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Research Paper

Inhibitory effects of 1,3-thiazine derivatives on melanogenesis

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Abstract

Objectives The aim of this study was to identify a novel skin-depigmenting agent from synthetic 1,3-thiazine derivatives.

Methods We investigated the inhibitory effects of six kinds of 1,3-thiazine derivative on melanogenesis by examining their effects on tyrosinase activity and melanin biosynthesis in melan-a cells and the zebrafish model.

Key findings Of the six compounds, 4-hydroxy-2,6-dimethyl-5,6-dihydro-4*H*-1,3-thiazine (TZ-6) had the strongest anti-melanogenic effects in cultured melan-a cells (30.4% inhibition at 100 μM). In addition, TZ-6 exhibited an inhibitory effect on mushroom and cellular tyrosinase. Based on the results of Western blotting, TZ-6 reduced the expression of tyrosinase at 100 μM. Additionally, TZ-6 reduced body pigmentation and inhibited tyrosinase activity in the zebrafish model.

Conclusions The results have provided useful information for the development of a skin whitening agent.

Keywords melanin; 1,3-thiazine derivatives; tyrosinase; zebrafish

Introduction

Melanogenesis is a multistage process involving melanin synthesis, melanin transport and melanosome release. Melanin is synthesized in melanosomes, which are membrane-bound granules within melanocytes, and is then transferred to keratinocytes. This process of melanogenesis is regulated by a variety of environmental, hormonal and genetic factors, including ultraviolet (UV) exposure, α-melanocyte-stimulating hormone (α-MSH), melanocortin 1 receptor (MC1R), and agouti-related protein.[1–5]

Melanin is a biological pigment that gives colour to the skin, hair and scales of many mammals. It is secreted by melanocyte cells, which are distributed in the basal layer of the epidermis.[6] Although the melanin pigment in human skin is a major defence mechanism against UV light from the sun, the production of abnormal pigmentation such as melasma, freckles, age spots (or liver spots), and other forms of melanin hyperpigmentation can be aesthetically undesirable.[7,8] Melanin is a heteropolymer of indole compounds that is produced inside melanosomes by the action of the tyrosinase enzyme on the tyrosinase precursor material in melanocytes. Tyrosinase is well known as a key enzyme for melanin biosynthesis in plants and animals. It is the copper-containing enzyme that catalyses the first two reactions of melanin synthesis: the conversion of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and of L-DOPA to dopaquinone.[9] Therefore, tyrosinase inhibitors may be useful for the treatment of some dermatological problems related to hyperpigmentation.

Thiazines are organic compounds containing one nitrogen and one sulfur atom in a ring of four carbons. Chemicals that include thiazine are used in marking dyes. The 1,3-thiazine derivatives have been reported to have antithyroid activity *in vivo,* as well as antimycobacterial effects.[10,11] However, their effect on melanin production in the skin

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is unknown. Thus, we have evaluated the effects of 1,3-thiazine derivatives on melanin biosynthesis and tyrosinase activity.

Materials and Methods

Materials

Mushroom tyrosinase, L-DOPA, and kojic acid (5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one) were purchased from Sigma-Aldrich Chemical Inc. (St Louis, MO, USA). Solvents for organic synthesis were redistilled. All other chemicals and solvents were of analytical grade and were used without further purification. The structures of the six 1,3-thiazine derivatives are provided in detail in Figure 1. These 1,3-thiazine derivatives were prepared using a previously described protocol.[11]

TZ-1

4-Hydroxy-2,4,5,6-tetramethyl-5,6-dihydro-4*H*-1,3-thiazine. 3-Methyl-3-peten-2-one (2.0 mmol) was added to a solution of thioacetamide (20 mmol) in dry dichloromethane (10 ml) at room temperature under an argon atmosphere. To this solution was added $BF_3{\cdot}Et_2O$ (1.2 mmol). The reaction mixture was stirred for 2 h, quenched with saturated sodium carbonate solution, and extracted with dichloromethane. The extracts were dried (Na_2SO_4) and evaporated to dryness. The residue was purified by flash chromatography on silica gel with dichloromethane to give a 77% yield of TZ-1 as a yellow solid with a melting point (mp) of 71.0–72.8°C; IR (KBr) 3172, 1618 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.84 (3H, d, J = 7.2 Hz), 1.26 (3H, d, J = 7.2 Hz), 1.33 (3H, s), 1.81–1.89 (1H, dq, J = 3.6, 7.2 Hz), 2.13 (3H, s), 3.62–3.67 (1H, dq, J = 3.6, 7.2 Hz), 4.44 (1H, br s); ^{13}C NMR ($CDCl_3$) δ 6.5, 18.2, 26.6, 37.6, 38.5, 87.1, 158.8; MS (CI): m/z = 174 [M^++1].

TZ-2

4-Hydroxy-2,4-dimethyl-6-pentyl-5,6-dihydro-4*H*-1,3-thiazine. Yield 74% (*cis/trans* = 72/28). White solid with mp 41.2–42.8°C; IR (KBr) 3156, 1614 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.89 (3H, t, J = 6.8 Hz), 1.29–1.33 (6H, m), 1.34 (3H, s), 1.35–1.43 (2H, m), 1.62–1.69 (1H, m), 2.09 (1H, dd, J = 4.0, 13.2 Hz), 2.13 (3H, s), 3.25–3.32 (1H, m), 5.55 (1H, br s); ^{13}C NMR ($CDCl_3$) δ 13.9, 22.4, 25.8, 26.6, 26.8, 31.5, 36.5, 38.3, 39.7, 84.8, 158.0; MS (CI): m/z = 216 [M^++1].

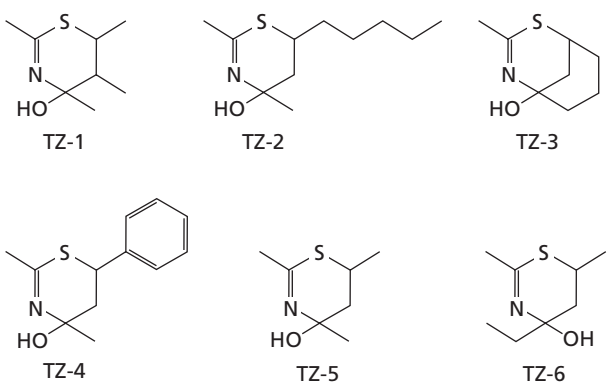

Figure 1 Structures of six 1,3-thiazine derivatives (TZ-1–TZ-6)

TZ-3

5-Hydroxy-3-methyl-4-aza-2-thiabicyclo[3.3.1]-3-nonene. Yield 36%. Yellow solid with mp 129.6–131.6°C; IR (KBr) 3147, 1618 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.60–1.74 (4H, m), 1.80–1.86 (2H, m), 2.03 (2H, d, J = 4.0 Hz), 2.19 (3H, s), 3.59 (1H, quint, J = 3.2 Hz), 5.82 (1H, br s); ^{13}C NMR ($CDCl_3$) δ 18.4, 26.7, 32.8, 33.2, 39.0, 39.7, 81.2, 160.3; MS (CI): m/z = 172 [M^++1].

TZ-4

4-Hydroxy-2,4-dimethyl-6-phenyl-5,6-dihydro-4*H*-1,3-thiazine. Yield 77% (*cis/trans* = 61/39), mp 114.6–116.2°C; IR (KBr) 3180, 1603 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.47 (3H, s), 1.87 (1H, t, J = 13.2 Hz), 2.18 (3H, s), 2.27 (1H, d, J = 4.0, 13.2 Hz), 4.42 (1H, d, J = 4.0, 13.2 Hz), 5.17 (1H, br s), 7.23–7.36 (5H, m), ^{13}C NMR ($CDCl_3$) δ 26.8, 31.8, 38.6, 44.0, 85.3, 127.6, 128.0, 128.9, 139.7, 158.0; MS (CI): m/z = 222 [M^++1].

TZ-5

4-Hydroxy-2,4,6-trimethyl-5,6-dihydro-4*H*-1,3-thiazine. Yield 51% (*cis/trans* = 70/30). ^1H NMR ($CDCl_3$) δ 1.26–1.28 (1H, m), 1.31 (3H, d, J = 6.8 Hz), 1.33 (3H, s), 2.08 (1H, dd, J = 4.0, 13.7 Hz), 2.14 (3H, s), 3.28–3.37 (1H, m), 5.14 (1H, br s); ^{13}C NMR ($CDCl_3$) δ 21.8, 26.8, 31.1, 34.2, 40.0, 85.0, 157.9.

TZ-6

4-Hydroxy-2,6-dimethyl-5,6-dihydro-4*H*-1,3-thiazine. (*cis/trans* = 70/30). ^1H NMR ($CDCl_3$) δ 0.96 (3H, t, J = 7.6 Hz), 1.1–1.3 (1H, m), 1.29 (3H, d, J = 6.2 Hz), 1.4–1.5 (1H, m), 1.7–1.8 (1H, m), 2.13 (3H, s), 3.23–3.27 (1H, m), 4.23 (1H, br s); ^{13}C NMR ($CDCl_3$) δ 6.9, 21.9, 26.7, 30.9, 33.7, 36.9, 87.0, 157.9.

General methods

Melting points were determined using a Yanagimoto micromelting point apparatus (Yanagimoto, Kyoto, Japan). IR spectra were obtained using a Perkin-Elmer 1600 spectrometer, (PerkinElmer, Waltham, MA, USA), and ^1H and ^{13}C NMR spectra were recorded on a JEOL-JNM-α400 (400 MHz) spectrometer (Jeol, Peabody, MA, USA). Mass spectra were obtained using a Shimadzu 9020-DF mass spectrometer (Shimadzu, Kyoto, Japan), and UV spectra using a Molecular Devices E09090 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cells

Murine melan-a melanocytes (melan-a cells) were originally derived from C57BL/6J (black) mice. The melan-a cells are close in character to melanocytes *in vivo* and have been widely used as a suitable substitute for normal primary mouse melanocytes in melanin metabolism tests.[12] The melan-a cell lines were obtained from Dr B. Lee at the Skin Research Institute, Amore-Pacific Corporation, Yongin, Korea.

Cell culture

Melan-a cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, and 200 nM phorbol 12-myristate 13-acetate. Cells were seeded in a 100-mm diameter tissue culture dish (5×10^5 cells/well). The cell cultures grew to confluence after three to four days at 37ºC in a 5% CO_2 atmosphere. They were seeded at 10^5 cells/well in a 24-well plate and incubated for 24 h. Each well was renewed with 990 μl RPMI1640 medium every day; treated with 10 μl of 10000, 1000 and 100 μM test sample for three days (solvent system: propylene glycol/EtOH/H_2O = 5/3/2); and then incubated for one day at 37ºC with 5% CO_2.

Determination of cell viability

The viability of melan-a cells was measured by staining the cell population with crystal violet. After incubation with several concentrations of test sample for 72 h, the culture medium was removed from each well, and the cells were washed with phosphate-buffered saline (PBS) and treated with 200 μl crystal violet (0.1% crystal violet, 10% EtOH, 89.9% PBS). The cells were stained at room temperature for 5 min and rinsed twice with water; 1 ml 95% ethanol was then added, and the solution was shaken at room temperature for 10 min. Crystal violet retained by adherent cells was extracted with 95% ethanol, and the absorption was measured at 590 nm (OD_{590}) using an ELISA reader.

Determination of melanin levels

Melanin production was measured using a modification of the methods reported by Hosoi *et al.*[13] After removing the RPMI1640 medium from each well, the cells were washed with PBS, and 1 ml 1 M NaOH was added to dissolve the melanin. The optical density was measured at 400 nm (OD_{400}) using an ELISA reader. The amount of melanin per well was calculated and represented as a percentage of the control. Phenylthiourea was used as a positive control.[14]

Mushroom tyrosinase activity

Each concentration of 1,3-thiazine derivative was dissolved in MeOH and 120 μl L-DOPA (8 mM, dissolved in 67 mM phosphate buffer, pH 6.8). A 40-μl sample of each 1,3-thiazine derivative solution was added to a 96-well microplate, followed by 40 μl mushroom tyrosinase (125 U). The amount of dopachrome in the reaction mixture was determined after incubation at 37°C for 20 min. Inhibitory activity was expressed as the concentration that inhibited 50% of tyrosinase activity (IC50), as determined by the optical density at 490 nm (OD_{490}).

Cellular tyrosinase activity

Melan-a cells were disrupted by resuspension in tyrosinase buffer (80 mM phosphate buffer, 1% Triton-X 100, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF)) followed by sonication in an ice bath. The supernatant was used for the enzyme assay after centrifugation at 12500 rev/min for 15 min; 150 μg protein was required for each reaction. The protein content was measured using the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA, USA), with bovine serum albumin as the standard. Tyrosinase activity was measured by its DOPA oxidase activity, as described previously.[15] All compounds were dissolved in MeOH and 120 μl L-DOPA (8 mM, dissolved in 67 mM phosphate buffer, pH 6.8), and 40 μl of each compound solution were added to a 96-well microplate, followed by 40 μl (150 μg protein) of cell-originated tyrosinase. After incubation at 37°C for 20 min, the amount of dopachrome was calculated. Inhibitory activity was expressed as the concentration that inhibited 50% of tyrosinase activity (IC50), as determined by the optical density at 490 nm (OD_{490}). Kojic acid was used as a standard tyrosinase inhibitor.

Intracellular tyrosinase activity was determined as previously described, with slight modification.[16] Briefly, melan-a cells were cultured in 100-mm dishes, and incubated with TZ-6 at various concentrations (1, 10 and 100 μM) for three days. L-DOPA (10 μl, 10 mM; dissolved in 67 mM phosphate buffer, pH 6.8) was placed in each well of a 96-well plate, and 90 μl of each lysate, containing 150 μg protein, was then added per well. After incubation at 37°C for 20 min, the amount of dopachrome was calculated by the optical density at 490 nm (OD_{490}).

Western blotting

Protein samples (40 μg) from the melan-a cell extract were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% skim milk and incubated with primary (tyrosinase; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and secondary (donkey anti-goat IgG; Santa Cruz Biotechnology) antibodies. The blots were developed using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Observation of melanin pigment on the surface of zebrafish embryos

Phenotype-based evaluations of zebrafish body pigmentation were carried out using the method reported by Choi *et al.*[17] Briefly, synchronized embryos were collected and arrayed by pipette with three to four embryos per well in 96-well plates containing 200 μl embryo medium. Compound TZ-6 was dissolved in 0.1% dimethylsulfoxide and added to the embryo medium. The effects on the pigmentation of zebrafish were observed under a stereomicroscope. Phenylthiourea (0.2 mM) was used as a standard positive control. Phenotype-based evaluations of body pigmentation were carried out at 35 h post-fertilization. Embryos were dechorionated using forceps, anaesthetized in tricaine methanesulfonate solution (Sigma, St Louis, MO, USA), mounted in 3% methylcellulose on a depression slide (Aquatic Eco-Systems, Apopka, FL, USA), and photographed under an MZ16 stereomicroscope (Leica Microsystems, Ernst-Leitz-Strasse, Germany).

Assay of tyrosinase activity and measurement of melanin content in zebrafish

Tyrosinase activity was determined spectrometrically, as described previously.[16] Briefly, approximately 100 zebrafish embryos were treated with compound TZ-6 from 9 to

48 h post-fertilization and then sonicated in Pro-Prep protein extraction solution (Intron, Daejeon, Korea). The lysate was cleared by centrifuging at 10000*g* for 5 min. After quantification, 250 μg total protein in 100 μl lysis buffer was transferred to a 96-well plate, and 100 μl 1 mM L-DOPA was added. The control well contained 100 μl lysis buffer and 100 μl 1 mM L-DOPA. After incubation for 60 min at 37ºC, the absorbance was measured at 475 nm using a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). The blank was removed from each absorbance value, and the final activity was expressed as a percentage of the water control. Phenylthiourea was used as a positive control. All experiments were replicated at least three times and yielded similar results.

To determine the melanin content, protein extract was prepared at 48 h post-fertilization. After centrifugation, the pellet was dissolved in 1 ml 1 M NaOH at 100ºC for 30 min. The sample was then vigorously vortexed to solubilize the melanin pigment. The optical density of the supernatant was measured at 490 nm, and the results were compared with a standard curve of known concentrations of synthetic melanin (0–300 μg/ml; Sigma, St Louis, MO, USA). The melanin content was calibrated by the amount of protein and expressed as a percentage of the control.

Statistical analysis

The data were analysed using statistical analysis system software (PRISM). All the data are expressed as mean ± SD of three independent experiments. Statistical comparisons between the different treatments were performed using one-way analysis of variance with Tukey's multiple comparison post test. P values of < 0.05 were considered to be statistically significant.

Results

Inhibitory effects on mushroom tyrosinase

The inhibitory effects of the six 1,3-thiazine derivatives and kojic acid on mushroom tyrosinase were examined (Table 1).

Table 1 Inhibitory effects of 1,3-thiazine derivatives and kojic acid on mushroom tyrosinase

Compound	Inhibition at 200 μM (%)[a]	IC50 (μM)
TZ-1	8.7 ± 3.5	> 400
TZ-2	12.3 ± 1.5	> 400
TZ-3	0.67 ± 2.5	> 400
TZ-4	22.2 ± 3.0*	363
TZ-5	39.5 ± 6.9*	245
TZ-6	33.4 ± 3.4*	278
Kojic acid	43.7 ± 5.9*	257

TZ-1, 4-hydroxy-2,4,5,6-tetramethyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-2, 4-hydroxy-2,4-dimethyl-6-pentyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-3, 5-hydroxy-3-methyl-4-aza-2-thiabicyclo[3.3.1]-3-nonene; TZ-4, 4-hydroxy-2,4-dimethyl-6-phenyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-5, 4-hydroxy-2,4,6-trimethyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-6, 4-hydroxy-2,6-dimethyl-5,6-dihydro-4*H*-1,3-thiazine. [a]Each value indicates the mean ± SD of three experiments. IC50, 50% inhibitory concentration. *$P < 0.05$ compared with control.

Compound TZ-5 had the highest inhibitory effect, with an IC50 of 245 μM. In addition, the inhibitory effect of compound TZ-5 on mushroom tyrosinase was similar to that of kojic acid (IC50 = 257 μM), which is a well-known tyrosinase inhibitor. Compounds TZ-4 and TZ-6 exhibited 22.2 and 33.4% inhibition at 200 μM, respectively.

Effects on melanin production and cell viability in cultured melan-a cells

We examined the inhibitory effects of the six 1,3-thiazine derivatives on melanin production and cell viability of melan-a cells. Cell viability was detected by crystal violet staining. Compounds TZ-1, TZ-5 and TZ-6 reduced the melanin pigment levels in melan-a cells (Table 2). Compound TZ-6 decreased the melanin content by 30.4%. Phenylthiourea was used as a positive control.

Inhibitory effects on tyrosinase activity

To confirm whether the down-regulatory effect of 1,3-thiazine derivatives on melanin synthesis was associated with the inhibition of tyrosinase activity, the compounds were examined using tyrosinase in a cell-free system. Compounds TZ-1, TZ-2, TZ-5 and TZ-6 had high levels of inhibition of tyrosinase activity (Table 3). The percent inhibition of

Table 2 Effect of each 1,3-thiazine derivative on cell growth and melanin production in melan-a cells

Compound	Concentration (μM)	Melanin production (%)	Cell viability (%)
TZ-1	1	101.7 ± 6.9	104.3 ± 2.5
	10	104.4 ± 6.2	105.4 ± 3.5
	100	82.9 ± 4.3*	101.6 ± 5.1
TZ-2	1	106.0 ± 5.5	106.9 ± 1.6
	10	89.2 ± 7.8	101.1 ± 9.7
	100	17.6 ± 6.8*	30.5 ± 6.1*
TZ-3	1	107.4 ± 9.8	105.2 ± 1.9
	10	106.2 ± 4.7	107.5 ± 8.1
	100	91.5 ± 3.5	99.8 ± 5.2
TZ-4	1	98.1 ± 6.2	100.7 ± 4.4
	10	83.4 ± 5.8*	96.5 ± 4.7
	100	20.8 ± 8.8*	29.9 ± 17.7*
TZ-5	1	93.9 ± 5.8	96.5 ± 6.6
	10	81.2 ± 5.9*	89.1 ± 6.6
	100	34.4 ± 7.9*	59.0 ± 8.9*
TZ-6	1	98.9 ± 6.2	99.5 ± 7.6
	10	87.1 ± 5.6	91.7 ± 7.9
	100	40.6 ± 9.1*	71.0 ± 8.1*
Phenylthiourea	1	95.4 ± 7.2	102.3 ± 2.6
	10	73.2 ± 4.3*	101.3 ± 4.2
	100	30.7 ± 5.8*	98.9 ± 3.1

Test sample and medium were renewed daily. Cell viability and the melanin contents of melan-a cells were determined after three days. TZ-1, 4-hydroxy-2,4,5,6-tetramethyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-2, 4-hydroxy-2,4-dimethyl-6-pentyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-3, 5-hydroxy-3-methyl-4-aza-2-thiabicyclo[3.3.1]-3-nonene; TZ-4, 4-hydroxy-2,4-dimethyl-6-phenyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-5, 4-hydroxy-2,4,6-trimethyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-6, 4-hydroxy-2,6-dimethyl-5,6-dihydro-4*H*-1,3-thiazine. The data shown represent the means ± SD of three independent experiments performed in duplicate. *$P < 0.05$ compared with control.

Table 3 Inhibitory effects of 1,3-thiazine derivatives and kojic acid on tyrosinase activity in a cell-free system

Compound	Inhibition at 200 μM (%)[a]	IC50 (μM)
TZ-1	22.8 ± 1.7*	>1000
TZ-2	30.3 ± 2.8*	447.1
TZ-3	2.07 ± 4.3	>1000
TZ-4	0.42 ± 1.8	_
TZ-5	24.5 ± 2.2*	680.72
TZ-6	26.9 ± 1.9*	653.62
Kojic acid	41.0 ± 2.2*	318.12

TZ-1, 4-hydroxy-2,4,5,6-tetramethyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-2, 4-hydroxy-2,4-dimethyl-6-pentyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-3, 5-hydroxy-3-methyl-4-aza-2-thiabicyclo[3.3.1]-3-nonene; TZ-4, 4-hydroxy-2,4-dimethyl-6-phenyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-5, 4-hydroxy-2,4,6-trimethyl-5,6-dihydro-4*H*-1,3-thiazine; TZ-6, 4-hydroxy-2,6-dimethyl-5,6-dihydro-4*H*-1,3-thiazine. [a]Each value indicates the mean ± SD of three experiments. IC50, 50% inhibitory concentration. *$P < 0.05$ compared with control.

compounds TZ-1, TZ-2, TZ-5 and TZ-6 against tyrosinase was 22.8, 30.3, 24.5, and 26.9% at 200 μM, respectively. To determine the effect on intracellular tyrosinase activity of TZ-6, which had the most significant inhibitory effect among the compounds tested, we examined the intracellular tyrosinase activity assay after treatment of TZ-6 for three days in melan-a cells. It revealed an inhibitory ratio of 19.3% at concentration 100 μM (Figure 2a).

Western blotting of tyrosinase treated with compound TZ-6

To determine whether compound TZ-6 had an inhibitory effect on melanin production as a result of the decreased expression of tyrosinase protein, we examined changes in tyrosinase at the protein level using Western blotting. Compound TZ-6 decreased tyrosinase expression at 100 μM (Figure 2b).

Melanin pigment production at the surface of zebrafish embryos

We examined changes in the melanin content of zebrafish embryos to verify the inhibitory effect of compound TZ-6 on melanin pigment production. The black spots situated on the eye and dorsal regions of the embryos comprised melanin pigment. Figure 3a shows a vehicle-treated zebrafish used as a control. Phenylthiourea, the positive control, was added to the embryo medium, and the level of zebrafish embryo melanin pigment at 35 h post-fertilization is shown in Figure 3b. The amount of melanin produced in embryos treated with phenylthiourea was significantly decreased compared with the control. In addition, compound TZ-6 significantly reduced the melanin pigment content of the eye and dorsal regions of the embryos in comparison with the control group (Figure 3c).

Effects of compound TZ-6 on tyrosinase activity and melanin production in zebrafish embryos

To confirm the molecular targets of compound TZ-6, the melanin content and tyrosinase activity were determined using whole extracts of zebrafish. We detected remarkable reductions in melanin content and tyrosinase activity after treatment with compound TZ-6 (Figure 4). Compound TZ-6 reduced the total melanin content and tyrosinase activity in a dose-dependent manner. Moreover, the inhibitory effect of compound TZ-6 at 0.6 mM was higher than that of 0.2 mM phenylthiourea, which is routinely used to inhibit pigment production in zebrafish.[18]

Discussion

Tyrosinase plays a critical regulatory role in melanin biosynthesis. Thus, numerous studies have examined the regulation of tyrosinase activity in melanocytes. Kojic acid is a representative tyrosinase inhibitor and has been used in many countries as a skin whitening agent. However, a recent study has shown that kojic acid has serious side effects, such as hepatocytotoxicity, skin cancer and dermatitis.[19] In addition to kojic acid, aloesin and arbutin have been reported to be potential inhibitors of tyrosinase activity.[20,21] However, the effects of these compounds are not sufficient for clinical use. Therefore, potent tyrosinase inhibitors that suppress melanogenesis have been studied with the aim of treating hyperpigmentation. However, there is still a commercial desire to develop effective skin whitening agents from natural products or synthetic compounds.

We investigated the inhibitory effects of synthetic 1,3-thiazine derivatives on mushroom tyrosinase. Compounds

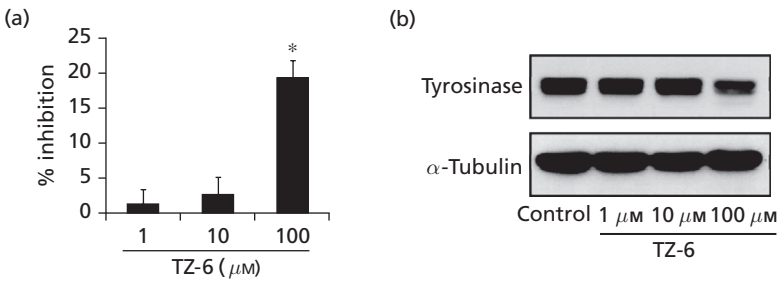

Figure 2 Effect of compound TZ-6 on intracellular tyrosinase activity and expression. (a) Tyrosinase activity; (b) tyrosinase expression. Cells were cultured with TZ-6 (4-hydroxy-2,6-dimethyl-5,6-dihydro-4*H*-1,3-thiazine; 100 μM) for 72 h. Intracellular tyrosinase activity and tyrosinase expression were measured, as described in Materials and Methods. The data are represented as mean ± SD of three independent experiments. *$P < 0.05$ compared with control.

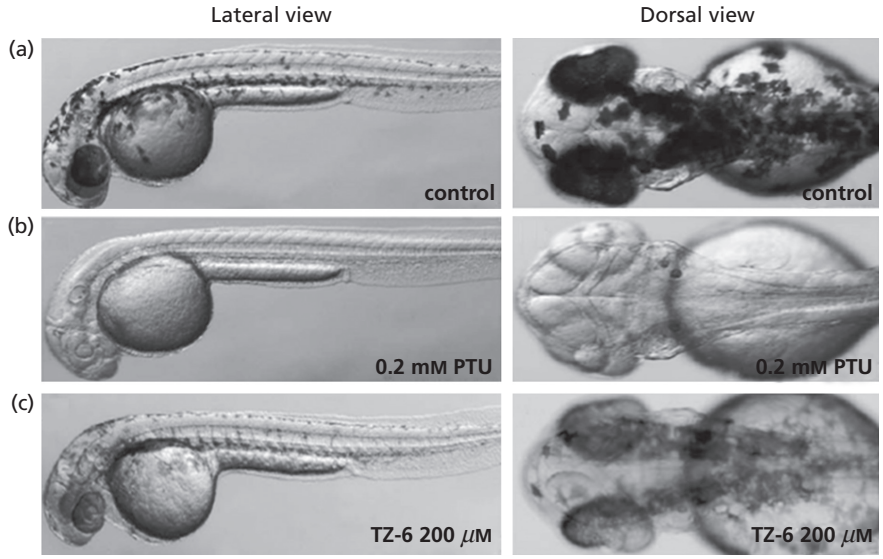

Figure 3 Effects of compound TZ-6 and phenylthiourea on the pigmentation of zebrafish. Synchronized embryos were treated with TZ-6 (4-hydroxy-2,6-dimethyl-5,6-dihydro-4*H*-1,3-thiazine) at the indicated concentrations. Test compounds were dissolved in 0.1% dimethylsulfoxide and added to the embryo medium. The effects on the pigmentation of zebrafish were observed under the stereomicroscope. (a) Control; (b) phenylthiourea (PTU) 200 μM; (c) TZ-6 200 μM. All experiments were repeated three times.

TZ-4, TZ-5 and TZ-6 significantly inhibited the oxidation of L-DOPA by mushroom tyrosinase. Of these, compound TZ-5 had the highest inhibitory effect on mushroom tyrosinase (Table 1), effects similar to those of kojic acid.

Based on these results, we tested the inhibitory effect of 1,3-thiazine derivatives on melanin production in melan-a cells. Of the 1,3-thiazine derivatives tested, compound TZ-6 exhibited the strongest effect on melanin biosynthesis in melan-a cells, whereas compounds TZ-4 and TZ-5 were more cytotoxic and exhibited lower inhibitory effects than did compound TZ-6.

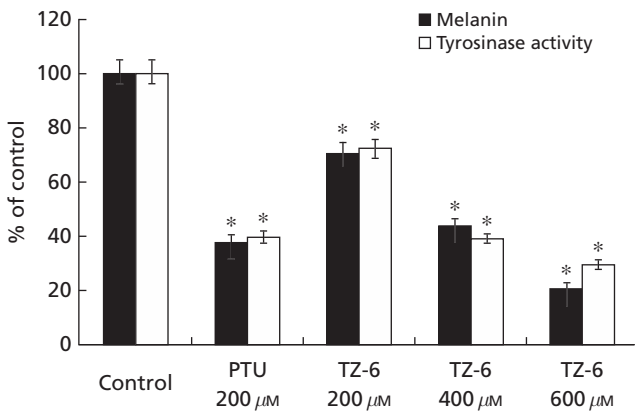

Figure 4 Effects of TZ-6 on melanin and tyrosinase in zebrafish. Approximately 100 embryos were collected and dissolved in lysis buffer. After centrifugation, melanin pigment was redissolved in 1 M NaOH. Total melanin content was quantified using a spectrometer. For measurement of tyrosinase activity, 250 μg total protein was incubated with 3,4-dihydroxyphenylalanine (final 0.5 mM), then quantified using a spectrometer. The data are represented as mean ± SD of three independent experiments. TZ-6, 4-hydroxy-2,6-dimethyl-5,6-dihydro-4*H*-1,3-thiazine; PTU, phenylthiourea. $^{*}P < 0.05$ compared with control.

We investigated the effect of 1,3-thiazine derivatives on tyrosinase enzyme extracted from melan-a cells. Compounds TZ-1, TZ-2, TZ-5 and TZ-6 had inhibitory effects on tyrosinase in a cell-free system. Interestingly, the inhibitory patterns of the 1,3-thiazine derivatives differed between mushroom tyrosinase and melan-a cell-originated tyrosinase. Specifically, compound TZ-4 had no inhibitory effect on cell-originated tyrosinase. This phenomenon may have been at least partially due to differences between species. Mushroom tyrosinase is popular among researchers because it is commercially available and inexpensive, and there are simple tools with which to investigate its characteristics. However, our evidence indicated that the inhibition of mushroom tyrosinase activity did not correlate with the inhibition of cellular tyrosinase in melan-a cells. To evaluate effective depigmenting agents, it was necessary to assess the inhibition of mammalian tyrosinase, rather than mushroom tyrosinase. Based on these results, our subsequent experiments focused on the compound TZ-6.

To study the mechanism of action of compound TZ-6 on melanogenesis, we evaluated the inhibitory effect of compound TZ-6 on intracellular tyrosinase activity and tyrosinase expression. Compound TZ-6 inhibited the intracellular activity and expression of tyrosinase in melan-a cells. Therefore, the depigmenting effect of compound TZ-6 was related to the suppression of tyrosinase expression and the catalytic inhibition of tyrosinase.

The zebrafish is a very useful vertebrate model organism, given its similar organ systems and gene sequences to those of humans.[22] The zebrafish has been used previously in research on whitening agents and to investigate the mechanism of inhibition of melanin production.[17] The zebrafish has melanocytes on the longitudinal dark stripes of the epidermis.

The striped pigment pattern of zebrafish comprises two types of pigment cells: melanophores (black chromatophores) and xanthophores (yellow chromatophores).[23] Skin melanin can be observed approximately 24 h post-fertilization. Zebrafish can be used as a screening model for melanogenic regulatory compounds.[17] We demonstrated that compound TZ-6 exhibited inhibitory effects on body pigmentation and tyrosinase activity in zebrafish.

Conclusions

In this study, compound TZ-6 was a melanogenic inhibitor in melan-a cells and zebrafish. Compound TZ-6 and its derivatives may be useful candidates as tyrosinase inhibitors, thereby exhibiting skin whitening activity. The synthesis modification of 1,3-thiazine derivatives may be helpful for the discovery of powerful tyrosinase inhibitors. Further investigations are required to elucidate clearly the inhibitory mechanism of TZ-6 on melanin synthesis.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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