig. 5D).

3.8 Reactive oxygen species (H_2O_2) formation

Our results indicate that 4-HEB at concentrations ranging from 75-1500 μ M at various time points showed a dose and time dependent increase in ROS formation in human melanocytic SK-MEL-28 melanoma cells (Fig. 6A). ROS formation observed in amelanotic C32 melanoma cells (Fig. 6B) which do not express functional tyrosinase [23] was significantly less than ROS formation in SK-MEL-28 cells, which express functional tyrosinase [23]. Our results indicate that when tested at even higher concentrations (750 μ M-5 mM), ethyl 4-methoxybenzoate (E4MB) caused significantly lower ROS formation in melanocytic SK-MEL-28 melanoma cells in comparison to 4-HEB, and caused negligible ROS formation in amelanotic C32 melanoma cells (Fig. 6C and D).

3.9 Cell cycle analyses of 4-HEB treatment on SK-MEL-28 cells

As summarized in Table 2, SK-MEL-28 cells treated with 4-HEB (75, 150, 250, and 500 μ M) caused corresponding increase in the percentage of sub G_0/G_1 cells undergoing apoptosis and an increase in the percentage of cells in the S phase in comparison to non-treated control cells. The total percentage increase in cells in the sub G_0/G_1 and S phase was accompanied by a corresponding decrease in cells in the G_1 phase.

3.10 Partition coefficient

The LogP value of 4-hydroxy phenolic compounds (Table 1) followed an order of ethyl 4-hydroxybenzoates > 4-hydroxytoluene > 4-hydroxyacetophenone and 4-hydroxyanisole > 4-hydroxybenzoic acid > 4-hydroxybenzyl alcohol which corresponds to phenols with the following functional groups at the para position to the phenolic group: $-\text{COOEt}$ > $-\text{CH}_3$ > $-\text{C}(\text{CH}_3)=\text{O}$, $-\text{OCH}_3$, > $-\text{COOH}$ > $-\text{CH}_2\text{OH}$. It should be noted that benzoic acid derivatives are ionized more than 99.99% at pH 7.4 and therefore LogP has a limited value in the prediction of toxicity for these phenolic compounds towards melanoma cell lines. 2-Hydroxy derivatives with $-\text{COOEt}$, $-\text{C}(\text{CH}_3)=\text{O}$, $-\text{COOH}$ and $-\text{CH}_2\text{OH}$ functional groups had a higher LogP value than 4-hydroxy and 3-hydroxy derivatives for phenolic compounds mainly due to an intramolecular hydrogen binding which hinders the molecule from interaction with aqueous environment. Toluene, anisole, benzyl alcohol, acetophenone, benzoic acid and ethyl benzoate that lacked a phenol demonstrated higher LogP value than their corresponding hydroxy derivatives.

4. Discussion

Riley's group had previously investigated the structure activity relationship of tyrosinase dependent cytotoxicity of a series of substituted linear alkoxyphenols [6]. However, their study did not investigate the metabolic bioactivation of alkoxyphenols by liver rat microsomal preparations enzymes. Moreover the influence of hydrophilic functional groups such as alcohol and carboxylic acid on the metabolism of phenols by melanoma tyrosinase was not addressed previously. Such information is invaluable in the design of a safer anti-melanoma phenolic based prodrug.

In this study, 24 phenolic analogs were investigated with heterogeneous functional groups (2-, 3-, and 4-hydroxy derivatives of anisole, toluene, benzyl alcohol, acetophenone, benzoic acid and ethyl benzoate) (Fig. 2) to identify compounds that are bioactivated by tyrosinase but not by rat liver microsomes and hence limiting their toxicity towards liver. In this study, we have found that 4-hydroxy derivatives generally demonstrated a higher metabolic bioactivation with tyrosinase with a lower metabolic bioactivation with CYP2E1 induced rat liver microsomal preparations. Our findings showed that 4-hydroxybenzyl alcohol, 4-hydroxybenzophenone, 4-hydroxybenzoic acid and its ethyl ester were metabolized substantially less than 4-HA and 4-hydroxytoluene by CYP2E1 induced rat liver microsomal preparation indicating that the introduction of a water soluble functional group such as hydroxyl group or carboxy group hindered their metabolic bioactivation by rat liver microsomal preparation substantially. CYP2E1 induced microsomes were used because our previous study showed that 4-HA was more cytotoxic towards CYP2E1 induced isolated rat hepatocytes in comparison with normal rat hepatocytes [10]. However, since induced rat microsomes were used as a source of CYP2E1, these results should be interpreted with caution as there may be species differences in the bioactivation of prodrugs by CYP2E1. Use of commercially available human CYP2E1 (supersomes) for investigation of bioactivation of prodrugs would constitute an important avenue of such research in the future.

Our study suggests that the oxidation state, presence of an electron donating/withdrawing group and position of the functional group on the phenol all play a major role in determining the extent of metabolism by tyrosinase and rat liver microsomal preparations, and the toxicity of these compounds towards melanoma, which is in keeping with earlier reports in literature [27,28].

In cytotoxicity studies, although 4-hydroxybenzoic acid was not toxic towards the melanoma cell lines its ethyl ester derivative was the second most toxic phenolic analog tested in the study after 4-HA. 4-Hydroxybenzoic acid showed an IC_{50} (48 h) of 11.2 mM in SK-MEL-28 cells, while its ethyl ester prodrug ethyl 4-hydroxybenzoate was demonstrated to have an IC_{50} (48 h) of 75 μ M. The lack of induced toxicity could be due to the extensive ionization of 4-hydroxybenzoic acid in culture media at pH 7.4, thereby limiting its entry into the melanoma cells. The presence of an ester group in chemical structure of ethyl 4-hydroxybenzoate prevents the ionization and thereby the molecule can diffuse across the cell unhindered and contribute towards cell toxicity leading to a 150-fold increase in the toxicity of ethyl 4-hydroxybenzoic acid in comparison to its carboxylic acid derivative.

In an earlier study, we observed a direct relationship between toxicity towards murine B16-F0 cells and the degree of lipophilicity was observed via a one parameter quantitative structure activity relationship (QSTR) equation [18]. However, in the current study some of the compounds did not follow the general rule of the greater lipid solubility the greater cytotoxicity. For instance even though 4-hydroxytoluene has similar lipid solubility and metabolic bioactivation by tyrosinase metabolism profiles to that of 4-HA, 4-hydroxytoluene was 40 fold less toxic towards both murine and human melanoma cell lines than 4-HA. The reason that 4-

HA was the more cytotoxic than 4-hydroxytoluene could be attributed to the presence of an electron donating group of methoxy at para position in 4-HA.

Despite being metabolized less by tyrosinase, ethyl 4-hydroxybenzoate demonstrated a comparable toxicity to that of 4-HA towards the melanoma cell lines. One reason could be that ethyl 4-hydroxybenzoate possesses a higher degree of lipid solubility than 4-HA (Table 1) which makes it easier for ethyl 4-hydroxybenzoate to enter the cells. The second reason could be that we have used mushroom tyrosinase for in vitro metabolic bioactivation because purified human tyrosinase is not available commercially. In addition, factors other than lipophilicity, electron donating/withdrawing groups, and metabolic bioactivation by tyrosinase may be responsible for toxicity of these phenolic compounds towards B16-F0 and SK-MEL-28 cells.

Additionally, in order to investigate the role of the 4-hydroxy functional group in cytotoxicity towards melanoma cells and their bioactivation with tyrosinase, ethyl 4-methoxybenzoate (E4MB) and 4-methoxyanisole (4-MA), in which the 4-hydroxy functional group is masked by a methoxy group, were used as controls for 4-HEB and 4-HA, respectively, because they lack hydroxy functional group. Table 3, summarizes the difference between 4-HEB and E4MB, and highlights that 4-HEB selectively undergoes metabolic bioactivation by tyrosinase, and leads to significant GSH depletion, NADH and AA oxidation mediated by tyrosinase bioactivation, and selective ROS and intracellular GSH depletion and cytotoxicity in SK-MEL-28 cells when compared to E4MB, which lacks a hydroxyl functional group.

In order to investigate the role of tyrosinase, the cytotoxicity of 4-HEB was also investigated in amelanotic C32 and SK-MEL-24 melanoma cell lines, which do not express tyrosinase [23]. It was found that 4-HEB demonstrated significantly less toxicity towards amelanotic C32 and SK-MEL-24 melanoma cell lines than toward melanocytic SK-MEL-28 melanoma cells, which express tyrosinase [23]. 4-HEB also leads to significantly less ROS formation and ICG depletion in amelanotic C32 than in melanocytic SK-MEL-28. Moreover, an earlier report, Smit et al. [29] showed that prolonged exposure to agents that exhibited tyrosinase-dependent toxicity resulted in reduction of cell numbers and cell damage, which was detectable by various methods. In addition, 4-HEB showed selective toxicity towards five melanocytic melanoma cell lines SK-MEL-28, SK-MEL-5, MeWo, B16-F0 and B16-F10 compared to four non-melanoma cells lines SW-620, Saos-2, PC3, and BJ cells. Our results also indicate that 4-HA and 4-HEB showed significantly less cell toxicity when tyrosinase was silenced by shRNA plasmid, thus providing further evidence that melanocytotoxicity was mediated in part by tyrosinase.

In summary, ethyl 4-hydroxybenzoate was identified to be the most suitable cytotoxic phenol from this series against murine B16 and human SK-MEL-28 melanoma cells with minimal metabolism by rat liver microsomal preparations. Therefore, similar to our previous work [12], our data suggests that ethyl 4-hydroxybenzoate may have potential as a lead anti-melanoma compound. However, further investigation into its in vivo efficacy against melanoma tumor model in mice and in vivo metabolism, toxicology and pharmacokinetic profiles is required. One should also note that 4-HEB may also cause toxicity towards cells having high tyrosinase activity including dopaminergic neurons, pigmented retinal epithelial cells, and melanocytes which should be also investigated.

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References

1. Ries, LAG.; Eisner, MP.; Kosary, CL.; Hankey, BF.; Miller, BA.; Clegg, L.; Mariotto, A.; Feuer, EJ.; Edwards, BK., editors. SEER Cancer Statistics Review, 1975-2001. National Cancer Institute; Bethesda, MD: 2004. http://seer.cancer.gov/csr/1975_2001/
2. Anderson CM, Buzaid AC, Legha SS. Systemic treatments for advanced cutaneous melanoma. *Oncology (Williston Park)* 1995;9(11):1149–1158. discussion 1163-1164, 1167-1168. [PubMed: 8703684]
3. Riley PA. Hydroxyanisole depigmentation: In-vitro studies. *J Pathol* 1969;97(2):193–206. [PubMed: 4982031]
4. Ojima I. Guided molecular missiles for tumor-targeting chemotherapy--case studies using the second-generation taxoids as warheads. *Acc Chem Res* 2008;41(1):108–119. [PubMed: 17663526]
5. Vielkind U, Schlage W, Anders F. Melanogenesis in genetically determined pigment cell tumors of platyfish and platyfish-swordtail hybrids: correlation between tyrosine activity and degree of malignancy. *Cancer Res Clin Oncol* 1977;90(3):285–299.
6. Jordan AM, Khan TH, Malkin H, Osborn HM, Photiou A, Riley PA. Melanocyte-directed enzyme prodrug therapy (MDEPT): Development of second generation prodrugs for targeted treatment of malignant melanoma. *Bioorg Med Chem* 2001;9(6):1549–1558. [PubMed: 11408174]
7. Chem YM, Chavin W. Tyrosinase activity in highly pigmented human melanoma and in negro skin. *Proc Soc Exp Biol Med* 1974;145:695–698. [PubMed: 4205290]
8. Naish S, Cooksey CJ, Riley PA. Initial mushroom tyrosinase-catalysed oxidation product of 4-hydroxyanisole is 4-methoxy-ortho-benzoquinone. *Pigment Cell Res* 1988;1(6):379–381. [PubMed: 3148921]
9. Naish S, Holden JL, Cooksey CJ, Riley PA. Major primary cytotoxic product of 4-hydroxyanisole oxidation by mushroom tyrosinase is 4-methoxy ortho benzoquinone. *Pigment Cell Res* 1988;1(6):382–385. [PubMed: 3148922]
10. Moridani MY, Cheon SS, Khan S, O'Brien PJ. Metabolic activation of 4-hydroxyanisole by isolated rat hepatocytes. *Drug Metab Dispos* 2002;30(10):1063–1069. [PubMed: 12228181]
11. Rustin GJ, Stratford MR, Lamont A, et al. Phase I study of intravenous 4-hydroxyanisole. *Eur J Cancer* 1992;28A(89):1362–1364. [PubMed: 1515252]
12. Vad NM, Shaik IH, Mehvar R, Moridani MY. Metabolic bioactivation and toxicity of ethyl 4-hydroxybenzoate in human SK-MEL-28 melanoma cells. *J Pharm Sci* 2008;97(5):1934–1945. [PubMed: 17847068]
13. Vad NM, Yount G, Moore D, Weidanz J, Moridani MY. Biochemical mechanism of acetaminophen (APAP) induced toxicity in melanoma cell lines. *J Pharm Sci* 2009;98(4):1409–1425. [PubMed: 18759348]
14. Vad NM, Yount G, Moridani MY. Biochemical mechanism of acetylsalicylic acid (aspirin) selective toxicity toward melanoma cell lines. *Melanoma Res* 2008;18(6):386–399. [PubMed: 18971789]
15. Naish-Byfield S, Cooksey CJ, Latter AM, Johnson CI, Riley PA. In vitro assessment of the structure-activity relationship of tyrosinase-dependent cytotoxicity of a series of substituted phenols. *Melanoma Res* 1991;1(4):273–287. [PubMed: 1823634]
16. Moridani MY. Biochemical basis of 4-hydroxyanisole induced cell toxicity towards B16-F0 melanoma cells. *Cancer Lett* 2006;243(2):235–245. [PubMed: 16427188]
17. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959;82(1):70–77. [PubMed: 13650640]
18. Moridani MY, Moore M, Bartsch RA, Yang Y, Heibati-Sadati S. Structural toxicity relationship of 4-alkoxyphenols' cytotoxicity towards murine B16-F0 melanoma cell line. *J Pharm Pharm Sci* 2005;8(2):348–360. [PubMed: 16124947]
19. Wu X, Zeng H, Zhang X, et al. Phosphatase of regenerating liver-3 promotes motility and metastasis of mouse melanoma cells. *Am J Pathol* 2004;164(6):2039–2054. [PubMed: 15161639]
20. Moridani MY, Cheon SS, Khan S, O'Brien PJ. Metabolic activation of 3-hydroxyanisole by isolated rat hepatocytes. *Chem Biol Interact* 2003;142(3):317–333. [PubMed: 12453669]

21. Johansen IE. Intron insertion facilitates amplification of cloned virus cDNA in escherichia coli while biological activity is reestablished after transcription in vivo. *Proc Natl Acad Sci USA* 1996;93(22): 12400–12405. [PubMed: 8901593]
22. Shaik IH, Mehvar R. Rapid determination of reduced and oxidized glutathione levels using a new thiol-masking reagent and the enzymatic recycling method: Application to the rat liver and bile samples. *Anal Bioanal Chem* 2006;385(1):105–113. [PubMed: 16547740]
23. Hiroaki K, Yamashita T, Wada I, Jin HY, Jimbow K. Tyrosinase and tyrosinase-related protein 1 require Rab7 for their intracellular transport. *J Invest Dermatol* 2002;119(2):475–480. [PubMed: 12190873]
24. Siraki AG, Chan TS, O'Brien PJ. Application of quantitative structure-toxicity relationships for the comparison of the cytotoxicity of 14 p-benzoquinone congeners in primary cultured rat hepatocytes versus PC12 cells. *Toxicol Sci* 2004;81(1):148–159. [PubMed: 15178806]
25. Lu M, Strohecker A, Chen F, et al. Aspirin sensitizes cancer cells to TRAIL-induced apoptosis by reducing survivin levels. *Clin Cancer Res* 2008;14(10):3168–3176. [PubMed: 18483385]
26. Moridani MY, Siraki A, O'Brien PJ. Quantitative structure toxicity relationships for phenols in isolated rat hepatocytes. *Chem Biol Interact* 2003;145(2):213–223. [PubMed: 12686497]
27. Kasraee B. Peroxidase-mediated mechanisms are involved in the melanocytotoxic and melanogenesis-inhibiting effects of chemical agents. *Dermatology* 2002;205(4):329–339. [PubMed: 12444326]
28. Passi S, Nazzaro-Porro M. Molecular basis of substrate and inhibitory specificity of tyrosinase: Phenolic compounds. *Br J Dermatol* 1981;104(6):659–665. [PubMed: 6788064]
29. Smit NP, Peters K, Menko W, Westerhof W, Pavel S, Riley PA. Cytotoxicity of a selected series of substituted phenols towards cultured melanoma cells. *Melanoma Res* 1992;2(56):295–304. [PubMed: 1292781]
30. Vad NM, Kudugunti SK, Graber D, Bailey N, Srivenugopal K, Moridani MY. Efficacy of acetaminophen in skin B16-F0 melanoma tumor-bearing C57BL/6 mice. *Int J Oncol* 2009;35(1): 193–204. [PubMed: 19513568]

Abbreviations list

DETAPAC	Diethylenetriaminepentaacetic acid
FBS	fetal bovine serum
MEM	Minimum essential Medium Alpha
DMEM	Dulbecco's modified eagle medium
DTNB	5'-Dithiobis-(2-nitrobenzoic acid)
AA	Ascorbic Acid
MTT	(3-(4, 5-Dimethylthiazolyl)-2)-5-diphenyl tetrazolium bromide)
shRNA	Short hairpin RNA
PMSF	phenylmethylsulphonyl fluoride
SSA	sulphosalicylic acid
ROS	Reactive oxygen species
MPT	Membrane permeability transition
4-HA	4-hydroxyanisole
4-HT	4-hydroxytoluene
4-HBAL	4-hydroxybenzyl alcohol
4-HAP	4-hydroxyacetophenone

4-HBA	4-hydroxybenzoic acid
4-HEB	ethyl 4-hydroxybenzoate
3-HA	3-hydroxyanisole
3-HT	3-hydroxytoluene
3-HBAL	3-hydroxybenzyl alcohol
3-HAP	3-hydroxyacetophenone
3-HBA	3-hydroxybenzoic acid
3-HEB	ethyl 3-hydroxybenzoate
2-HA	2-hydroxyanisole
2-HT	2-hydroxytoluene
2-HBAL	2-hydroxybenzyl alcohol
2-HAP	2-hydroxyacetophenone
2-HBA	2-hydroxybenzoic acid
2-HEB	ethyl 2-hydroxybenzoate
E-4MB	ethyl 4-methoxybenzoate

Fig. 1.
Postulated biochemical mechanism of action for phenolic prodrug toxicity in melanoma cells.

Fig. 2.
Chemical structures of phenolic analogs.

Fig. 3.

Enzymatic oxidation of 4-HEB and E4MB at pH 7.4 by tyrosinase. AA and NADH oxidations were monitored at 266 nm and 340 nm, respectively. Ethyl 4-hydroxybenzoate (4-HEB); Ethyl 4-methoxybenzoate (E4MB); n=3.

Fig. 4. Toxicity correlation between IC_{50} of phenolic agents in murine B16-F0 and human SK-MEL-28 melanoma cell lines. 2-Hydroxyanisole, 3-hydroxyanisole, 4-hydroxybenzoic acid, 4-hydroxyacetophenone were considered as outliers, $n=3$.

Fig. 5.

Intracellular GSH depletion in human melanocytic SK-MEL-28 and amelanotic C32 melanoma cells. (A) 4-HEB (75 μ M-300 μ M) showed dose and time dependent intracellular GSH depletion in human melanocytic SK-MEL-28 melanoma cells. (B) 4-HEB (75 μ M-300 μ M) caused significantly less GSH depletion in amelanotic C32 melanoma cells. (C-D) E4MB (0.75, 1.0, and 1.5 mM) caused significantly less intracellular GSH depletion in SK-MEL-28 and C32 melanoma cells. The IC_{50} (48 h) for 4-HEB and E4MB in SK-MEL-28 cells was 75 μ M and 750 μ M, respectively. Ethyl 4-hydroxybenzoate (4-HEB); Ethyl 4-methoxybenzoate (E4MB), n=3.

(A) 4-HEB induced ROS formation in melanocytic human SK-MEL-28 cells

(B) 4-HEB induced ROS formation in amelanotic human C32 cells

(C) E4MB induced ROS formation in melanocytic human SK-MEL-28 cells

(D) E4MB induced ROS formation in amelanotic human C32 cells

Fig. 6. ROS formation in human melanocytic SK-MEL-28 and amelanotic C32 melanoma cells. (A) 4-HEB (75-1500 μ M) led to a time- and dose dependent escalation in ROS formation in human melanocytic SK-MEL-28 cells. (B) 4-HEB (75-1500 μ M) led to significantly less ROS formation in amelanotic C32 melanoma cells. (C-D) E4MB (750 μ M -5 mM) lead to significantly less ROS formation in human melanocytic SK-MEL-28 and amelanotic C32 melanoma cells, n=6.

Fig. 7. 4-HEB toxicity in melanocytic melanoma cells (black bars) and non-melanoma (open bars) cell lines. * significantly different, n=3.

Fig. 8.
4-HEB toxicity in melanocytic melanoma and amelanotic melanoma cell lines. * significantly different, n=3.

Fig. 9.

4-HEB toxicity in the presence and absence of tyrosinase shRNA silencing plasmid. (A) Tyrosinase protein levels were detected by western blotting with a specific anti-tyrosinase monoclonal antibody. Transfection with shRNA3 clone curtailed tyrosinase expression. (B) 4-HEB (75 μ M) caused significantly less toxicity in SK-MEL-28 cells transfected with shRNA plasmid directed against tyrosinase. * significantly different when compared to control, n=3.

Table 1

Metabolism of phenolic compounds by tyrosinase and rat liver microsomal preparations and their cytotoxicity IC₅₀ (48 h) towards murine B16-F0 and human SK-MEL-28 cell lines.

Phenolic agent	Tyrosinase/O ₂	% GSH depletion (2 h)	IC ₅₀ (48 h)	LogP
4-hydroxy analogs				
4-hydroxytoluene	88±4	60±7	1.9	1.93
4-hydroxyanisole	95±3 [†]	44±8	0.06	1.56
4-hydroxybenzyl alcohol	70±6	18±2	2.2	0.58
4-hydroxyacetophenone	56±6	19±3	3.0	1.63
4-hydroxybenzoic acid	30±5	11±4	4.2	1.36
Ethyl 4-hydroxybenzoate	24±4	15±2	0.190	2.11
3-hydroxy analogs				
3-hydroxytoluene	79±8	51±4	2.1	1.88
3-hydroxyanisole	24±3	21±3	0.043	1.54
3-hydroxybenzyl alcohol	29±3	8±2	4.6	0.37
3-hydroxyacetophenone	60±5	48±4	1.4	1.50
3-hydroxybenzoic acid	10±1	24±3	1.8	1.29
Ethyl 3-hydroxybenzoate	9±3	16±4	1.9	1.97
2-hydroxy analogs				
2-hydroxytoluene	11±3	39±4	3.2	1.80
2-hydroxyanisole	25±2	24±5	0.051	1.52
2-hydroxybenzyl alcohol	0	0	1.1	0.77
2-hydroxyacetophenone	82±5	50±6	3.5	2.02
2-hydroxybenzoic acid	0	12±3	5.2	1.88
Ethyl 2-hydroxybenzoate	22±2	4±1	2.6	2.48
Non-hydroxy analogs				
Toluene	26±2	11±1	>10	2.66
Anisole	21±3	20±3	>5	2.03
Benzyl alcohol	0	36±4	8.0	1.24

Phenolic agent	% GSH depletion (2 h)			IC ₅₀ (48 h)	LogP
	Tyrosinase/O ₂	Rat liver microsomes	B16-F0 (mM)	SK-MEL-28 (mM)	
Acetophenone	44±3	18±3	>12	>10	1.74
Benzoic acid	12±2	7±2	11	>10	1.63
Ethyl benzoate	26±3	45±7	>10	7.0	2.30

^fThe GSH depletion measurement conducted at 30 min.
The Log P values were estimated using the LogP program available at www.logp.com

SK-MEL-28 cell cycle analysis after treatment with 4-HEB (48 h). 4-HEB (75, 150, 250, and 500 μ M) caused corresponding increase in the percentage of sub G₀/G₁ cells undergoing apoptosis and an increase in the percentage of cells undergoing S phase cell cycle arrest in comparison to non-treated control cells as analyzed by flow cytometry.

Table 2

SK-MEL-28 Cells					
	Treatment	Apoptosis (Sub G ₀ /G ₁)	G ₁	S	G ₂ /M
Control 4-HEB	0.1% DMSO	1.6±0.1	76.9±0.3	11.1±0.3	10.2±0.2
	75 μ M	2.9±0.3*	74.7±0.8	12.4±0.7	9.9±0.1
	150 μ M	3.1±0.2*	72.1±0.7	13.2±1.2	10.6±0.9
	250 μ M	5.0±0.9*	63.4±1.8*	20.1±2.6*	10.7±0.7
	500 μ M	6.2±0.8*	59.8±1.6*	22.1±0.9*	10.9±0.4

* significantly different, n=3.

Table 3

Summary data for 4-HEB and E4MB. 4-HEB selectively undergoes metabolic bioactivation by tyrosinase, and leads to significant GSH depletion, NADH and AA oxidation mediated by tyrosinase bioactivation, and selective ROS and intracellular GSH depletion and cytotoxicity in SK-MEL-28 cells when compared to E4MB, which lacks a hydroxy functional group.

	Tyrosinase metabolism mediated % GSH depletion (2 h)	Tyrosinase Kinetics [*]		IC ₅₀ (48 h) SK-MEL-28 cells	ICG ⁺ depletion (2 h) at IC ₅₀	Fold ROS ⁺ formation (40 min) at IC ₅₀
		NADH	AA
4-HEB	24±4%	389 nM [*]	143 nM [*]	75 μM	8%	6
					C32 cells ^d	
				SK-MEL-28 cells ^d	37%	24

Tyrosinase metabolism mediated % GSH depletion (2 h)	Tyrosinase Kinetics ^a		IC ₅₀ (48 h) SK- MEL-28 cells	SK-MEL-28 cells ^b	ICG ⁺ depletion (2 h) at IC ₅₀	Fold ROS ⁺ formation (40 min) at IC ₅₀
	NADH	AA				
5±3%	50 nM ^{**}	8 nM ^{**}	750 µM	C32 cells ^b	6%	4
					10%	9

* nM of AA or NADH/4-HEB (μM)/tyrosinase (U/ml)/min.

** nM of AA or NADH/E4MB (μM)/tyrosinase (U/ml)/min.

[†] ICG, Intracellular GSH; ROS, Reactive oxygen species

^a 4-HEB was tested at its IC₅₀ (48 h) of 75 μM

^b E4MB was tested at its IC₅₀ (48 h) of 750 μM