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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

The tyrosinase-inhibitory effects of 2-phenyl-1,4-naphthoquinone analogs: importance of the (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold of an endomethylene type

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Abstract

In order to investigate the effect of the (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold of an endomethylene type on tyrosinase inhibition, 2-phenyl-1,4-naphthoquinone derivatives were synthesized by Michael addition of substituted benzenes to 1,4-naphthoquinone and subsequently an auto-oxidation. Most of the derivatives potently inhibited mushroom tyrosinase and four derivatives, including **1c** ($IC_{50} = 22.00 \pm 1.63$ μM), more potently inhibited mushroom tyrosinase than kojic acid ($IC_{50} = 37.86 \pm 2.21$ μM). Cell-based assays using B16F10 cells (a melanoma cell line) showed **1c** dose-dependently suppressed melanin production and more potently inhibited tyrosinase activity than kojic acid. Docking simulation results between **1c** and tyrosinase and a kinetic study suggest that **1c** is a competitive inhibitor that binds to the active site of tyrosinase. These results support that anti-melanogenic effect of naphthoquinone derivatives results from their tyrosinase inhibiting activity and the (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold of an endomethylene type can confer tyrosinase-inhibitory activity.

Keywords Tyrosinase inhibitor · (*E*)-β-phenyl-α,β-unsaturated carbonyl · 2-phenyl-1 · 4-naphthoquinone · HMBC · Docking simulation

Introduction

Tyrosinase has attracted attention because it plays an important role in the biosynthesis of melanin (Solano 2014), a core determinant of the colours of hair, pupils and skin and the browning of vegetables and fruits (Mayer and Harel 1979). Tyrosinase is a metalloenzyme that contains two copper(II) ions at its active site, and is involved in the consecutive oxidation reactions responsible for converting L-tyrosine into dopaquinone via L-dopa (Hearing and Tsukamoto 1991). Dopaquinone is an unstable entity but is transformed in vivo to eumelanin (a type of melanin responsible for brown and black colours) by reactions involving mercapto-containing substances, such as, cysteine and glutathione or to phenomelanin, which is responsible for yellow and red colours. Furthermore, eumelanin to pheomelanin ratio in skin is primary determinant of skin colour. Melanins protect skin cells from ultraviolet radiation, but excessive accumulations of melanin can cause hyperpigmentation, such as senile lentigines, melasma and freckles and aesthetic problems.

Over past several decades, a large number of naturally occurring substances and synthetic compounds have been tested with respect to their abilities to inhibit tyrosinase for the purpose of discovering novel whitening agents. Although many compounds have been found to inhibit tyrosinase activity, relatively few are used for clinical purposes due to a lack potency and side effects, which include

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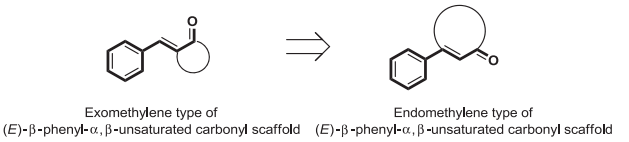


Fig. 1 Exomethylene and endomethylene types of the (*E*)-β-phenyl-α, β-unsaturated carbonyl scaffold

cytotoxicity and mutagenicity. The skin lightning agents containing salicylhydroxamic acid, kojic acid (Gonçalez et al. 2013, Ki et al. 2013, Kumar et al. 2013), magnesium L-ascorbyl-2-phosphate, phenols (Jimbow 1991), hydroxyanisole, azelaic acid (Breathnach et al. 1989, Garcia-Lopez 1989), tretinoin, mercury salts, corticosteroids (Neering 1975), retinoid (Griffiths et al. 1993, Kimbrough-Green et al. 1994), *N*-acetyl-4-*S*-cysteaminylphenol, arbutin (hydroquinone-β-D-glucopyranoside), hydroquinone (HQ) (Arndt and Fitzpatrick 1965, Fitzpatrick et al. 1966, Heilgemeir and Balda 1981, Kligman and Willis 1975) and monobenzyl hydroquinone are generally used in cosmetics. They are accepted globally, although having certain drawbacks and side effects. The application of kojic acid has been limited due to its carcinogenic effect and issue of instability during storage. HQ is considered a toxic agent to mammalian cells and revealed a series of unwanted effects such as irritation, hypochromia, contact dermatitis, ochronosis, burning and chestnut spots on the nails (Curto et al. 1999, Engasser 1984, Fisher 1983, Romaguera and Grimalt 1985). Due to side effects, the EU Cosmetic Regulation completely bans the application of corticosteroids, HQ, monobenzyl hydroquinone, mercury salts and tretinoin as whitening agents. Although many tyrosinase inhibitors have proved effective in in vitro studies, only a few of them showed good results in clinical trials. Therefore, there is a need to identify novel, safe tyrosinase inhibitors with greater anti-melanogenic efficacies and better selectivities.

For the past several years, our laboratory has been searching for potent tyrosinase inhibitors capable of being used as skin-lightening agents. Based on the chemical structures of L-tyrosine and L-dopa, which are both natural substrates of tyrosinase, we have designed and synthesized several compounds including MHY498 (Kim et al. 2013), 5-(substituted benzylidene)hydantoins (Ha et al. 2011), benzylidene-linked thiohydantoins (Kim et al. 2014) and MHY2081 (Kang et al. 2015) as competitive tyrosinase inhibitors, which inhibit tyrosinase-inhibitory activity more potently than kojic acid, a well-known strong tyrosinase inhibitor. An analysis of the chemical structures of these active compounds revealed they all possessed the (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold, which indicated that this scaffold importantly confers tyrosinase-inhibitory activity. The scaffold of the above-mentioned tyrosinase inhibitors is an exomethylene type, which shown in Fig. 1. Therefore, in this study, we investigated whether compounds with an endomethylene type of the scaffold still exhibited large tyrosinase inhibiting activity. For this purpose, a series of 2-(substituted phenyl)-1,4-naphthoquinone derivatives containing the endomethylene type of the (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold were designed and synthesized by a Michael reaction followed by an auto-oxidation. The synthesized naphthoquinones were assessed for tyrosinase-inhibitory activity using mushroom tyrosinase and their abilities to inhibit melanin biosynthesis, and tyrosinase activity was examined using B16F10 cells (a murine melanoma cell line).

Materials and methods

Chemistry

All chemical reagents were commercially available and used without further purification. Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) data were obtained on an Expression CMS (Advion, Ithaca, NY, USA) and a 6530 Accurate Mass quadrupole time-of-flight liquid-chromatography mass spectrometer (Agilent), respectively. Nuclear Magnetic Resonance (NMR) data were recorded on a Varian Unity INOVA 400 spectrometer and Varian Unity AS500 spectrometer (Agilent Technologies, Santa Clara, CA, USA), using DMSO-d_6 and chemical shifts were reported in parts per million (ppm) with reference to the respective residual solvent or deuterated peaks (δ_H 7.24 and δ_C 77.0 for $CDCl_3$, δ_H 2.50 and δ_C 39.7 for DMSO-d_6). Coupling constants are reported in hertz. The abbreviations used are as follows: s (singlet), brs (broad singlet), d (doublet), t (triplets), q (quartet), td (triplet of doublets), dd (doublet of doublets) or m (multiplet). All the reactions described below were performed under nitrogen atmosphere and monitored by thin-layer chromatography (TLC). TLC was performed on Merck precoated $60F_{254}$ plates. All anhydrous solvents were distilled over CaH_2 or Na/benzophenone prior to use. Identity of all final compounds was confirmed by 1D (^1H and ^{13}C) and 2D (NOESY) NMR and mass spectrometry.

The general procedure for the preparation of 2-(substituted phenyl)naphthalene-1,4-dione derivatives (1a–1f)

Appropriate phenols (50 mg) in acetic acid (1.0 mL) were added to a stirred solution of 1,4-naphthoquinone (2.0 eq.) in acetic acid (2.0 mL) in a 25 mL round bottom flask and then 2M-H_2SO_4 (0.5 mL) was added. The reaction mixture was stirred at room temperature for 2–10 h under a nitrogen

atmosphere. After addition of water, the reaction mixture was neutralized with saturated $NaHCO_3$ solution and partitioned between ethyl acetate and water. The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. The resulting residue was purified by column chromatography (hexane:ethyl acetate = 7:1–1:1) to give solids at yields of 17–86%.

2-(2,4-Dihydroxyphenyl)naphthalene-1,4-dione (1a)

(Redaelli et al. 2015). Yield: 86%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.67 (s, 1H, OH), 9.66 (s, 1H, OH), 8.02–7.97 (m, 2H, 6-, 7-H), 7.86–7.84 (m, 2H, 5-, 8-H), 7.05 (d, 1H, J = 8.5 Hz, 6′-H), 7.00 (s, 1H, 3-H), 6.37 (d, 1H, J = 1.5 Hz, 3′-H), 6.30 (dd, 1H, J = 8.5, 1.5 Hz, 5′-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 185.4, 184.5, 160.6, 157.5, 147.9, 135.4, 134.7, 134.6, 133.0, 132.9, 132.2, 127.1, 126.0, 112.7, 107.3, 103.3 (Redaelli et al., 2015).

2-(4-Hydroxy-3-methoxyphenyl)naphthalene-1,4-dione (1b)

Yield: 37%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.17–8.07 (m, 2H, 6-, 7-H), 7.76–7.74 (m, 2H, 5-, 8-H), 7.16 (m, 2H, 2′-, 6′-H), 7.03 (s, 1H, 3-H), 6.98 (d, 1H, J = 8.0 Hz, 5′-H), 3.94 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 185.5, 185.1, 148.1, 147.7, 146.6, 133.9 (×2), 132.8, 132.3, 127.2, 126.1, 125.5, 123.6, 114.9, 112.4, 56.3; LRMS (ESI-) m/z 279 $(M\text{-}H)^-$; HRMS (ESI-) m/z $C_{17}H_{12}O_4$ $(M\text{-}H)^-$ calcd 279.0663, obsd 279.0660.

2-(4-Hydroxy-2-methoxyphenyl)naphthalene-1,4-dione (1c)

Yield: 37%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.88 (s, 1H, OH), 8.02–7.98 (m, 2H, 6-, 7-H), 7.87–7.85 (m, 2H, 5-, 8-H), 7.10 (d, 1H, J = 8.0 Hz, 6′-H), 6.95 (s, 1H, 3-H), 6.49 (s, 1H, 3′-H), 6.43 (d, 1H, J = 8.0 Hz, 5′-H), 3.66 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 185.3, 184.1, 161.0, 159.1, 148.2, 135.7, 134.6, 134.5, 132.8, 132.2, 132.1, 127.0, 126.0, 114.4, 107.8, 99.9, 55.9; LRMS (ESI-) m/z 279 $(M\text{-}H)^-$; HRMS (ESI-) m/z $C_{17}H_{11}O_4$ $(M\text{-}H)^-$ calcd 279.0663, obsd 279.0665.

2-(2-Hydroxy-4-methoxyphenyl)naphthalene-1,4-dione (1d)

Yield: 17%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.84 (s, 1H, OH), 8.03–7.98 (m, 2H, 6-, 7-H), 7.87–7.85 (m, 2H, 5-, 8-H), 7.16 (d, 1H, J = 8.0 Hz, 6′-H), 7.02 (s, 1H, 3-H), 6.48 (dd, 1H, J = 8.0, 2.0 Hz, 5′-H), 6.43 (d, 1H, J = 2.0 Hz, 3′-H), 3.74 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 185.4, 184.3, 162.1, 157.3, 147.7, 136.0, 134.7, 134.6, 133.0, 132.7, 132.2, 127.1, 126.1, 114.4, 105.5, 102.0, 55.8; LRMS (ESI-) m/z 279 $(M\text{-}H)^-$; HRMS (ESI-) m/z $C_{17}H_{11}O_4$ $(M\text{-}H)^-$ calcd 279.0663, obsd 279.0664.

2-(3,4-Dihydroxyphenyl)naphthalene-1,4-dione (1e)

Yield: 42%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.33 (brs, 2H, OH), 8.06–8.04 (m, 1H, 6-H, or 7-H), 7.99–7.97 (m, 1H, 6-H, or 7-H), 7.88–7.84 (m, 2H, 5-, 8-H), 7.08 (d, 1H, J = 1.5 Hz, 2′-H), 6.98 (dd, 1H, J = 8.0, 2.0 Hz, 6′-H), 6.97 (s, 1H, 3-H), 6.81 (d, 1H, J = 8.0 Hz, 5′-H); ^{13}C NMR (100 MHz, CD_3OD+$CDCl_3$) δ 185.7, 185.2, 147.8, 147.4, 144.5, 133.9, 133.4, 132.7, 132.1, 127.1, 125.9, 125.3, 122.7, 120.6, 116.5, 115.3; LRMS (ESI-) m/z 265 $(M\text{-}H)^-$ (Janeczko et al. 2016).

2-(2,4-Dimethoxyphenyl)naphthalene-1,4-dione (1f)

Yield: 84%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15–8.10 (m, 2H, 6-, 7-H), 7.76–7.74 (m, 2H, 5-, 8-H), 7.21 (d, 1H, J = 8.5 Hz, 6′-H), 7.03 (s, 1H, 3-H), 6.58 (dd, 1H, J = 8.5, 2.0 Hz, 5′-H), 6.43 (d, 1H, J = 2.0 Hz, 3′-H), 3.86 (s, 3H, CH_3), 3.78 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 185.6, 184.2, 162.5, 158.8, 147.6, 136.5, 133.8, 133.7, 132.9, 132.4, 131.8, 127.1, 126.1, 116.0, 104.9, 99.2, 55.9, 55.7.

Biological evaluation

Mushroom tyrosinase inhibition assay

The mushroom tyrosinase-inhibitory assay for the synthesized compounds **1a**–**1f** was carried out according to the known method with slight modifications (Hyun et al. 2008). To a 96-well microplate, a total of 200 µL mixture was added, containing 20 µL mushroom tyrosinase solution (1000 U/mL), 10 µL of each test compounds (final concentration 30 µM) and 170 µL of substrate solution (293 µM L-tyrosine solution and 14.7 mM potassium phosphate buffer (pH 6.5) solution (1:1, v/v)). The microplate was incubated for 30 min at 37 °C. A VersaMax^{TM} microplate reader (Molecular Devices, Sunnylvale, CA, USA) was used to measure the absorbance of dopachrome contents produced during incubation at 450 nm. A volume of 50 µM of kojic acid and 500 µM of arbutin were used as positive controls, respectively. All experiments were repeated for three times. Formula [Inhibition (%) = [1−(A/B)] × 100(A = the absorbance of test compounds and B = the absorbance of the blank control with no test compound)] was used for the calculation of tyrosinase inhibition. The IC_{50} value of compound **1c** was determined in three different concentrations.

Kinetic analysis of tyrosinase inhibition

Kinetic analysis study was performed to determine the nature of inhibition of compound **1c** using mushroom

tyrosinase. Different concentration of a substrate, L- tyrosine (0.5 mM, 1 mM, 2 mM or 4 mM), a tyrosinase enzyme (20 µL or 1000 U/mL) and test samples (0 µL, 5 µL, 10 µL or 25 µL) were mixed together and added to a 96-well microplate in total of 200 µL final solution. The 96-well microplates were incubated at 37 °C for 30 min. An ELISA reader (Tecan, Austria) was used to measure the absorbance of dopachrome produced during incubation at 450 nm. Km (Michaelis constant) and Vmax (maximum velocity) were calculated using a Lineweaver–Burk plot and a nature of enzyme inhibition of the test compound **1c** was determined. The experiments were repeated for three times.

In silico docking simulation of compound 1c and tyrosinase

Docking studies were carried out between tyrosinase, and kojic acid, or compound **1c**. First of all, 3D structure of compound **1c** was created with the help of Chem3D pro 12.0. From PDB (Protein Data Bank), the 3D structure of *Agaricus bisporus* tyrosinase (PDB ID: 2Y9X) was obtained. The docking score of compound **1c** was determined by AutoDock Vina and Chimera software according to the known procedure (Morris et al. 1998, Moustakas et al. 2006). A pharmacophore between **1c** and tyrosinase were created by LigandScout software, which showed the binding interactions between the tyrosinase and compound **1c**.

Cell culture

Mouse melanoma cells B16F10 were obtained from a Korean Cell Line Bank. In a humidified atmosphere (5% CO_2) and at 37 °C temperature, B16F10 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) having FBS (10% heat-inactivated fetal bovine serum) and streptomycin/penicillin (100 IU/50 µg/mL). To determine cell viability, melanin content and tyrosinase activity, cells were cultured in 24-well plates. All experiments were repeated for three times.

Viability assay of compound 1c in B16F10 melanoma cells

MTT assay was performed for the determination of cell viabilities (Shin et al. 2008). The cells were cultured in a 24-well plate in the amount of 5×10^4 cells per well. The 24-well plates were stored in a humidified environment containing 5% CO_2 at 37 °C for 24 h. Different concentrations (0, 5, 10, 25 µM) of test compound **1c** were added to the cells and further incubated for 24 h under the same conditions. Then the cells were treated with MTT solution and incubated for 2 h at 37 °C. After supernatant were removed, formazan crystals were dissolved in DMSO/EtOH (1:1, 200 µL) and moved to a 96-well ELISA plate. At 570 nm, well absorbances were detected by an Elisa reader. Experiments were replicated for three times.

Tyrosinase activity of compound 1c in B16F10 melanoma cells

The standard procedure with slight modifications was followed for the measuring of tyrosinase activity in B16F10 cells (Chen et al. 2009). The cells were cultured in a 24-well plate in the amount of 5×10^4 cells per well. The 24-well plates were stored in a humidified environment containing 5% CO_2 at 37 °C for 24 h. Test compound **1c** (0, 5, 10 or 25 µM), α-MSH (1 µM) and kojic acid (25 µM) were added to the cells and further incubated for 24 h under the same conditions. The cells were washed with PBS twice and lysed with lysis buffer containing 50 mM PBS (90 µL, pH 6.8), 0.1 mM PMSF (5 µL) and 1% Triton X-100 (5 µL). The lysed cells were frozen at −80 °C for 30 min. Frozen lysates were centrifuged at 12,000 rpm for 30 min at 4 °C to give a supernatant. A volume of 20 µL of L-dopa (10 mM) was mixed with 80 µL of the supernatant in 96-well ELISA plates and incubated at 37 °C for 30 min. Optical densities were obtained at 500 nm. All experiments were repeated for three times.

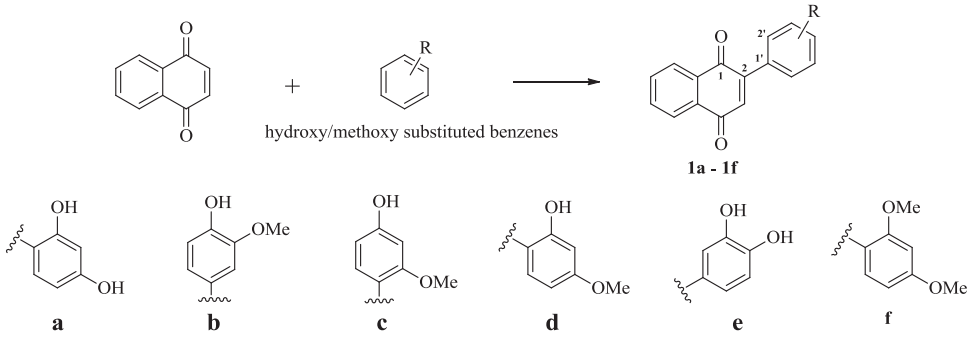
Melanin production in B16F10 melanoma cells

For the determination of inhibitory effect of compound **1c** on melanin biosynthesis, a melanin content assay was carried out according to the known method with slight modifications (Chen et al. 2009). B16F10 cells were distributed in a 24-well plate (5×10^4 cells/well). Then the cells were stored in 5% CO_2 incubator for an overnight at 37 °C. Test compound **1c** (0, 5, 10 or 25 µM), α-MSH (1 µM) and kojic acid (25 µM) were added to the cells and further incubated for 24 h under the same conditions. After cleaning of the cells with PBS buffer, 1 N NaOH solution (200 µL) was added to the cells. Lysates were transferred to 96-well plates to measure the dissolved melanin amounts and the absorbances were checked at 405 nm using an enzyme-linked immunosorbent assay reader. All experiments were repeated for three times.

DPPH radical scavenging activity assay

A known method with slight modifications was followed to determine the DPPH radical scavenging abilities of the test compounds **1a**–**1f** (Matos et al. 2015). A volume of 20 µL of the test compounds **1a**–**1f** (10 mM in DMSO) was added to a 96-well plate, and then 180 µL of a DPPH methanol solution (0.2 mM) was added. L-Ascorbic acid was used as

Scheme 1 Reagents and conditions: 4N-H_2SO_4 aqueous solution/acetic acid (v/v = 1:4)



a standard. The 96-well plate were incubated in the dark for 30 min and absorbances were measured at 517 nm using VersaMax^{TM} microplate reader. All experiments were performed in triplicates. The following formula was used for the calculation of radical scavenging activities of **1a**–**1f**.

$$\text{Scavenging activity}(\%) = [(\text{Ac} - \text{As})/\text{Ac}] \times 100$$

where Ac is the optical density of the non-treated control and As is the optical density of the test sample.

Statistical analysis

GraphPad software (La Jolla, CA, USA) was used for the statistical analysis. All experiments were performed three times. Results were calculated as means ± SEMs. The significance of intergroup differences was measured using one-way INOVA and Tukey's test. Two-sided *P*-values of <0.05 were considered statistically significant.

Results and discussion

Chemistry

According to our previous results, the presence of a hydroxyl group on the β-phenyl ring of the (*E*)-β-phenyl-α,β unsaturated carbonyl scaffold generally enhances tyrosinase-inhibitory activity. Therefore, the synthesis of 2-phenyl-1,4-naphthoquinone derivatives **1a**–**1e** with at least one hydroxyl substituent on the 2-phenyl ring was carried out (Scheme 1). The synthetic method based on a Pd-catalyzed coupling reaction (the Suzuki–Miyaura reaction) (Maluenda and Navarro 2015), between 2-halo-1,4-naphthoquinone and substituted phenylboronic acid was excluded due to cost considerations. Instead, we focused on the chemical properties of 1,4-naphthoquinone, because it can serve as a good Michael acceptor and hydroxy-substituted benzenes can act as good nucleophiles due to the strong electron-donating property of the hydroxyl substituent. Thus, we speculated that 2-(hydroxy-/methoxy-substituted phenyl)naphthoquinone derivatives **1a**–**1f** could be prepared from 1,4-naphthoquinone and hydroxyl-substituted benzenes using the Michael reaction and subsequent spontaneous oxidation, as depicted in Scheme 1. Reactions between 1,4-naphthoquinone and resorcinol (1,3-dihydroxybenzene), 2-methoxyphenol, 3-methoxyphenol, catechol (1,2-dihydroxybenzene) or 1,3-dimethoxybenzene in an acidic co-solvent (4N-H_2SO_4 plus acetic acid; v/v = 1:4) afforded the desired 2-(hydroxyl-/methoxy-substituted phenyl)naphthoquinone derivatives, **1a**–**1f**. It was noteworthy that reaction with 3-methoxyphenol produced two regioisomers, 2-(4-hydroxy-2-methoxyphenyl)naphthalene-1,4-dione (**1c**) and 2-(2-hydroxy-4-methoxyphenyl)naphthalene-1,4-dione (**1d**), respectively, in a ratio of 4:1, presumably due to the ortho- and para-orientations of the hydroxyl substituent on the β-phenyl ring. We used 1D (^{1}H and ^{13}C) and 2D NMR spectra (COSY, HSQC and HMBC) to assign these two regioisomers.

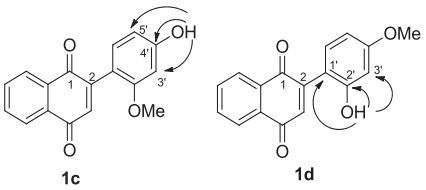


Fig. 2 Key HMBC correlations of compounds **1c** and **1d**

According to the HMBC data of **1c** (Supporting information), the proton (9.88 ppm) of 4′-hydroxyl group showed correlations with three carbons (C′-3, 99.9 ppm; C′-4, 161.0 ppm; C′-5 and 107.8 ppm) (Fig. 2), indicating that compound **1c** was 2-(4-hydroxy-2-methoxyphenyl) naphthalene-1,4-dione. The structure of compound **1d** was determined to be 2-(2-hydroxy-4-methoxyphenyl)naphthalene-1,4-dione based on HMBC data (Supporting information). In particular, the proton (9.84 ppm) of 2′-hydroxyl group showed correlations with three carbons (C′-1, 114.4 ppm; C′-2, 157.3 ppm and C′-3, 102.0 ppm). The reactions between 1,4-naphthoquinone and 2-methoxyphenol and 1,2-dihydroxybenzene each produced only one detectable regioisomer, that is, compounds **1b** and **1e**, respectively, the structures of which were easily determined by analysing the splitting patterns of protons on the 2-phenyl ring of the

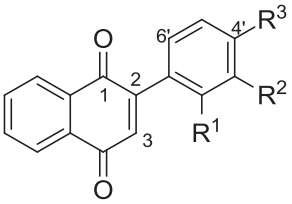
regioisomers. On the other hand, reaction between 1,4-naphthoquinone and phloroglucinol (1,3,5-trihydroxybenzene) gave a compound consisting of two 1,4-naphthoquinones and one phloroglucinol under the same reaction conditions (data not shown), instead of the desired 2-(1,3,5-trihydroxyphenyl)naphthoquinone; although ^{1}H and ^{13}C NMR and mass data of the compound were obtained, its chemical structure could not be determined. Of the six 2-phenylnaphthoquinone derivatives produced, 2-(4-hydroxy-3-methoxyphenyl)naphthalene-1,4-dione (**1b**), 2-(4-hydroxy-2-methoxyphenyl)naphthalene-1,4-dione (**1c**), and 2-(2-hydroxy-4-methoxyphenyl)naphthalene-1,4-dione (**1d**) were unknown compounds. In all products, the presence of only one vinylic proton peak (3-H) of the 1,4-naphthoquinone moiety indicated that only one phenyl groups were attached to the 2-position of 1,4-naphthoquinone and that auto-oxidation had occurred after Michael addition in each case. The vinylic proton peaks (H-3) of all six 2-phenyl-1,4-naphthoquinone derivatives (**1a**–**1f**) revealed in the 6.90–7.03 ppm range.

Biological evaluation

Mushroom tyrosinase assay

The tyrosinase-inhibitory effects of the synthesized 2-phenyl-1,4-naphthoquinone derivatives on commercially available mushroom tyrosinase were assayed at 30 μM and arbutin (Cui et al. 2005) (500 μM) and kojic acid (30 μM) were used as positive controls. As shown in Table 1, compounds **1a**–**1c** and **1f** were found to more potently inhibit mushroom tyrosinase activity than the positive controls. Compound **1e**, which contained a 2-catechol group failed to inhibit tyrosinase. Notably, **1f** with no hydroxyl group on its β-phenyl ring, inhibited mushroom tyrosinase as potently as kojic acid (49.40 ± 3.28% vs. 47.49 ± 3.32% inhibition, respectively). Although **1a** exhibited potent tyrosinase-inhibitory activity at 30 μM, it also showed appreciable toxicity in B16F10 cells at a concentration of ≥ 10 μM (data not shown). Compound **1d** having a 2-hydroxy-4-methyoxyphenyl group did not exhibit a tyrosinase-inhibitory activity, but compound **1c** in which the 4-methoxy group of **1d** was substituted with a 4-hydroxy group showed a large inhibition. On the basis of IC_{50} values, compound **1c** ($IC_{50} = 22.00 \pm 1.63$ μM) inhibited greater tyrosinase inhibitory than **1b** ($IC_{50} = 24.91 \pm 2.12$ μM) or kojic acid ($IC_{50} = 37.86 \pm 2.21$ μM), and neither showed appreciable cytotoxicity at a concentration of ≤10 μM. To the best of our knowledge, these compounds are the first naphthoquinone derivatives with tyrosinase-inhibitory activity. Because of its greater tyrosinase-inhibitory activity than kojic acid based on IC_{50} values, compound **1c** was used in following studies.

Table 1 Substitution patterns, reaction time and tyrosinase-inhibitory activities by the prepared 2-(substituted phenyl)-1,4-naphthoquinone derivatives, **1a**–**1f**, arbutin and kojic acid



Compound	R^1	R^2	R^3	Reaction time[a] (h)	Tyrosinase inhibition[b] (%)
1a	OH	H	OH	12	51.39 ± 6.99
1b	H	OMe	OH	12	51.48 ± 5.30
1c	OMe	H	OH	10	54.28 ± 5.30
1d	OH	H	OMe	10	NI[d]
1e	H	OH	OH	11	NI
1f	OMe	H	OMe	12	49.40 ± 3.28
Arbutin[c]					32.65 ± 1.77
Kojic acid					47.49 ± 3.32

NI no inhibition

[a]Michael addition and autoxidation reaction time

[b]Tyrosinase inhibition was performed using L-tyrosine at 30 μM of each inhibitor or 30 μM of kojic acid

[c]The assays were carried out at 500 μM of arbutin

[d]Results are indicated as mean ± standard error of three experiments' mean

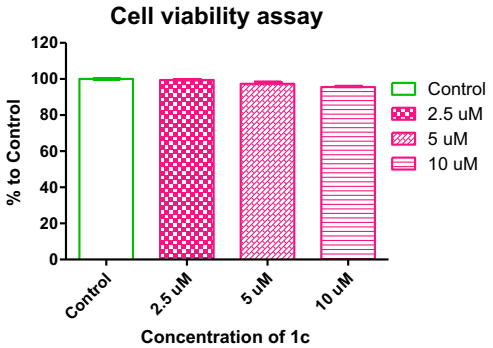


Fig. 3 Effect of **1c** on B16F10 cell viability. The viabilities of cells treated with **1c** (2.5 μM, 5 μM or 10 μM) are indicated as percentage viabilities vs. untreated controls

Cell studies

The effect of compound **1c** on B16F10 cell viability was examined using an MTT assay. The obtained results showed that exposure to compound **1c** at concentrations of ≤ 10 μM for 24 h had no appreciable effect (Fig. 3).

The inhibitory effect of compound **1c** on melanogenesis in B16F10 melanoma cells was evaluated by measuring intracellular melanin contents in cells co-treated with **1c** and

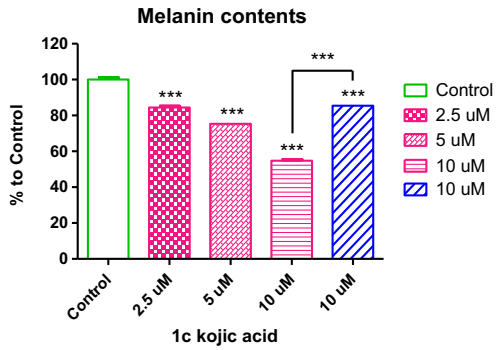


Fig. 4 Effects of **1c** on melanogenesis. B16F10 cells were treated with α-MSH and **1c** or kojic acid for 24 h. The asterisk indicates significant differences between cells treated with **1c** or kojic acid and non-treated control cells: ***$p < 0.001$

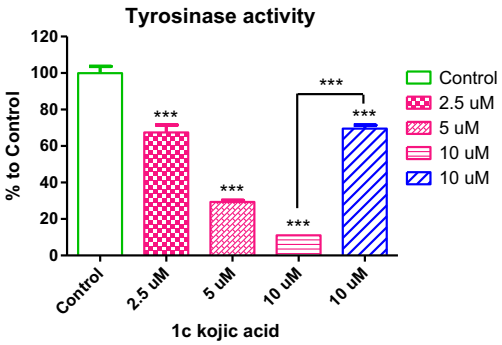


Fig. 5 Effects of compound **1c** on cellular tyrosinase activity. B16F10 cells were treated with α-MSH and **1c** or kojic acid for 24 h. The asterisk denotes significant differences between cells treated with **1c** or kojic acid and non-treated control cells: ***$p < 0.001$

α-MSH (α-melanocyte-stimulating hormone) and cells treated with α-MSH only. Melanin contents were found to dose-dependently decrease as the concentration of **1c** increased (Fig. 4). Furthermore, compound **1c** inhibited melanogenesis more effectively than kojic acid at 10 μM.

When we examined the inhibitory effect of compound **1c** on tyrosinase activity in B16F10 cells treated with α-MSH, it was found **1c** dose-dependently inhibited tyrosinase activity (Fig. 5). In particular, at 10 μM compound **1c** suppressed tyrosinase activity much more strongly than kojic acid. The similar patterns of tyrosinase activity inhibition and melanin content reduction by **1c**, suggest its anti-melanogenic effect was probably due to the inhibition of tyrosinase activity.

DPPH radical scavenging activity

It has been reported that ROS (reactive oxygen species) induce melanogenesis by activating tyrosinase (Gillbro and Olsson 2011, Valverde et al. 1996) and thus, we investigated the scavenging effects of the synthesized 2-(hydroxyl/methoxy-substituted phenyl)naphthoquinone derivatives using a DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. As shown in Table 2, most of the derivatives, including **1c**, exhibited radical scavenging activity similar to that of L-ascorbic acid (positive control), suggesting the inhibitory effect of **1c** on melanin production might be partially due to its radical scavenging activity.

Table 2 The effect of 2-(substituted phenyl)-1,4-naphthoquinone derivatives, **1a**–**1f**, on the DPPH radical scavenging ability

Compound	DPPH radical scavenging activity (%)[a]
1a	87.27 ± 0.44
1b	78.19 ± 0.81
1c	87.10 ± 0.70
1d	83.58 ± 1.70
1e	87.62 ± 0.29
1f	25.02 ± 0.58
L-Ascorbic acid	87.51 ± 0.18

[a]At 30 min after treatment of a DPPH methanol solution, the radical scavenging activity of each compound at a concentration of 1.0 mM was examined. All experiments were independently carried out in triplicate. Results are shown as mean ± standard error of the mean of three experiments

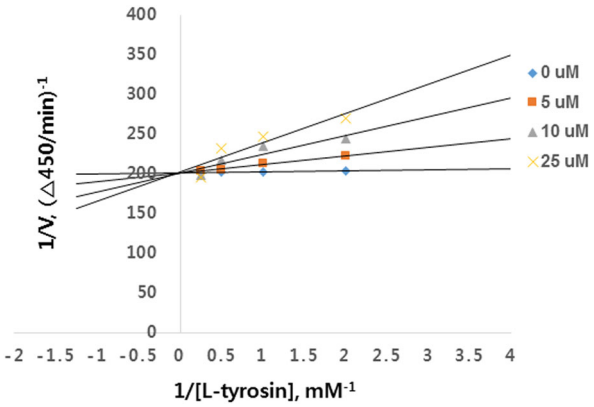
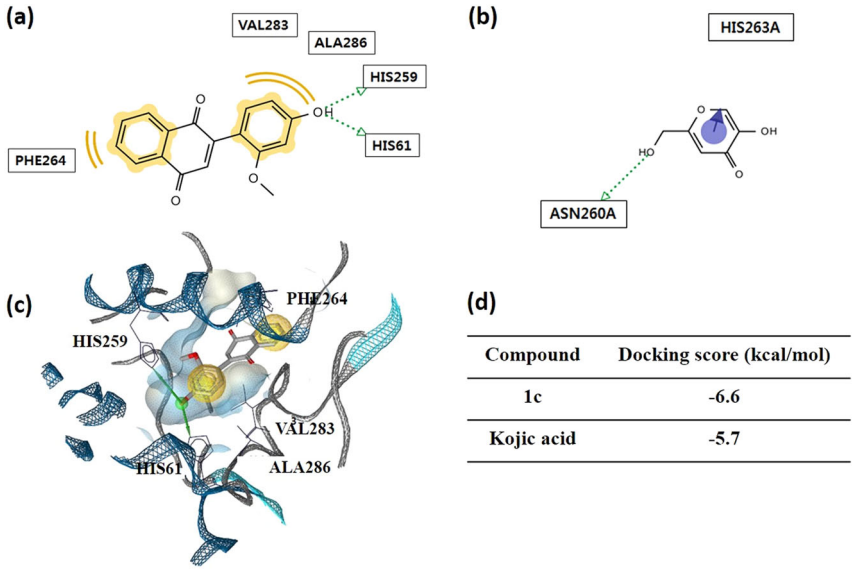


Fig. 6 Mode of mushroom tyrosinase inhibition by compound **1c**. The type of inhibition caused by **1c** was investigated using Lineweaver–Burk plots. The results are mean of 1/V values, where V is the increase in O.D. per minute at different L-tyrosine concentrations. The modified Michaelis–Menten equation was used: $1/Vmax = 1/Km(1+[S]/Ki)$, where Ki is the inhibition constant, V the reaction velocity, Km Michaelis constant and S the concentration of L-tyrosine

Mechanism of action

A Lineweaver–Burk plot was used to investigate the mode of mushroom tyrosinase inhibition by **1c**. As shown in Fig. 6, four straight lines with different slopes that intersected the *y*-axis at the same point were obtained, which indicated Vmax values were not affected by the concentration of **1c**. As the concentration of **1c** increased, Km values also increased gradually. These observations showed compound **1c** competitively inhibits mushroom tyrosinase activity in a dose-dependent manner similar to other competitive



Compound	Docking score (kcal/mol)
1c	-6.6
Kojic acid	-5.7

Fig. 7 Docking simulation of interactions between tyrosinase and **1c** or kojic acid and pharmacophore analysis. **a**, **b** Pharmacophore results for **1c** and kojic acid. **c** Docking result for the interaction between **1c** and tyrosinase. **d** Tyrosinase docking scores for **1c** and kojic acid. Green arrow: hydrogen-bonding, violet arrow: π–π stacking, yellow: hydrophobic interaction

tyrosinase inhibitors (Yi et al. 2010, Xie et al. 2017, Pintus et al. 2017).

Docking studies

To determine whether **1c** binds directly to the active site of tyrosinase, docking simulation was carried out with AutoDock Vina (Di Muzio et al. 2017) using the 3D structure of mushroom tyrosinase (PDB: 2Y9X), **1c** as a ligand, and kojic acid as a positive reference ligand. Compound **1c** was found to have a stronger binding affinity (−6.6 kcal/mol) for the active site than kojic acid (−5.7 kcal/mol) (Fig. 7d). LigandScout 3.1.2 was used to identify the binding residues of **1c** and kojic acid responsible for their binding with tyrosinase. The results obtained showed that **1c** directly hydrogen bonds to the active site of tyrosinase using its 4′-hydroxyl group at two amino acid residues (His61 and His259) (Fig. 7a,c). In addition, the results showed the benzene ring of the 1,4-naphthoquinone of **1c** interacts hydrophobically with Phe264 of tyrosinase and that its 2-phenyl ring interacts hydrophobically with the two amino acid residues Val283 and Val286. However, LigandScout results indicated kojic acid interacts with tyrosinase by forming one hydrogen bond with Asn260 and by π–π stacking with His263 (Fig. 7b). Thus, the greater binding energy of **1c** to tyrosinase than of kojic acid to tyrosinase appears to explain the greater interaction between **1c** and tyrosinase.

Conclusion

In conclusion, in order to identify whether an (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold of an endomethylene type can confer tyrosinase-inhibitory activity, 2-(hydroxyl-/methoxy-substituted phenyl)naphthoquinone derivatives **1a**–**1f** were synthesized by Michael reaction and subsequent spontaneous oxidation. Of the six compounds prepared, four showed either matched or bettered the mushroom tyrosinase-inhibitory effect of kojic acid ($IC_{50} = 37.86 \pm 2.21$ μM) and **1c** exhibited the strongest inhibitory effect ($IC_{50} = 22.00 \pm 1.63$ μM). Furthermore, this observation was supported by the results of our kinetic study and in silico docking simulation, which showed compound **1c** competitively inhibits tyrosinase. In addition, cell-based assays using B16F10 cells showed that **1c** exerted its anti-melanogenic effect by inhibiting cellular tyrosinase without appreciable cytotoxicity. These results support that the (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold of the endomethylene type has an intrinsic ability to facilitate the inhibition of tyrosinase.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Arndt KA, Fitzpatrick TB (1965) Topical use of hydroquinone as a depigmenting agent. JAMA 194:965–967

Breathnach AC, Nazzaro-Porro M, Passi S, Zina G (1989) Azelaic acid therapy in disorders of pigmentation. Clin Dermatol 7:106–119

Chen L-G, Chang W-L, Lee C-J, Lee L-T, Shih C-M, Wang C-C (2009) Melanogenesis inhibition by gallotannins from Chinese galls in B16 mouse melanoma cells. Biol Pharm Bull 32:1447–1452

Cui T, Nakamura K, Ma L, Li J-Z, Kayahara H (2005) Analyses of arbutin and chlorogenic acid, the major phenolic constituents in oriental pear. J Agric Food Chem 53:3882–3887

Curto EV, Kwong C, Hermersdörfer H, Glatt H, Santis C, Virador V, Hearing Jr VJ, Dooley TP (1999) Inhibitors of mammalian melanocyte tyrosinase: in vitro comparisons of alkyl esters of gentisic acid with other putative inhibitors. Biochem Pharmacol 57:663–672

Engasser PG (1984) Ochronosis caused by bleaching creams. J Am Acad Dermatol 10:1072–1073

Fisher A (1983) Current contact news. Hydroquinone uses and abnormal reactions. Cutis 31:240–244. 250

Fitzpatrick T, Arndt K, El Mofty A, Pathak M (1966) Hydroquinone and psoralens in the therapy of hypermelanosis and vitiligo. Arch Dermatol 93:589–600

Garcia-Lopez M (1989) Double-blind comparison of azelaic acid and hydroquinone in the treatment of melasma. Acta Derm Venereol (Stockh) 143:58–61

Gillbro J, Olsson M (2011) The melanogenesis and mechanisms of skin-lightening agents–existing and new approaches. Int J Cosmet Sci 33:210–221

Gonçalez M, Correa MA, Chorilli M (2013) Skin delivery of kojic acid-loaded nanotechnology-based drug delivery systems for the treatment of skin aging. BioMed Res Int 2013:9

Griffiths C, Finkel L, Ditre C, Hamilton T, Ellis C, Voorhees J (1993) Topical tretinoin (retinoic acid) improves melasma. A vehicle-controlled, clinical trial. Br J Dermatol 129:415–421

Ha YM, Kim J-A, Park YJ, Park D, Kim JM, Chung KW, Lee EK, Park JY, Lee JY, Lee HJ (2011) Analogs of 5-(substituted benzylidene) hydantoin as inhibitors of tyrosinase and melanin formation. Biochim Et Biophys Acta (BBA)-General Subj 1810:612–619

Hearing VJ, Tsukamoto K (1991) Enzymatic control of pigmentation in mammals. FASEB J 5:2902–2909

Heilgemeir G, Balda B (1981) Irreversible toxic depigmentation. Observations following use of hydroquinonemonobenzylether-containing skin bleaching preparations. MMW Munch Med Wochenschr 123:47

Hyun SK, Lee W-H, Jeong DM, Kim Y, Choi JS (2008) Inhibitory effects of kurarinol, kuraridinol, and trifolirhizin from Sophora flavescens on tyrosinase and melanin synthesis. Biol Pharm Bull 31:154–158

Janeczko M, Demchuk OM, Strzelecka D, Kubiński K, Masłyk M (2016) New family of antimicrobial agents derived from 1, 4-naphthoquinone. Eur J Med Chem 124:1019–1025

Jimbow K (1991) N-acetyl-4-S-cysteaminylphenol as a new type of depigmenting agent for the melanoderma of patients with melasma. Arch Dermatol 127:1528–1534

Kang KH, Lee B, Son S, Yun HY, Moon KM, Jeong HO, Kim DH, Lee EK, Choi YJ, Do HK (2015) (Z)-2-(Benzo [d] thiazol-2-ylamino)-5-(substituted benzylidene) thiazol-4 (5H)-one Derivatives as Novel Tyrosinase Inhibitors. Biol Pharm Bull 38:1227–1233

Ki D-H, Jung H-C, Noh Y-W, Thanigaimalai P, Kim B-H, Shin S-C, Jung S-H, Cho C-W (2013) Preformulation and formulation of newly synthesized QNT3-18 for development of a skin whitening agent. Drug Dev Ind Pharm 39:526–533

Kim HR, Lee HJ, Choi YJ, Park YJ, Woo Y, Kim SJ, Park MH, Lee HW, Chun P, Chung HY (2014) Benzylidene-linked thiohydantoin derivatives as inhibitors of tyrosinase and melanogenesis: importance of the β-phenyl-α, β-unsaturated carbonyl functionality. MedChemComm 5:1410–1417

Kim SH, Ha YM, Moon KM, Choi YJ, Park YJ, Jeong HO, Chung KW, Lee HJ, Chun P, Moon HR (2013) Anti-melanogenic effect of (Z)-5-(2, 4-dihydroxybenzylidene) thiazolidine-2, 4-dione, a novel tyrosinase inhibitor. Arch Pharm Res 36:1189–1197

Kimbrough-Green CK, Griffiths CE, Finkel LJ, Hamilton TA, Bulengo-Ransby SM, Ellis CN, Voorhees JJ (1994) Topical retinoic acid (tretinoin) for melasma in black patients: a vehicle-controlled clinical trial. Arch Dermatol 130:727–733

Kligman AM, Willis I (1975) A new formula for depigmenting human skin. Arch Dermatol 111:40–48

Kumar KS, Vani MG, Wang SY, Liao JW, Hsu LS, Yang HL, Hseu YC (2013) In vitro and in vivo studies disclosed the depigmenting effects of gallic acid: A novel skin lightening agent for hyperpigmentary skin diseases. Biofactors 39:259–270

Maluenda I, Navarro O (2015) Recent developments in the Suzuki-Miyaura reaction: 2010–2014. Molecules 20:7528–7557

Matos M, Varela C, Vilar S, Hripcsak G, Borges F, Santana L, Uriarte E, Fais A, Di Petrillo A, Pintus F (2015) Design and discovery of tyrosinase inhibitors based on a coumarin scaffold. RSC Adv 5:94227–94235

Mayer AM, Harel E (1979) Polyphenol oxidases in plants. Phytochemistry 18:193–215

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ (1998) Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. J Comput Chem 19:1639–1662

Moustakas DT, Lang PT, Pegg S, Pettersen E, Kuntz ID, Brooijmans N, Rizzo RC (2006) Development and validation of a modular, extensible docking program: DOCK 5. J Comput Aided Mol Des 20:601–619

Di Muzio E, Toti D, Polticelli F (2017) DockingApp: a user friendly interface for facilitated docking simulations with AutoDock Vina. J Comput Aided Mol Des 31:213–218

Neering H (1975) Treatment of melasma (chloasma) by local application of a steroid cream. Dermatology 151:349–353

Pintus F, Matos MJ, Vilar S, Hripcsak G, Varela C, Uriarte E, Santana L, Borges F, Medda R, Di Petrillo A (2017) New insights into highly potent tyrosinase inhibitors based on 3-heteroarylcoumarins: Anti-melanogenesis and antioxidant activities, and computational molecular modeling studies. Biorg Med Chem 25:1687–1695

Redaelli M, Mucignat-Caretta C, Isse AA, Gennaro A, Pezzani R, Pasquale R, Pavan V, Crisma M, Ribaudo G, Zagotto G (2015) New naphthoquinone derivatives against glioma cells. Eur J Med Chem 96:458–466

Romaguera C, Grimalt F (1985) Leukoderma from hydroquinone. Contact Dermat 12:183–183

Shin D-H, Kim O-H, Jun H-S, Kang M-K (2008) Inhibitory effect of capsaicin on B16-F10 melanoma cell migration via the phosphatidylinositol 3-kinase/Akt/Rac1 signal pathway. Exp Mol Med 40:486

Solano F (2014) Melanins: skin pigments and much more-types, structural models, biological functions, and formation routes. New J Sci 2014:28

Valverde P, Manning P, Todd C, McNeil C, Thody A (1996) Tyrosinase may protect human melanocytes from the cytotoxic effects of the superoxide an ion. Exp Dermatol 5:247–253

Xie W, Zhang H, He J, Zhang J, Yu Q, Luo C, Li S (2017) Synthesis and biological evaluation of novel hydroxybenzaldehyde-based kojic acid analogues as inhibitors of mushroom tyrosinase. Bioorg Med Chem Lett 27:530–532

Yi W, Cao R, Peng W, Wen H, Yan Q, Zhou B, Ma L, Song H (2010) Synthesis and biological evaluation of novel 4-hydroxybenzaldehyde derivatives as tyrosinase inhibitors. Eur J Med Chem 45:639–646