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Synthesis and Antityrosinase Mechanism of Benzaldehyde Thiosemicarbazones: Novel Tyrosinase Inhibitors

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ABSTRACT: *p*-Hydroxybenzaldehyde thiosemicarbazone (HBT) and *p*-methoxybenzaldehyde thiosemicarbazone (MBT) were synthesized and established by ^{1}H NMR and mass spectra. Both compounds were evaluated for their inhibition activities on mushroom tyrosinase and free-cell tyrosinase and melanoma production from B_{16} mouse melanoma cells. Results showed that both compounds exhibited significant inhibitory effects on the enzyme activities. HBT and MBT decreased the steady state of the monophenolase activity sharply, and the IC_{50} values were estimated as 0.76 and 7.0 μM, respectively. MBT lengthened the lag time, but HBT could not. HBT and MBT inhibited diphenolase activity dose-dependently, and their IC_{50} values were estimated as 3.80 and 2.62 μM, respectively. Kinetic analyses showed that inhibition type by both compounds was reversible and their mechanisms were mixed-type. Their inhibition constants were also determined and compared. The research may supply the basis for the development of new food preservatives and cosmetic additives.

KEYWORDS: *benzaldehyde thiosemicarbazones, chemistry synthesis, anti-tyrosinase, inhibitory kinetics*

INTRODUCTION

Tyrosinase (EC 1.14.18.1), ubiquitously distributed in organisms, is known to be a multifunctional copper-containing enzyme from the oxidase superfamily.[1] It possesses monophenolase activity and diphenolase activity. Tyrosinase is responsible for the hydroxylation of tyrosine into *o*-quinone via *o*-diphenols. That is, tyrosinase catalyzes the hydroxylation of tyrosine and the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA). The former is called monophenolase, and the latter is called diphenolase. Tyrosinase is known to be involved in the browning of fruits and vegetables, the formation of brown pigments, and the molting process of insects. Therefore, tyrosinase inhibitors have become increasingly important in agriculture,[2,3] the cosmetic industry,[4] and medication,[5] which makes the development and screening of potent inhibitors of tyrosinase extremely important.

Many efforts have been addressed to screening efficient and safe tyrosinase inhibitors from natural materials and synthetic methods.[6] However, most of them are not potent enough to put into practical use due to their weak individual activities or safety concerns. Undoubtedly, this is still a need to search for and develop novel tyrosinase inhibitors with better activities together with lower side effects.[7] In recent decades, benzaldehyde, especially its hydroxylated analogues, has been extensively investigated as a tyrosinase inhibitors due to favorable interaction with the hydrophobic protein pocket surrounding the binuclear copper active site of tyrosinase. In our pervious research, a great many tyrosinase inhibitors were screened from natural materials and synthetic methods. We have reported some tyrosinase inhibitors, such as hinokitiol;[8] fatty acids;[9] 2-phenylethanol, 2-phenylacetaldehyde, and 2-phenylacetic acid;[10] α-cyano-4-hydroxycinnamic acid;[11] cefazolin and cefodizime;[12] methyl *trans*-cinnamate;[13] *trans*-cinnamaldehyde thiosemicarbazone;[14] and alkyl-3,4-dihydrobenzoates.[15] The inhibitory effect of thiosemicarbazone derivatives on tyrosinase exhibited potent inhibitory activity, the reason being that the sulfur atom of the thiosemicarbazide moiety was able to chelate the two copper ions in the active site of tyrosinase.[14] More recently, our groups described the inhibitory effects of chlorobenzaldehyde thiosemicarbazones on mushroom tyrosinase.[16] We speculated that condensation products of hydroxy- or methoxy-substituted benzaldehyde with thiosemicarbazide might exhibit potent tyrosinase inhibitiory activity. The aim of the current paper is, therefore, to synthesize the novel tyrosinase inhibitors *p*-hydroxybenzaldehyde thiosemicarbazone (HBT) and *p*-methoxybenzaldehyde thiosemicarbazone (MBT), to evaluate their inhibitory effects on tyrosinase activities, to investigate the kinetic parameters and the inhibition mechanisms, and to study their antioxidation activities. The results should supply the basis for the development of new food preservatives and food additives.

MATERIALS AND METHODS

Reagents. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), L-tyrosine (L-Tyr), and L-3,4-dihydroxyphenylalanine (L-DOPA) were obtained from Sigma-Aldrich (USA). Tyrosinase (EC 1.14.18.1) from mush-

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room with the specific activity of the enzyme of about 6680 U/mg was also the product of Sigma-Aldrich. Trypsin–EDTA, penicillin, streptomycin, and RPMI-1640 (Medium 1640 has a wide range of applications for mammalian cells, developed by Roswell Park Memorial Institute; 1640 is medium code) were purchased from Gibco (Germany). Diethyl pyrocarbonate (DEPC), dimethyl sulfoxide (DMSO), Triton X-100, and 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) were purchased from Amresco (USA). All other reagents were of analytical grade. The water used was redistillated and ion-free.

Synthesis. Compounds were prepared by the reaction of corresponding benzaldehyde with thiosemicarbazide in an acidic solution of ethanol, as previous described.[16] A mixture of corresponding benzaldehyde (20 mmol) with thiosemicarbazide (20 mmol) in 60 mL of ethanol with 4 mL of acetic acid solution was refluxed for 2–5 h and then cooled to room temperature. The precipitates were collected and washed with cold ethanol. The products were purified by recrystallization from ethanol and were identified by ESI-MS and NMR analyses. ESI-MS data were obtained on a Bruker ESQUIRE-LC (Germany), and NMR data were acquired on a 400 MHz NMR spectrometer (AV400) from Bruker (Germany).

Assay of the Enzyme Activity and Inhibitory Effects. The monophenolase activity and the diphenolase activity assays were performed as previously reported with modification.[12] In this investigation, L-Tyr was used as the substrate for the monophenolase activity assay and L-DOPA as the substrate for the diphenolase activity assay. The reaction media (3 mL) for activity assay contained 0.6 mM L-Tyr or 0.5 mM L-DOPA in 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.8) and 0.1 mL of different concentrations of inhibitor (dissolved in DMSO as previously). They were first incubated at 30 °C for 10 min, and then 0.1 mL of the aqueous solution of mushroom tyrosinase was added to the mixture. The final concentrations of mushroom tyrosinase were 222.66 U/mL for monophenolase activity and 44.56 U/mL for diphenolase activity. The enzyme activity was determined by following the increasing absorbance at 475 nm accompanying the oxidation of the substrates with a molar absorption coefficient of 3700 (M^{-1} cm^{-1}) by using a Beckman UV-650 spectrophotometer. The temperature was controlled at 30 °C. The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}). Controls, without inhibitor but containing 3.3% DMSO, were routinely carried out.

Determination of the Inhibition Type and Inhibition Constant. The inhibition type was assayed by Lineweaver–Burk plot, and the inhibition constant was determined by the second plots of the apparent K_m/V_{mapp} or $1/V_{mapp}$ versus the concentration of the inhibitor.

Radical-Scavenging Activities. Radical-scavenging activities of the samples on DPPH were estimated according to the modified procedure described by Iwai et al.[17] First, 1 mL of 100 mM acetate buffer (pH 5.5), 1.80 mL of ethanol, and 0.1 mL of ethanolic solution of 3 mM DPPH were put into a test tube. Then, 0.1 mL of the different concentration of the sample solution (dissolved in DMSO) was added to the tube and incubated at 25 °C for 20 min. As control, 0.1 mL of DMSO was added to the tube. From the decrease of the absorbance, the scavenging activity was calculated.

Cell Culture. B_{16} mouse melanoma cells were obtained from Shanghai Institute of Biochemistry and Cell Biology (IBCB, Shanghai, China). The cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2/95% air controlled incubator at 37 °C. Cell viability was detected with MTT assay.

Intracellular Tyrosinase Activity Assay in B_{16} Cells. Tyrosinase activity in B_{16} cells was detected as previously described by Mallick et al.[18] Cells were plated in 6-well plates at a density of 150 cells/mL. After B_{16} cells were incubated in the presence or absence of samples (0.05–1 mg/mL) for 72 h, cells were washed with PBS and then treated with lysis buffer (containing 1% Nonidet P-40, 0.01% SDS, and 0.02% proteinase inhibitor cocktail). Cellular lysates were centrifuged at 10000*g* at 4 °C for 20 min. The supernatants were collected, and the protein contents were determined by using the BCA protein assay kit (Pierce, Rockford, IL). The reaction mixture, consisting of cell extract supernatant (0.1 mL) and 0.9 mL of L-DOPA (3.8 mM) in 25 mM phosphate buffer (pH 6.8), was mixed, and the tyrosinase activity was read at 475 nm for 30 min. The reaction was performed at 25 °C.

Determination of Intracellular Melanin Levels. Melanin assay was performed using procedures described previously by Tsuboi et al.[19] Samples were added to the cell culture at different concentrations (0.05–1 mg/mL). After culturing for 72 h, cells were harvested. An aliquot was used for protein determination, and the remaining cells were centrifugation at 10000*g* for 15 min. The protein contents were determined by using the BCA protein assay kit, and the remaining cells were lysed in 0.5 mL of 5 M NaOH at 100 °C for 1 h; 0.2 mL portions of crude cell extract were transferred to 96-well plates. Melanin concentrations were calculated by comparison of the OD value at 405 nm.

Measurement of Cell Viability. Cell viability was determined using the MTT assay. MTT is a tetrazolium salt and is converted to insoluble formazan by mitochondrial dehydrogenase of living cells. Briefly, after cells were incubated with samples (0.05–1 mg/mL) for 72 h, 20 μL of MTT (5 mg/mL stock solution) was added to each well. After 1 h of incubation, the reaction was terminated by the addition of dimethyl sulfoxide. The optical density of each well was measured at 570 nm. Each treatment was performed in triplicate, and each experiment was repeated three times.

RESULTS AND DISCUSSION

Chemical Synthesis of HBT and MBT. HBT and MBT were synthesized and crystallized with cold ethanol. The products obtained were white acicular crystals with yields of 84.6 and 89.5%, respectively. The products were identified by ESI-MS and ^1H NMR. HBT has the characters m/z 196 ($M + H^+$, DMSO); ^1H NMR (DMSO-d_6, TMS, 400 MHz) δ 11.25 (HN, s), 9.89 (OH, s), 7.95 (CH, s), 8.08, 7.82 (NH_2, d), 7.62 (C_6H_2, 2H, d), 6.76 (C_6H_2, 2H, d). MBT has the characters m/z 210 ($M + H^+$, DMSO); ^1H NMR (DMSO-d_6, TMS, 400 MHz) δ 11.31 (HN, s), 8.00 (CH, s), 8.13, 7.92 (NH_2, d), 7.72 (C_6H_2, 2H, d), 6.95 (C_6H_2, 2H, d), 3.76 (OCH_3, 3H, s). Their structures are shown in Figure 1.

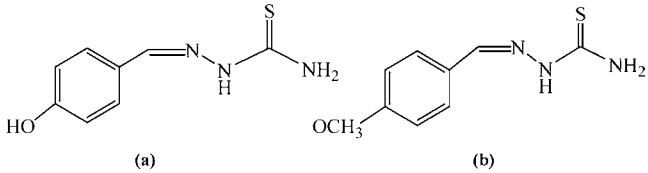

Figure 1. Chemical structures of HBT (a) and MBT (b).

Effect of HBT and MBT on the Monophenolase Activity of Tyrosinase. The kinetic courses of the oxidation of L-Tyr by mushroom tyrosinase in different concentrations of HBT or MBT were tested, and the results are shown in panels A and B, respectively, of Figure 2. When the monophenolase activity of tyrosinase was assayed using L-Tyr as substrate, a lag time, characteristic of monophenolase activity, was observed simultaneously with the appearance of the first stable product, dopachrome. The system reached a constant rate after the lag time, which was estimated by extrapolation of the linear portion of the product accumulation curve to the abscissa. After the reaction system reached the steady state, the curve of the product increased linearly with increasing reaction time. The slope of the line denoted the steady-state rate and the lag time.

The steady-state rate was influenced by HBT as shown in Figure 2A-I. With increasing inhibitor concentration, the steady-state rate descended distinctly and dose-dependently.

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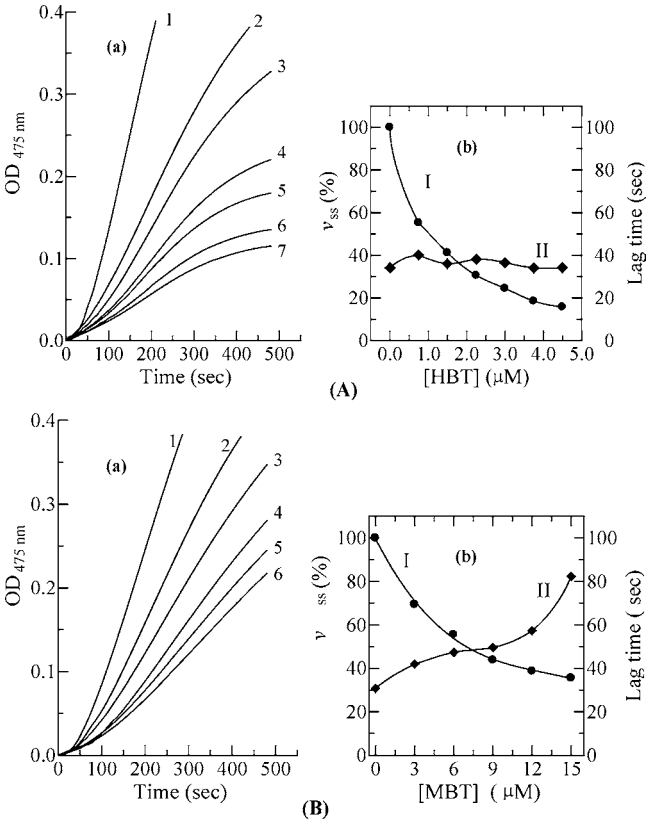

Figure 2. Inhibition effects of HBT (A) and MBT (B) on monophenolase activity of mushroom tyosinase: (a) progress curves for the oxidation of L-Tyr by the enzyme; (b) effects on the steady-state rate (curve I) and on the lag time (curve II) for the oxidation of tyrosine. The concentrations of HBT for curves 1−7 were 0, 0.75, 1.5, 2.25, 3.0, 3.75, and 4.5 μM, respectively. The concentrations of MBT for curves 1−7 were 0, 3.0, 6.0, 9.0, 12.0, and 15.0 μM, respectively.

The IC_{50} value for the monophenolase activity inhibited by HBT was determined to be 0.76 μM. HBT had no influence on the lag time of the enzyme as shown in Figure 2A-II. When using MBT as inhibitor, both the steady-state rate and the lag time were influenced. With increasing inhibitor concentration, the steady-state rate descended distinctly and dose- dependently with an IC_{50} value of 7.0 μM as shown in Figure 2B-I, whereas the lag time changed from 30 s in its absence to 71.3 s in the presence of 15 μM MBT as shown in Figure 2B-II. The results indicated that MBT could inhibit the monophenolase activity of tyrosine not only by decreasing the steady-state rates of the enzyme but also by prolonging the lag time.

In comparison, the IC_{50} values of HBT and MBT for the monophenolase activity were obviously distinguished. The former was about 10 times larger than the latter, indicating the inhibitory intensity of HBT was stronger than that of MBT on the monophenolase activities of the enzyme.

Effect of HBT and MBT on the Diphenolase Activity of Tyrosinase. L-DOPA was used as substrate for the assay to the diphenolase activity. The progress curve of enzyme reaction was a family of lines passing through the origin with different slopes, which indicated the diphenolase activity. The results showed that the lag period did not exist in the progress of enzyme catalyzing the oxidation of L-DOPA. HBT and MBT exhibited potent inhibitory effect on diphenolase activity with dose dependence (Figure 3). The IC_{50} values of both compounds on the diphenolase activity of the enzyme obtained

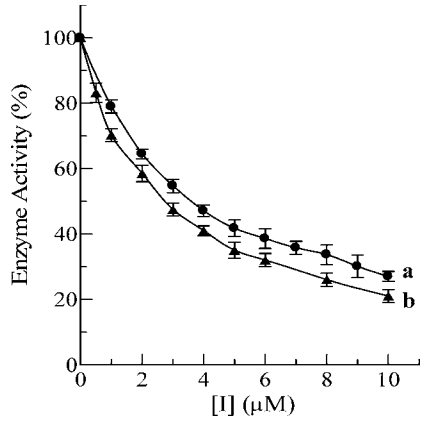

Figure 3. Inhibition effects of HBT (a) and MBT (b) on the diphenolase activity of mushroom tyrosinase.

were 3.80 and 2.62 μM, respectively; therefore, both synthesized combination compounds had obvious inhibitory effects on the diphenolase activity of mushroom tyrosinase. HBT and MBT displayed more potent tyrosinase inhibitory activities than the parent compounds or analogue, 4-hydroxybenzaldehyde (IC_{50}= 1200 μM)[20] and 2-hydroxy-4-methoxybenzaldehyde (IC_{50}= 30 μM).[21] These results indicated the thiosemicarbazone moiety and the electron-rich aromatic ring may have contributed to their tyrosinase inhibition activity, which was consistent with the literature[7] reported.

Inhibition Mechanism of HBT and MBT on the Diphenolase Activity of Tyrosinase. The inhibitory mechanism of HBT and MBT on tyrosinase for oxidation of L-DOPA was studied. Both inhibitors showed the same behavior. The relationship between enzyme activity and its concentration in the presence of HBT and MBT was tested. As shown in Figure 4 for the inhibitor of HBT, the plots of the

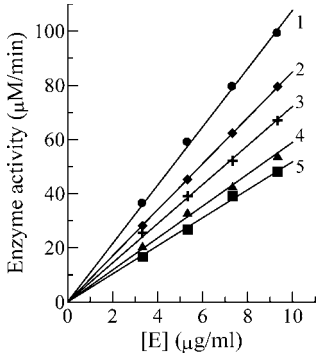

Figure 4. Determination of the inhibitory mechanism of HBT on mushroom tyrosinase. The concentrations of HBT for curves 1−5 were 0, 0.5, 1.0, 1.5, and 2.0 μM, respectively.

remaining enzyme activity versus the concentration of enzyme at different inhibitor concentrations gave a family of straight lines, which all passed through the origin. The presence of inhibitor did not reduce the amount of enzyme, but just resulted in the inhibition of enzyme activity. The results showed that both HBT and MBT were reversible inhibitors of tyrosinase for the oxidation of L-DOPA. The inhibition type by HBT and MBT on tyrosinase for the oxidation of L-DOPA was investigated by Lineweaver−Burk double-reciprocal plots. In

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the presence of HBT, the kinetics of the enzyme is shown in Figure 5. The plots of $1/v$ versus $1/[S]$ gave a family of straight

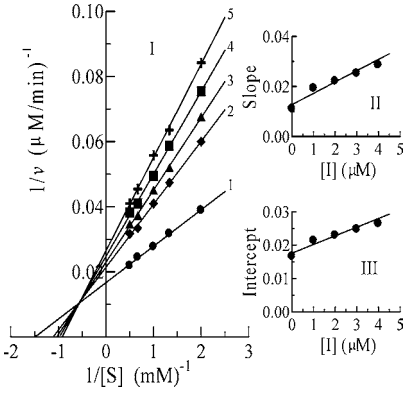

Figure 5. Lineweaver–Burk plots for the inhibition of HBT on mushroom tyrosinase for the oxidation of L-DOPA. The concentrations of HBT for curves 1–5 were 0, 0.5, 1.0, 1.5, and 2.0 μM, respectively. Inset II represents the plot of slope versus the concentration of HBT for determining the inhibition constants K_I. Inset III represents the plot of intercept versus the concentration of HBT for determining the inhibition constant K_{IS}.

lines with different slopes that intercept in the second quadrant, indicating that HBT was a competitive–uncompetitive mixed-type inhibitor. It can be presumed that both compounds can not only combine with free enzymes but also combine with enzyme–substrate complexes. The equilibrium constant for inhibitor binding with free enzyme (K_I) was obtained from a plot of the slope (K_m/V_{mapp}) versus the concentration of the inhibitor, and the enzyme–substrate complex (K_{IS}) was obtained from a plot of the vertical intercept ($1/V_{mapp}$) versus the concentration of inhibitor. The values of K_I and K_{IS} were determained as 2.82 and 6.79 μM, respectively. In contrast, MBT was the same inhibitor type with HBT, and the inhibitor constants (K_I and K_{IS}) were estimated to be 1.47 and 15.10 μM, respectively. Because $K_{IS} > K_I$ in both inhibitors, the affinity of the inhibitors for the free enzyme is greater than that of the inhibitor for the enzyme–substrate complex.

Radical-Scavenging Activities of HBT and MBT against DPPH. Tyrosinase is a kind of oxidase, so some reducers such as cysteine and vitamin C with strong radical-scavenging activity are all potent inhibitors of tyrosinase. The mechanism is that inhibitors cleared the peroxy radicals, terminated radical chain initiation, and weakened the role of oxygen of tyrosinase, which weakened the role of tyrosinase. The radical-scavenging activities of HBT and MBT were determined. The results (Figure 6) showed that HBT had radical-scavenging activity, whereas MBT did not. When the concentration was 100 μM, scavenging of oxygen free radicals produced against 0.1 mM DPPH by HBT and MBT was determined to be 46.7 and 5.5%, respectively. The results showed that the different substituent groups on the *p*-position of benzene ring have a certain influence on the ability to scavenge oxygen free radicals.

Effects of HBT and MBT on the Activity of Tyrosinase in B_{16} Mouse Melanoma Cells. The inhibition effect on the cellular tyrosinase in B_{16} cell by HBT and MBT was studied. The diphenolase activity of tyrosinase was assayed by using L-DOPA as substrate. Figure 7 shows the relationship between the remaining enzyme activity and inhibitor concentration.

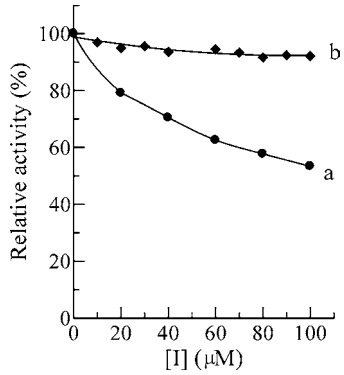

Figure 6. DPPH radical-scavenging activities of HBT (a) and MBT (b). Different dilutions of sample were added to 0.1 mL of DPPH (3 mM, dissolved in ethanol) and incubated at 25 °C for 20 min. Absorbance was measured at 517 nm.

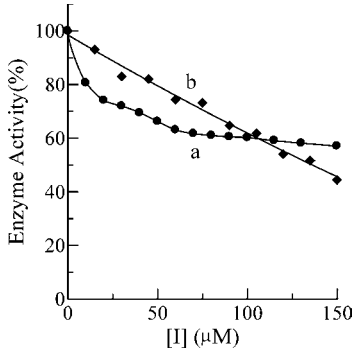

Figure 7. Inhibition effects of HBT (a) and MBT (b) on the activity of cellular tyrosinase from B_{16} cells for the oxidation of L-DOPA.

Both compounds could inhibit the diphenolase activity of cellular tyrosinase dose dependently. When the concentration of HBT was <150 μM, the inhibition rate of tyrosinase enzyme activity was <50%. However, MBT was more potent inhibition than HBT. The inhibitor concentration leading to 50% activity lost (IC_{50}) was estimated to be 139 μM.

Cytological Effects of HBT and MBT on B_{16} Mouse Melanoma Cells. Using DMSO as a negative control, murine B_{16} melanoma cells was treated for 72 h by HBT and MBT in 0.1, 1.0, 10, and 100 μM, respectively. The changes of tyrosine activity, melanin content, and rate of cell proliferation of the B_{16} cell were measured. With increasing concentration of HBT, the rate of cell proliferation gently decreased as shown in Figure 8a. When the concentration of HBT was <100 μM, it had little effect on the cellular tyrosinase activity. When the concentration added was 100 μM, the melanin contents of B_{16} cell were inhibited and the remaining melanin contents were 56.5%. As for MBT, when its concentration was <100 μM, it had weak influence on the tyrosinase activity and melanin content in the B_{16} cell. When the MBT concentration reached 100 μM, the cell proliferation rate, tyrosinase activity, and melanin content in B_{16} cell decreased to 77.3, 49.5, and 60.5%, respectively. The results are shown in Figure 8b. These data indicate that HBT and MBT could inhibit cellular tyrosinase activity and melanin production in mouse melanoma cells.

Tyrosinase is composed of four subunits and contains two binuclear coppers in its active sites per tetramer.[22] Park et al.[23] reported that according to the three-dimensional structure of tyrosinase, the copper ions were of great importance for

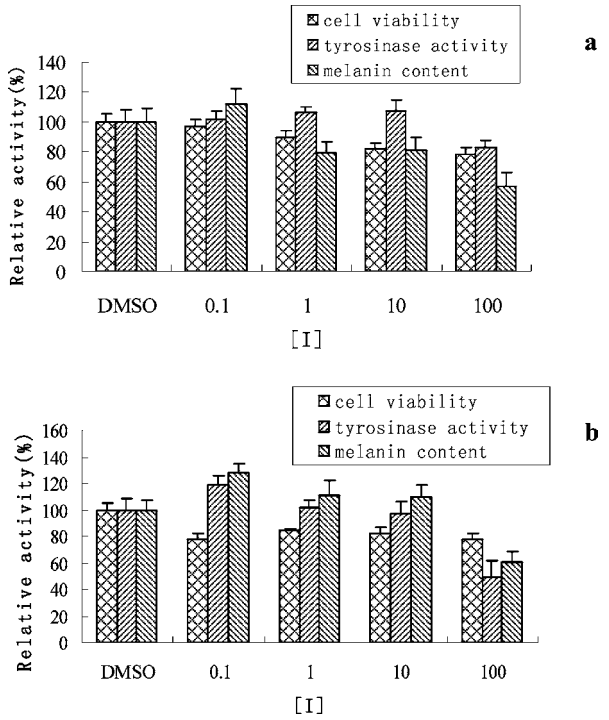

Figure 8. Effects of HBT (a) and MBT (b) on tyrosinase activity and cellular melanin content in B_{16} cells. Cells were treated with compounds for 72 h at various concentrations. Data represent the mean ± SD of three different experiments.

tyrosinase activity, and a slight change in the dicopper center might lead to activity loss. Benzylaldehyde thiosemicarbazone is a kind of Schiff base compound that exhibits strong affinity to copper ions. Thus, when mixed with tyrosinase and its substrate, a benzylaldehyde thiosemicarbazone derivative may form a complex with a tyrosinase molecule by its sulfur atom and nitrogen atom through a hydrogen bond and a coordinate bond.[24] This tight complex could make the free oxygen molecule decrease its reaction ability and even unable to take part in the hydroxylation with monophenols and in the oxidation with *o*-diphenols, as the free oxygen molecule was surrounded closely by two copper ions from tyrosinase and two hydrogen atoms from two benzylaldehyde thiosemicarbazone molecules. Therefore, tyrosinase would lose its catalyzing ability.[25]

In conclusion, our groups designed the combination compounds of benzaldehyde and thiosemicarbazone analogue for the study of tyrosinase inhibitors. Hydroxy- and methoxy-substituted benzaldehyde thiosemicarbazone, HBT and MBT, were synthesized. HBT and MBT exhibited strong inhibition on the activity of mushroom tyrosinase and demonstrated potent melanogenic activity in B_{16} cells. Further investigations are warranted to explore their potential application in the management of hypopigmentation disorders.

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Author Contributions

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ABBREVIATIONS USED

HBT, *p*-hydroxybenzaldehyde thiosemicarbazone; MBT, *p*-methoxybenzaldehyde thiosemicarbazone; L-Tyr, L-tyrosine; L-DOPA, L-3,4-dihydroxyphenylalanine; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DEPC, diethylpyrocarbonate; BCA, bicinchoninic acid; IC_{50}, inhibitor concentrations leading to 50% activity lost; K_{IS}, equilibrium constant of the inhibitor combining with the enzyme−substrate complex.

REFERENCES

(1) Chase, M. R.; Raina, K.; Bruno, J.; Sugumaran, M. Purification, characterization and molecular cloning of prophenoloxidases from sarcophaga bullata. *Insect Biochem. Mol. Biol.* **2000**, *30*, 953−967.

(2) Kubo, I.; Chen, Q. X.; Nihei, K. Molecular design of antibrowning agents: antioxidative tyrosinase inhibitors. *Food Chem.* **2003**, *81*, 241−247.

(3) Guerrero, A.; Rosell, G. Biorational approaches for insect control by enzymatic inhibition. *Curr. Med. Chem.* **2005**, *12*, 461−469.

(4) Cho, Y. H.; Kim, J. H.; Park, S. M.; Lee, B. C.; Pyo, H. B.; Park, H. D. New cosmetic agents for skin whitening from *Angelica dahurica*. *J. Cosmet. Sci.* **2006**, *57*, 11−21.

(5) Gasowska-Bajger, B.; Wojtasek, H. Indirect oxidation of the antitumor agent procarbazine by tyrosinase possible application in designing antimelanoma prodrugs. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3296−3300.

(6) Solano, F.; Briganti, S.; Picardo, M.; Ghanem, G. Hypopigmenting agents: an updated review on biological, chemical and clinical aspects. *Pigment Cell Res.* **2006**, *19*, 550−571.

(7) Yi, W.; Cao, R. H.; Chen, Z. Y.; Yu, L.; Ma, L.; Song, H. C. Design, synthesis and biological evaluation of hydroxy- or methoxy-substituted phenylmethylene thiosemicarbazones as tyrosinase inhibitors. *Chem. Pharm. Bull.* **2009**, *57*, 1273−1277.

(8) Zhu, Y. J.; Qiu, L.; Zhou, J. J.; Guo, H. Y.; Hu, Y. H.; Li, Z. C.; Wang, Q.; Chen, Q. X.; Liu, B. Inhibitory effects of hinokitiol on tyrosinase activity and melanin biosynthesis and its antimicrobial activities. *J. Enzyme Inhib. Med. Chem.* **2010**, *25* (6), 798−803.

(9) Guo, Y. J.; Pan, Z. Z.; Chen, C. Q.; Hu, Y. H.; Liu, F. J.; Shi, Y.; Yan, J. H.; Chen, Q. X. Inhibitory effects of fatty acid on the activity of mushroom tyrosinase. *Appl. Biochem. Bitotechnol.* **2010**, *162* (6), 1564−1573.

(10) Zhu, Y. J.; Zhou, H. T.; Hu, Y. H.; Tang, J. Y.; Su, M. X.; Guo, Y. J.; Chen, Q. X.; Liu, B. Antityrosinase and antimicrobial activities of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid. *Food Chem.* **2011**, *124* (1), 298−302.

(11) Qiu, L.; Chen, Q. H.; Zhuang, J. X.; Zhong, X.; Zhou, J. J.; Guo, Y. J.; Chen, Q. X. Inhibitory effects of α-cyano-4-hydroxycinnamic acid on the activity of mushroom tyrosinase. *Food Chem.* **2009**, *112* (3), 609−613.

(12) Zhuang, J. X.; Li, W. G.; Qiu, L.; Zhong, X.; Zhou, J. J.; Chen, Q. X. Inhibitory effects of cefazolin and cefodizime on the activity of mushroom tyrosinase. *J. Enzyme Inhib. Med. Chem.* **2009**, *24* (1), 251−256.

(13) Huang, Q. S.; Zhu, Y. J.; Li, H. L.; Zhuang, J. X.; Zhang, C. L.; Zhou, J. J.; Li, W. G.; Chen, Q. X. Inhibitory effects of methyl *trans*-cinnamate on mushroom tyrosinase and its antimicrobial activities. *J. Agric. Food Chem.* **2009**, *57* (6), 2565−2569.

(14) Zhu, Y. J.; Song, K. K.; Li, Z. C.; Pan, Z. Z.; Guo, Y. J.; Zhou, J. J.; Wang, Q.; Liu, B.; Chen, Q. X. Antityrosinase and antimicrobial activities of *trans*-cinnamaldehyde thiosemicarbazone. *J. Agric. Food Chem.* **2009**, *57*, 5518−5523.

(15) Pan, Z. Z.; Li, H. L.; Yu, X. J.; Zuo, Q. X.; Zheng, G. X.; Shi, Y.; Liu, X.; Lin, Y. M.; Liang, G.; Wang, Q.; Chen, Q. X. Synthesis and antityrosinase activities of alkyl 3,4-dihydroxybenzoates. *J. Agric. Food Chem.* **2011**, *59*, 6645−6649.
(16) Li, Z. C.; Chen, L. H.; Yu, X. J.; Hu, Y. H.; Song, K. K.; Zhou, X. W.; Chen, Q. X. Inhibition kinetics of chlorobenzaldehyde thiosemicarbazones on mushroom tyrosinase. *J. Agric. Food Chem.* **2010**, *58*, 12537−12540.
(17) Iwai, K.; Kishimoto, N.; Kakino, Y.; Mochida, K.; Fujita, T. In vitro antioxidative effects and tyrosinase inhibitory activities of seven hydroxycinnamoyl derivatives in green coffee beans. *J. Agric. Food Chem.* **2004**, *52*, 4893−4898.
(18) Mallick, S.; Singh, S. K.; Sarkar, C.; Saha, B.; Bhadra, R. Human placental lipid induces melanogenesis by increasing the expression of tyrosinase and its related proteins in vitro. *Pigment. Cell Res.* **2005**, *18*, 25−33.
(19) Tsuboi, T.; Kondoh, H.; Hiratsuka, J.; Mishima, Y. Enhanced melanogenesis induced by tyrosinase gene-transfer increases boron-uptake and killing effect of boron neutron capture therapy for amelanotic melanoma. *Pigment. Cell Res.* **1998**, *11*, 275−282.
(20) Yi, W.; Cao, R.; H., Peng, W. L.; Wen, H.; Yan, Q.; Zhou, B. H.; Ma, L.; Song, H. C. Synthesis and biological evaluation of novel 4-hydroxybenzaldehyde derivatives as tyrosinase inhibitors. *Eur. J. Med. Chem.* **2010**, *45*, 639−646.
(21) Kubo, I.; Kinst-Hori, I. 2-Hydroxy-4-methoxybenzaldehyde: a potent tyrosinase inhibitor from african medicinal plants. *Planta Med.* **1999**, *65*, 19−22.
(22) Matoba, Y.; Kumagai, T.; Yamamoto, A.; Yoshitsu, H.; Sugiyama, M. Crystallographic evidence that the binuclear copper center of tyrosinase is flexible during catalysis. *J. Biol. Chem.* **2006**, *281*, 8981−8990.
(23) Park, J.; Sung, N. D. 3D-QSAR analysis and molecular docking of thiosemicarbazone analogues as a potent tyrosinase inhibitor. *Bull. Korean Chem. Soc.* **2011**, *32* (4), 1241−1248.
(24) Xue, C. B.; Zhang, L.; Luo, W. C.; Xie, X. Y.; Jiang, L.; Xiao, T. 3D-QSAR and molecular docking studies of benzaldehyde thiosemicarbazone, benzaldehyde, benzoic acid, and their derivatives as phenoolxidase inhibitors. *Bioorg. Med. Chem.* **2007**, *15*, 2006−2015.
(25) Liu, J. B.; Yi, W.; Wan, Y. Q.; Ma, L.; Song, H. C. 1-(1-Arylethylidene) thiosemicarbazide derivatives: a new class of tyrosinase inhibitors. *Bioorg. Med. Chem.* **2008**, *16*, 1096−1102.