

Characterization of inhibitory effects of the potential therapeutic inhibitors, benzoic acid and pyridine derivatives, on the monophenolase and diphenolase activities of tyrosinase

Nematollah Gheibi ¹, Negar Taherkhani ², Abolfazl Ahmadi ², Kamahldin Haghbeen ³, Dariush Ilghari ^{4*}

¹ Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, Iran

² Department of Biology, Faculty of Basic Sciences, Islamic Azad University of Science and Research, Tehran, Iran

³ Biochemistry and Biophysics Department, National Institute for Genetic Engineering and Biotechnology, Tehran, Iran

⁴ Department of Clinical Biochemistry and Genetics, Qazvin University of Medical Science, Qazvin, Iran

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ABSTRACT

Objective(s): Involvement of tyrosinase in the synthesis of melanin and cell signaling pathway has made it an attractive target in the search for therapeutic inhibitors for treatment of different skin hyperpigmentation disorders and melanoma cancers.

Materials and Methods: In the present study, we conducted a comprehensive kinetic analysis to understand the mechanisms of inhibition imposed by 2-amino benzoic acid, 4-amino benzoic acid, nicotinic acid, and picolinic acid on the monophenolase and diphenolase activities of the mushroom tyrosinase, and then MTT assay was exploited to evaluate their toxicity on the melanoma cells.

Results: Kinetic analysis revealed that nicotinic acid and picolinic acid competitively restricted the monophenolase activity with inhibition constants (K_i) of 1.21 mM and 1.97 mM and the diphenolase activity with K_i s of 2.4 mM and 2.93 mM, respectively. 2-aminobenzoic acid and 4-aminobenzoic acid inhibited the monophenolase activity in a non-competitive fashion with K_i s of 5.15 μ M and 3.8 μ M and the diphenolase activity with K_i s of 4.72 μ M and 20 μ M, respectively.

Conclusion: Our cell-based data revealed that only the pyridine derivatives imposed cytotoxicity in melanoma cells. Importantly, the concentrations of the inhibitors leading to 50% decrease in the cell density (IC_{50}) were comparable to those causing 50% drop in the enzyme activity, implying that the observed cytotoxicity is highly likely due to the tyrosinase inhibition. Moreover, our cell-based data exhibited that the pyridine derivatives acted as anti-proliferative agents, perhaps inducing cytotoxicity in the melanoma cells through inhibition of the tyrosinase activities.

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Introduction

Tyrosinase (EC 1.14.18.1) is a copper-containing bifunctional enzyme, widely distributed in mammals, plants and micro-organisms (1). This enzyme catalyzes hydroxylation and oxidation of monophenols and diphenols, respectively (2). In fact, it catalyzes the hydroxylation of tyrosine to form 3,4-dihydroxyphenylalanine (L-DOPA), and L-DOPA to DOPA quinine (3). Quinones, in turn, develop chemically to melanins and other polyphenolic compounds (4).

Engagement of tyrosinase in important cell signaling pathways has made the enzyme an attractive target in the search for therapeutic inhibitors for prevention and treatment of different disorders including skin hyperpigmentation and

cancers (5, 6). In addition, inhibition of tyrosinase with various kinds of inhibitors has been a useful tool for gaining better understanding of the mechanism of action of the enzyme (7, 8). In the past decade, a large number of compounds such as benzaldehyde and benzoate derivatives have been identified that inhibit the enzyme activity (9–16). It is known that the aldehyde group in the benzaldehyde derivatives react with functional groups including hydroxyl, amino and sulfhydryl group (9, 10). Previous studies have indicated that benzaldehyde inhibitors restrict the enzyme activity by forming a Schiff base with a primary amino group in the enzyme structure (14, 15). It has also been suggested that benzoate inhibits tyrosinase by a copper chelating mechanism (16). It is thought that

*Corresponding author: Dariush Ilghari. Department of Clinical Biochemistry and Genetics, Qazvin University of Medical Science, Qazvin, Iran. email: d.ilghari@gmail.com

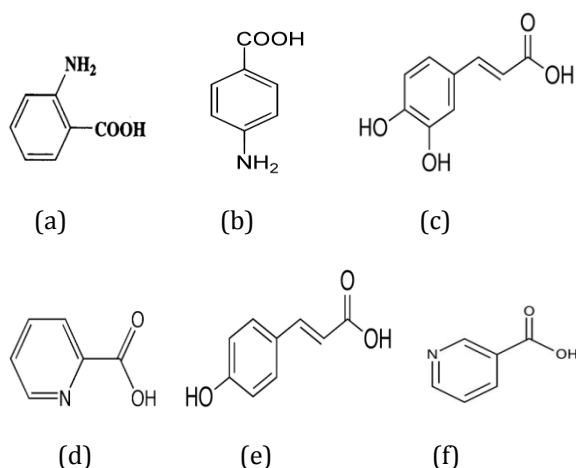


Figure 1. The chemical structures of substrates and inhibitors of Mushroom tyrosinase MT used in this study. a) 2-amino benzoic acid b) 4-amino benzoic acid c) caffeic acid d) picolinic acid e) p-coumaric acid f) nicotinic acid

carboxyl group in the molecular structure of benzoate acts as a nucleophile, perhaps chelating Cu^{2+} , the essential cofactor of tyrosinase (17).

Although aromatic derivatives, including benzoate and pyridine ones, reversibly restrict tyrosinase activity (9, 10), the mechanisms of inhibition through which they exert their inhibitory effects are not fully understood. In the present work, we, therefore, accomplished comprehensive kinetic analysis to gain better understanding of the mechanisms by which some aromatic derivatives including 2-amino benzoic acid, 4-amino benzoic acid, nicotinic acid, and picolinic acid restrict the mushroom tyrosinase activity. To do so, we kinetically analyzed both monophenolase and diphenolase activities of the enzyme in presence and absence of the inhibitors using spectroscopic methods. In addition, to check whether these inhibitors were able to induce cytotoxicity, the melanoma cell lines were treated with the inhibitors at the concentration range that they imposed their inhibitory effects on the purified mushroom tyrosinase. Our data clearly demonstrated that the aromatic derivatives reversibly inhibited monophenolase and diphenolase activities of the enzyme and despite the close structural similarity (Figure 1), these analogues showed differences in mechanism of inhibition. Importantly, our data clearly showed that only nicotinic acid and picolinic acid had cytotoxic effects on the melanoma cells.

Materials and Methods

Materials

MT (mushroom tyrosinase: EC 1.14.18.1) were prepared as previously described (18). P-hydroxycinnamic (p-coumaric acid $\lambda_{\text{max}} = 288 \text{ nm}$, $\epsilon = 19400 \text{ M}^{-1} \cdot \text{cm}^{-1}$), 3,4-dihydroxycinnamic acid (caffeic acid, $\lambda_{\text{max}} = 311 \text{ nm}$, $\epsilon = 12000 \text{ M}^{-1} \cdot \text{cm}^{-1}$), 4-amino benzoic acid, 2-amino benzoic acid, nicotinic

acid, and picolinic acid were purchased from Sigma. All other reagents were homemade analytical grade. The water used was re-distilled and ion-free; spectrophotometric measurements were carried out using a Rayleigh model 2100 spectrophotometer. The chemical formulas of all compounds (substrates and inhibitors) used in this research are given in (Figure 1). The buffer used throughout this research was 15 mM phosphate buffer solution (PBS), pH=6.8, and the corresponding salts were obtained from Merck. All experiments were carried out at 293 K and all solutions prepared in doubly distilled water. Freshly prepared enzyme and substrate solutions were used in this work.

Enzyme reactions

All enzymatic reactions were run in phosphate buffer (10 mM) at pH=6.8, in a conventional quartz cell, thermostated to maintain the temperature at $20 \pm 0.1^\circ\text{C}$. The selected conditions of solvent, buffer, pH, temperature, and enzyme concentration were applied for assaying the oxidase activity of MT according to our previous studies (19-20). Cresolase reactions were measured by depletion of p-coumaric acid for 10 min at $\lambda_{\text{max}} = 288 \text{ nm}$ and enzyme concentration of $70.8 \mu\text{M}$, 240 unit/ml. The reactions were followed using six different fixed concentrations of substrate (5–50 μM) at different concentrations of acids as inhibitors. Catecholase activity was followed by depletion of caffeic acid for 2 min at $\lambda_{\text{max}} = 311 \text{ nm}$ and enzyme concentration of $11.8 \mu\text{M}$, 40 unit/ml at different concentrations of acids as inhibitors. The reactions were carried out using fixed concentration of substrate (50 μM) at different concentrations of acids. All assays were repeated at least three times. Substrate was added when the incubation time (2 min) for the mixture of enzyme with each concentration of inhibitors was completed. Definitions of units were given by the vender. Accordingly, one unit cresolase activity was equal to 0.001 change in optical density of L-tyrosine per minute at 280 nm in 3 ml of the reaction mixture at 25°C and pH= 6.5. Similarly, one unit of catecholase activity was equal to 0.001 change in optical density of ascorbic acid per minute at 265 nm in 3 ml of the reaction mixture (25°C and pH = 6.5), when catechol or L-DOPA were used as substrates.

Inhibition analysis

The Michaelis constant (K_m) and maximum velocity (V_{max}) of the tyrosinase were determined by the Lineweaver-Burk plots. The velocity equation for the competitive inhibition in reciprocal form is: $1/V = K_m/V_{\text{max}}(1 + [I]/K_i)1/[S] + 1/V_{\text{max}}$.

The inhibition constants (K_i) of the competitive inhibitors were calculated by the following equation: $K_{m, \text{app}} = K_m [1 + ([I]/K_i)]$, where $K_{m, \text{app}}$ is the apparent K_m in the presence of an inhibitor. The reciprocal equation for the noncompetitive inhibition is:

$1/V = K_m/V_{max}(1 + [I]/K_i)1/[S] + 1/V_{max}(1 + [I]/K_i)$. K_i of the noncompetitive inhibitors were calculated using the following equation: $1/V_{maxapp} = (1 + [I]/K_i) / V_{max}$, where V_{maxapp} is the apparent V_{max} in the presence of an inhibitor.

Cytotoxicity assay

Melanoma cell lines (NCBI Code: C136; human skin) were grown in RPMI1640 (Gibco, No 51800-019) medium supplemented with 10% fetal bovine serum (FBS). The stock solutions of 2-amino benzoic acid, 4-amino benzoic acid, nicotinic acid, and picolinic acid were prepared by dissolving the compound in 100 μ l isopropanol and then dissolved by PBS buffer. The different concentrations of aromatic acids were prepared and treated with the melanoma cell lines. Cells were plated in the appropriate media on 24-well micro plates in a 500 μ l total volume at a density of 10^4 cell/ml. Triplicate wells were treated with media containing different concentrations of the extracts. The plates were incubated at 37°C in 5% CO₂ for time course of 24 hr. Cell viability was evaluated by the MTT colorimetric technique (21). The OD570 was determined using a microplate reader. Media- only treated cells served

as the indicator of 100% cell viability. The required concentration (IC₅₀) of CAPE that can lead to 50% decrease in melanoma cell viability 24 hr after incubation was calculated from the logarithmic regression equation derived from graphing the viability of the cells at 24 hr versus the concentration of aromatic acids. The calculated concentration was then used to guide further selection of aromatic acid concentrations for fine tuning of IC₅₀ determination, experimentally. Viability percentage was evaluated as (OD treatment/ OD control) \times 100 (22).

Results

Characterization of inhibitory effect of the benzoic acid and pyridine derivatives on the monophenolase activity of mushroom tyrosinase

The inhibition kinetics of benzoic acid (2-amino benzoic acid and 4-amino benzoic acid) and pyridine derivatives (nicotinic acid and picolinic acid) on the monophenolase activity of the mushroom tyrosinase were investigated using p-coumaric acid as substrate and then the kinetic parameters for the enzyme were acquired from Lineweaver-Burk double reciprocal plots and the secondary plots of the slope versus concentration of inhibitors (Figures 2A-2D).

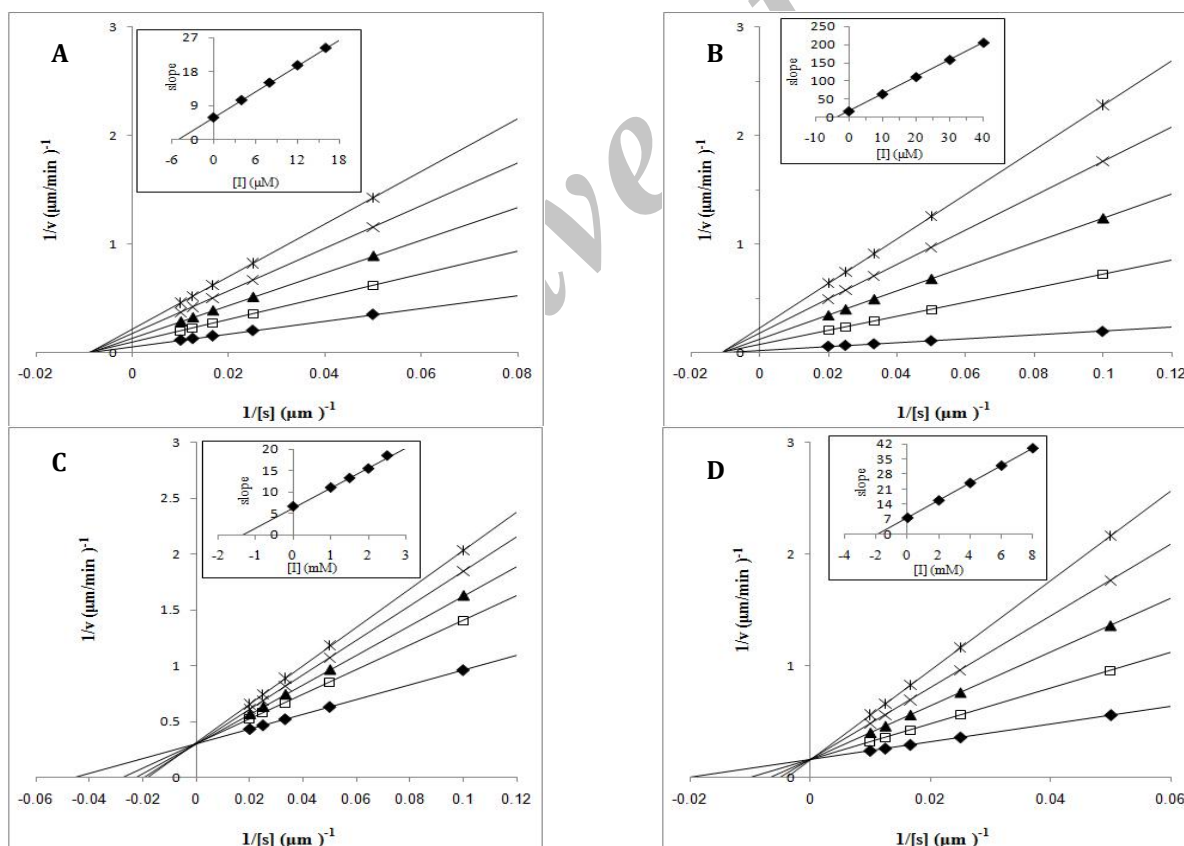


Figure 2. Double reciprocal Lineweaver-Burk plots of MT kinetic assay for monophenolase reactions. p-coumaric acid was a substrate. The reaction was done in 10 mM PBS, pH 6.8, at 20°C and 70.8 μ M enzyme, in the presence of different concentrations of (A) 2-amino benzoic acid: 0 μ M (■), 4 μ M (▲), 8 μ M (×), 12 μ M (⋈), 16 μ M (◆); (B) 4-amino benzoic acid: 0 μ M (■), 5 μ M (▲), 10 μ M (×), 20 μ M (⋈), 30 μ M (◆); (C) nicotinic acid: 0 mM (■), 1 mM (▲), 1.5 mM (×), 2 mM (⋈), 2.5 mM (◆) and (D) picolinic acid: 0 mM (■), 2 mM (▲), 4 mM (×), 6 mM (⋈), 8 mM (◆). Inserts: secondary plots, the *slope* against different concentrations of inhibitor, which gives the inhibition constant ($-K_i$) from the abscissa-intercepts

Our kinetic data showed that, under the conditions used in this study, the monophenolase activity followed the Michaelis-Menten kinetics (Figures 2A–2D). These data also demonstrated that, similar to 2-amino benzoic acid (Figure 2A), 4-amino benzoic acid inhibited the monophenolase activity reversibly in a noncompetitive manner (Figure 2B). This is evidenced by the fact that increasing the concentration of the inhibitors led to a set of lines with different slopes, intercepting one another at a common point on the X- axis (Figures 2A and 2B). The equilibrium constants for binding of inhibitors to the free enzyme or enzyme-substrate complex, K_i , were obtained from the plots of the slope lines versus the concentration of the inhibitors, which were linear (Figures 2A and 2B; inserts). As summarized in Table 1, the calculated constants were 5.15 μM and 3.8 μM for 2-amino benzoic acid and 4-amino benzoic acid, respectively.

Similar kinetic analysis was performed to characterize inhibitory effects of nicotinic acid and picolinic acid on the enzyme's monophenolase activity. From our kinetic data, it appeared that both inhibitors restricted the monophenolase activity reversibly in a competitive fashion (Figures 2C and 2D). This is confirmed by the Lineweaver-Burk

double reciprocal plots, showing that increasing the concentration of the inhibitors results in a family of lines with different slopes, intercepting one another at a common point on the Y-axis (Figures 2C and 2D). The inhibition constants for binding of these inhibitors to the free enzyme (E) were calculated from the secondary plots of the slope versus concentrations of the inhibitors (Figures 2C and 2D; inserts), which were also linear. As tabulated in Table 1, the obtained K_i s were 1.21 mM and 1.97 mM for nicotinic acid and picolinic acid, respectively. Consistent with the calculated K_i s, the inhibitory concentration of the inhibitors, leading to 50% monophenolase activity lost (IC_{50}), were estimated to be 5.15 μM , 3.8 μM , 3.41 mM and 3.55 mM for 2-amino benzoic acid, 4-amino benzoic acid, nicotinic acid, and picolinic acid, respectively (Table 1).

Characterization of inhibitory effects of the benzoic acid and pyridine derivatives on the diphenolase activity of mushroom tyrosinase

The kinetic behavior of mushroom tyrosinase was studied during the oxidation of caffeic acid as substrate. Under the conditions employed in this work, the oxidation reaction of caffeic acid by mushroom tyrosinase followed the Michaelis-Menten

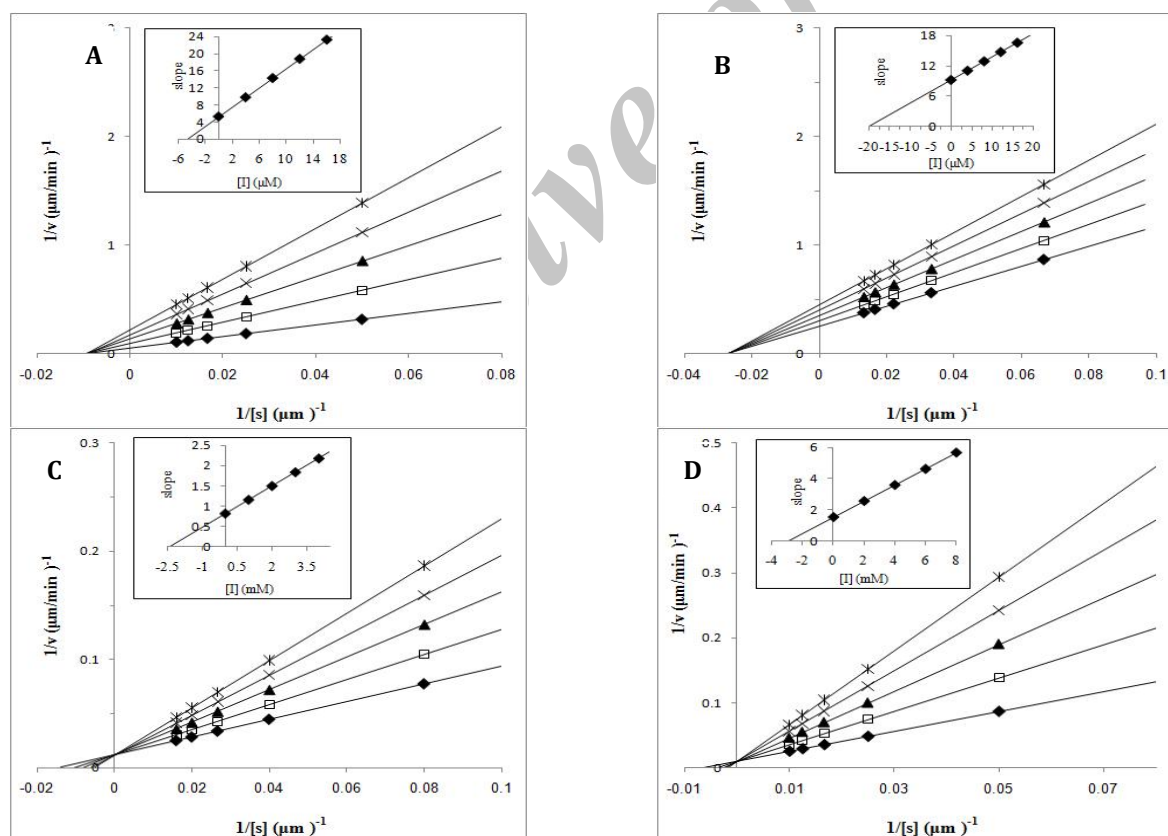


Figure 3. Double reciprocal Lineweaver-Burk plots of MT kinetic assay for diphenolase reactions. caffeic acid was a substrate. The reaction was done in 10 mM PBS, pH 6.8, at 20°C and 11.8 μM enzyme, in the presence of different concentrations of (A) 2-amino benzoic acid: 0 μM (■), 4 μM (▲), 8 μM (×), 12 μM (⋈), 16 μM (◆); (B) 4-amino benzoic acid: 0 μM (■), 4 μM (▲), 8 μM (×), 12 μM (⋈), 16 μM (◆); (C) nicotinic acid: 0 mM (■), 1 mM (▲), 2 mM (×), 3 mM (⋈), 4 mM (◆), and (D) picolinic acid: 0 mM (■), 2 mM (▲), 4 mM (×), 6 mM (⋈), 8 mM (◆). Inserts: secondary plots, the slope against different concentrations of inhibitor, which gives the inhibition constant ($-K_i$) from the abscissa-intercepts

Table 1. Inhibition parameters obtained from kinetic investigations of mushroom tyrosinase with and without presence of 2- and 4-amino benzoic, nicotinic and picolinic acids

Constants	2-amino benzoic acid	4-amino benzoic acid	Nicotinic acid	Picolinic acid
IC ₅₀ (Monophenolase)	5.15 μ M	3.8 μ M	3.41 mM	3.54 mM
IC ₅₀ (Diphenolase)	4.72 μ M	20 μ M	3.8 mM	3.69 mM
K _i (Monophenolase)	5.15 μ M	3.8 μ M	1.21 mM	1.97 mM
K _i (Diphenolase)	4.72 μ M	20 μ M	2.4 mM	2.93 mM
Inhibition	reversible	reversible	reversible	reversible
Inhibition type	noncompetitive	noncompetitive	competitive	competitive

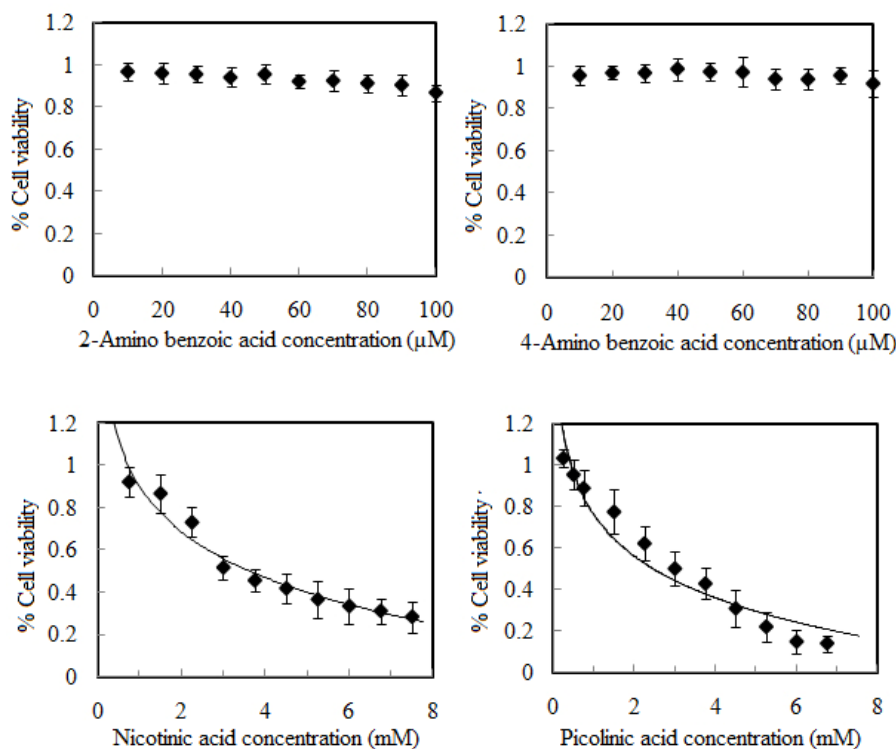
kinetics (Figures 3A-3D). The results illustrated in Figures 3A and 3B show that 2-amino benzoic acid and 4-amino benzoic acid inhibited the tyrosinase diphenolase activity reversibly in a noncompetitive way. This is evident by the fact that increasing the concentration of the inhibitors resulted in a family of lines with different slopes, intercepting one another at a common point on the X axis (Figures 3A and 3B). The equilibrium constants for binding of inhibitors with free enzyme or enzyme-substrate complex were obtained from a plot of the slope lines versus the inhibitor concentration, which were linear (Figures 3A and 3B; inserts). The obtained K_is were 4.72 μ M and 20 μ M for 2-amino benzoic acid and 4-amino benzoic acid, respectively (Table 1). The K_is were fully consistent with the estimated IC₅₀s, which were about 4.72 μ M and 20 μ M for 2-amino benzoic acid and 4-amino benzoic acid, respectively (Table 1).

Further kinetic analysis revealed that both nicotinic acid and picolinic acid restricted reversibly the diphenolase activity of the enzyme in a

competitive manner (Figures 3C and 3D). This is evidenced by the Lineweaver-Burk double reciprocal plots, showing that increasing the concentration of the inhibitors led to a family of lines with different slopes, intercepting one another at a common point on the Y-axis (Figures 3C and 3D). K_is for binding of these inhibitors to the free enzyme (E) were also estimated from the secondary plots of the slope versus concentrations of the inhibitors, which were linear (Figures 3C and 3D; inserts). The calculated K_is were 2.4 mM and 2.93 mM for nicotinic acid and picolinic acid, respectively (Table 1). In line with the K_is, IC₅₀s were estimated to be 3.8 mM and 3.7 mM for nicotinic acid and picolinic acid, respectively (Table 1).

Cytotoxicity on the melanoma cells

To evaluate whether the inhibitors are able to contain proliferation of melanoma cells, the melanoma cell lines were treated with various concentrations of 2-amino benzoic acid, 4-amino

**Figure 4.** Cell viability of melanoma cell line after 24 hr treatment with different concentrations of 2- and 4-amino benzoic acid, nicotinic acid, and picolinic acid

benzoic acid, nicotinic acid and picolinic acid and then the cell viability was analyzed by measuring the microwell cell density using a microplate reader (Figure 4). Media-only treated cells served as the indicator of 100% cell viability. As shown in Figure 4, treatment of the cell lines by 2-amino benzoic acid and 4-amino benzoic acid caused no significant change in the cell viability. On the contrary, presence of nicotinic acid and picolinic acid in the microwells declined remarkably the viability of the melanoma cells in a dose-dependent manner (Figure 4). This is evidenced by the fact that increasing the concentrations of nicotinic and picolinic acids from 0 to 8 mM led to an 80% drop in the melanoma cell viability (Figure 4). The concentrations at which the inhibitors reduced the cell density up to 50% (IC_{50}) were 3.61 mM and 2.42 mM for nicotinic acid and picolinic acid, respectively.

Discussion

In the present work, we characterized the inhibitory effects of the potential therapeutic inhibitors, i.e. benzoic acid (2-amino benzoic and 4-amino benzoic acids) and pyridine (nicotinic and picolinic acids) derivatives, on the diphenolase and monophenolase activities of mushroom tyrosinase. Consistent with previous studies (9, 10, 17, 23), our comprehensive kinetic analyses explicitly showed that the inhibitors restricted reversibly both diphenolase and monophenolase activities of the enzyme. For the monophenolase activity, 2-amino benzoic acid and 4-amino benzoic acid inhibited the enzyme activity in a noncompetitive fashion, whereas nicotinic acid and picolinic acid competitively restricted the enzyme activity. Additionally, comparison of the calculated K_i s suggested that the inhibitors imposed their inhibitory effect on the monophenolase activity with the potencies ranking as follows: picolinic acid \leq nicotinic acid \ll 2-amino benzoic acid $<$ 4-amino benzoic acid. Similarly, the benzoic acid and pyridine derivatives inhibited the diphenolase activity in noncompetitive and competitive manners, respectively, with inhibitory potencies ranking as follows: picolinic acid \leq nicotinic acid \ll 4-amino benzoic acid \leq 2-amino benzoic acid. The observed trends in inhibitory potencies of the inhibitors were supported by the estimated IC_{50} s of the monophenolase and diphenolase activities, confirming that the benzoic acid derivatives have significantly greater inhibitory effects on the enzyme activities as compared to the pyridine compounds. The non-competitive inhibitions exerted by the benzoic acid derivatives suggest that they bind to a site other than the active site of the enzyme and interact with either free enzyme (E) or the enzyme-substrate complex (ES), thus forming a non-productive complex (ESI). Furthermore, the competitive inhibitions imposed by the pyridine derivatives indicate that the inhibitors combine only

with the free enzyme, competing with the substrate for binding to the active site of the enzyme.

Search for nontoxic, potent tyrosinase inhibitors has been the subject of extensive studies (24). Among the nontoxic and potentially therapeutic inhibitors of tyrosinase, aromatic compounds have gained much attention due to their potential applications in the cosmetic industry and medicinal purposes (8, 24–27). It has been previously suggested that some of these compounds impair melanin synthesis and DNA repair, and even act as anti-proliferative agents by interfering with tyrosinase and the melanocyte stimulating hormone receptor (MC1R) activity (8, 26–27). Given that the benzoic acid and pyridine derivatives used in this study inhibit potentially the tyrosinase activity; it is tempting to hypothesize that these particular aromatic derivatives could also restrict proliferation of the melanoma cells. To check our hypothesis, we treated the melanoma cell lines with increasing concentrations of inhibitors and then the cell viability was assessed using MTT assay (Figure 4). Our data showed that only pyridine derivatives were able to induce cytotoxicity in the melanoma cell lines (Figure 4). This finding was fully in agreement with those from previous reports, suggesting nicotinic acid and picolinic acid as anti-proliferative agents (28–30). Raising concentrations of nicotinic acid and picolinic acid (from 0 to 8 mM) resulted in a significant decrease (about 80%) in the melanoma cell viability. The concentrations of the inhibitors, leading to 50% decrease in the cell density (IC_{50}), were very close and comparable to those resulting in 50% drop in the monophenolase and diphenolase activities, suggesting that the observed cytotoxicity is likely due to the tyrosinase inhibition. In line with previous studies (31), our cell-based analysis showed that the particular benzoic acid derivatives applied in the present work led to no significant cytotoxic effect on the growth of the melanoma cells (Figure 4). Since the applied benzoic acid derivatives can be absorbed by melanoma cell lines (31), it is reasonable to speculate that these compounds were not reachable by the cellular tyrosinase, hence were unable to impose their inhibitory effects on the enzyme activity. It is well-known that the benzoic acid can be converted to nontoxic hippuric acid in the liver and kidney cells (32). Similarly, it is possible that 2-amino and 4-amino benzoic acids are transformed to various ineffective metabolites, which are unable to limit the enzyme activities within melanoma cells. This, along with the possibility that the benzoic acid derivatives interact with high affinity with cellular proteins other than tyrosinase, perhaps reduce significantly effective concentrations of these derivatives required for inhibition of the enzyme activities.

Conclusion

Collectively, we characterized mechanisms of inhibition by which the benzoic acid and pyridine

derivatives exert their inhibitory effects on the monophenolase and diphenolase activities of mushroom tyrosinase. Despite similar molecular structures, our kinetic data demonstrated that the applied benzoic acid and pyridine derivatives inhibited the tyrosinase activities with different mechanisms. In fact, the pyridine derivative restricted the enzyme activities in a competitive fashion, whereas their benzoic acid counterparts imposed their inhibitory effects in a non-competitive manner. Moreover, our cell-based data showed that the pyridine derivatives acted as anti-proliferative agents, inducing cytotoxicity in melanoma cell lines probably by inhibition of the tyrosinase activities. Given these findings, nicotinic acid and picolinic acid could be considered as the potential therapeutic inhibitors for the treatment and prevention of melanoma and skin hyperpigmentation disorders.

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