p-Coumaric Acid Potently Down-regulates Zebrafish Embryo Pigmentation: Comparison of in vivo Assay and Computational Molecular Modeling with Phenylthiourea

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p-Coumaric acid is an organic compound that is a hydroxyl derivative of cinnamic acid. Due to its multiple biological activities p-coumaric acid has been widely studied in biochemical and cellular systems and is also considered as a useful therapeutic candidate for various neuronal diseases. However, the efficacy of p-coumaric acid on zebrafish developmental regulation has not been fully explored. In this study, therefore, we first investigated the action mechanism of the p-coumaric acid on the zebrafish development in a whole-organism model. p-Coumaric acid treated group significantly inhibited the pigmentation of the developing zebrafish embryos compared with control embryos without any severe side effects. In addition, p-coumaric acid down-regulated more effectively in a lower concentration than the well-known zebrafish's melanogenic inhibitor, phenylthiourea. We also compared the molecular docking property of p-coumaric acid with phenylthiourea on the tyrosinase's kojic acid binding site, which is the key enzyme of zebrafish embryo pigmentation. Interestingly, p-coumaric acid interacted with higher numbers of the amino acid residues and exhibited a tight binding affinity to the enzyme than phenylthiourea. Taken all together, these results strongly suggest that p-coumaric acid inhibits the activity of tyrosinase, consequently down-regulating zebrafish embryo pigmentation, and might play an important role in the reduction of dermal pigmentation. Thus, p-coumaric acid can be an effective and non-toxic ingredient for anti-melanogenesis functional materials.

Key Words: Melanogenesis, p-coumaric acid, Pigmentation, Tyrosinase, Zebrafish

INTRODUCTION

Since 1902 when Cuenot L. first used crosses of albino (unpigmented) mice to confirm Mendel's Laws (Cuénot, 1902), genetics has been playing an important role in enhancing our comprehension of melanocyte biology mechanism. There are almost 200 rodent genes that are crucial in melanogenesis (Halaban and Moellmann, 1992). Not only rodents, zebrafish also have emerged as an attractive model for the characterization of genetic regulation in melanin pigmentation, and 100 pigmentation mutants have been investigated in multiple genetic screens in zebrafish (Odenthal et al., 1996). The recent extensive genome data from zebrafish enabled us to define the genes and proteins which are related in pigment biologically (Logan et al., 2006).
Zebrafish had been a fascinating model also in the pharmacological research for the identification of active small molecules for regulating melanin pigmentation (Nguyen et al., 2013). Zebrafish embryo pigmentation occurs in the cytoplasmic organelles, called melanosomes, which contain tyrosinase enzymes. Tyrosinase is a multifunctional copper-containing enzyme which is mainly involved in the first two steps of melanin biosynthesis: (i) the hydroxylation of L-tyrosine and (ii) the oxidation of the product of this reaction, the L-DOPA. The X-ray crystallographic analysis with the tyrosinase, a pigmentation pivotal protein, provided us many insights into the regulation of melanogenesis (Decker et al., 2006; Matoba et al., 2006), and the three-dimensional (3D) molecular docking study of tyrosinase enhanced our understanding on the interaction mechanism of the enzyme action during the melanogenesis (Nokinsee et al., 2015).

Various tyrosinase inhibitors regulate melanin synthesis by modulating tyrosinase activity in two ways: (i) inactivation of the tyrosinase and (ii) competitive displacement of the tyrosinase substrates, L-tyrosine and L-DOPA. Thus, tyrosinase is not only responsible for skin pigmentation, but also linked to Parkinson’s disease and other neurodegenerative diseases (Senol et al., 2014). A well-known inhibitory compound of tyrosinase is kojic acid (Chang, 2009). However, kojic acid has been regarded as one of harmful agents because of their toxicity and side effects, such as skin cancer and dermatitis (Burnett et al., 2010). Thus, safe and novel tyrosinase inhibitors have been required for the depigmentation as well as for the neurodegeneration prevention.

In previous data, p-coumaric acid has exhibited significant neuroprotective effects without severe toxicity (Kim and Kim, 2000; Vauzour et al., 2010; Hong et al., 2012). But the effect of p-coumaric acid on the zebrafish embryo development and molecular binding study on the tyrosinase enzyme have not been sufficiently investigated yet. In this study, the p-coumaric acid was evaluated and compared with phenylthiourea (PTU), a well-known zebrafish tyrosinase inhibitor (Parker et al., 2013), for their inhibitory effects on zebrafish embryo pigmentation and for whole organism toxicity. We also performed molecular docking studies using the Autodock 4 and Discovery Studio program with the aim of explaining the differences in molecular binding activity of the p-coumaric acid on the tyrosinase enzyme with PTU.

MATERIALS AND METHODS

Maintenance of zebrafish

Adult zebrafish were purchased from a commercial aquarium store and 10–20 fish were reared in a water circulation tank. Fish were maintained at 28.5°C temperature and in 14/10 hour light/dark cycle. Zebrafish embryos were obtained from natural mating and developed in embryonic medium, 60 μg/ml Sea Salt (Sigma-Aldrich) in distilled water.

Chemical treatment of zebrafish embryos and imaging

Phenylthiourea (PTU, Sigma-Aldrich) was dissolved in embryonic medium and 200 μM PTU was used as positive control in all experiments. p-Coumaric acid (trans-4-Hydroxycinnamic acid, Sigma-Aldrich) was dissolved in DMSO (5,5-dimethyl-1-pyrrole-N-oxide). For anti-melanogenic effect test, 10 hpf (hour post fertilization) zebrafish embryos were arrayed in a 24-well plate (eight individuals per well) containing 1 ml embryonic medium. Then DMSO, PTU or p-coumaric acid was added to each well containing zebrafish embryos in a 24-well plate (eight individuals per well) containing 1 ml embryonic medium. Then DMSO, PTU or p-coumaric acid was added to each well containing zebrafish embryos in 28.5°C incubator. For early developmental toxicity test, 4 hpf zebrafish embryos were arrayed in the 24-well plate and examined their normal development at 24 hpf. For the heartbeats measurement, the number of atrium and ventricle contraction was counted for one minute in each embryo at 48 hpf (Milan et al., 2003). For imaging, embryos were dechorionated by forceps, washed with embryonic medium, anesthetized with tricaine (MS-220, Sigma-Aldrich) and mounted on 3% methyl cellulose (Kanto Chemical). Mounted embryos were imaged with a Leica MZ APO stereomicroscope and the DC300 FX system.

Quantitative measurement of melanocytes and statistical analysis

Proportion of melanocytes was determined with the ImageJ software (NIH), using equal-sized boxes for the dorsal view of whole embryos. Quantitative value was calculated as a percentage of black proportion per whole image. To assess
the significance of differences between control and experimental group, all statistical data was obtained from one-way ANOVA with Dunnett's post-test using IBM SPSS Statistics Data Editor software (Version 19). The significance level was set at $*P \leq 0.05$ versus DMSO control group and the data were represented as the means ± SEM (standard error of mean).

**Molecular modeling**

Models of the $p$-coumaric acid and PTU in complex with tyrosinase chain A were generated through molecular docking analysis. In order to predict and compare the binding positions of $p$-coumaric acid and PTU to the kojic acid binding site on the tyrosinase, we employed a docking program called PyRx which is a software using Autodock 4 (http://pyrx.sourceforge.net, Scripps Institute) (Trott and Olson, 2010). We downloaded the three dimensional (3D) crystal structure of tyrosinase (PDB ID = 3NQ1) from PDB (http://www.rcsb.org/pdb/) and chose the tyrosinase chain A. We also obtained the 3D structure of all single compounds from the PubChem Project (http://pubchem.ncbi.nlm.nih.gov/, pubchem CID: kojic acid = 3840, $p$-coumaric acid = 637542, PTU = 676454). Before docking, all water molecules and the cofactors were removed. The 3D structures were optimized by energy minimization using Discovery Studio (DS, Accelrys Software Inc., USA) (Lei et al., 2015). The X, Y, Z grid of the kojic acid binding site on the tyrosinase chain A was identified and calculated with the 'centroid program' in the DS 4.0 (X=-9.158, Y=-19.725, Z=6.981) (Rohmah et al., 2015). The PyRx program was used to dock $p$-coumaric acid and PTU into the X/Y/Z grid of the tyrosinase with the flexible docking option turned on. To examine the docking conformational space comprehensively, the search efficiency was set at 100%. The highest binding affinity (the lowest docking energy) score was chosen to explore the binding mode of docked compound in the tyrosinase protein using the PyRx program. For the analysis of the docking calculations, 8 conformers were considered for each ligand-macromolecule complex and the resulting docking clusters were calculated with the 2.0 Å root mean squared deviation (RMSD) tolerance on the heavy atoms (Sambasivarao et al., 2014). The two-dimensional (2D) and 3D molecular interaction models of the docked compound on the tyrosinase protein and receptor surface shape modeling (involving hydrogen bonding) were displayed using the DS v. 3.5 (Usha et al., 2014) and the ramachandran plot of the tyrosinase chain A was plotted by DS v.4.0 (Fig. 2c). The residue-property plot of the tyrosinase chain A was from the PDB ID 3NQ1's structure validation report (www.rcsb.org) (Fig. 2b). The significance level of the $p$-coumaric acid binding affinity on the tyrosinase was set at $*P \leq 0.05$ versus kojic acid and $**P \leq 0.05$ versus PTU. All data were represented as the means ± SEM (standard error of mean).

**RESULTS**

$p$-coumaric acid inhibits melanogenesis in zebrafish embryos

Zebrafish in vivo assay was conducted according to the previous method (May et al., 2015). To demonstrate the effect of $p$-coumaric acid on melanogenesis in animal model, we tested zebrafish embryos. Zebrafish embryos at 10 hpf were treated with various concentrations of $p$-coumaric acid and observed at 48 hpf (Fig. 1e). As a positive control, we used 200 μM PTU, which is a well-known tyrosinase inhibitor. As compared to DMSO treatment, PTU showed a clear inhibition in melanogenesis in developing zebrafish embryos (Fig. 1a and 1b). Interestingly, $p$-coumaric acid also inhibited melanogenesis of zebrafish embryos in a dose-dependent manner (Fig. 1c and 1d). For quantitative measurement of melanogenesis, we took picture of dorsal view of whole zebrafish embryos and calculated the percentage of black proportion (melanocytes) in each embryo by using the image analysis software, ImageJ (Fig. 1f). These results indicate that $p$-coumaric acid can be used as an inhibitory agent for melanogenesis in zebrafish embryos.

**Molecular docking analysis**

The PyRx Autodock 4 docking analysis was applied to investigate the molecular binding interactions of $p$-coumaric acid, PTU and kojic acid molecules, respectively with tyrosinase chain A (Fig. 2a) and to elucidate the possible molecular mechanism. Prior to the molecular docking, we plotted the ramachandran plot of the tyrosinase chain A structure in
Fig. 1. Anti-melanogenic effect of p-coumaric acid in developing zebrafish embryos. (a) 0.1% DMSO treated-embryo as a control. (b) Phenylthiourea (PTU) treatment at 200 μM concentration as a positive control. (c) p-coumaric acid (p-CA) treatment at 50 μM and (d) 100 μM concentration, respectively. (a-d) Lateral view of embryo at 48 hpf. Scale bar: 0.2 mm. (e) A schematic representation for the time of chemical treatment during zebrafish embryo development. (f) Quantitative measurement of melanocyte proportion in zebrafish embryo using ImageJ program. n=8. Error bars indicates Standard Error of the Mean (SEM). *P ≤ 0.05 versus DMSO control group using one-way ANOVA with Dunnett's post-test. p-Coumaric acid did not affect the normal development and heart beats of zebrafish embryo. (g-h) Control embryos treated with DMSO or PTU at 0.1% and 200 μM concentration, respectively. (i-j) p-coumaric acid treated embryos at 50 μM or 100 μM, respectively. All images are lateral view of embryos at 24 hpf. Scale bar, 0.2 mm (k) Heartbeat measurement of zebrafish embryos at 48 hpf. The number of heartbeat per minute was counted. *P ≤ 0.05 versus DMSO control group. n=8. Normal heartbeats of untreated zebrafish embryos was similar to DMSO control group. (l) Body length measurement of treated embryos at 48 hpf. n=8.
Fig. 2. (a) 3D structure of the tyrosinase enzyme chain A (b) Sequence view of the tyrosinase chain A annotated by issues in geometry and electron density. (c) Ramachandran plot of the tyrosinase chain A. (d) Docked conformation of ligand structures in the binding site of tyrosinase. Kojic acid (yellow), \( p \)-coumaric acid (green), and PTU (purple). 2D molecular interaction of the docked conformation of kojic acid (e), \( p \)-coumaric acid (f) and PTU (g) within the binding pocket of tyrosinase enzyme (PDB: 3NQ1). The binding affinity (docking energy) comparison bar graph (h) of the ligands on the tyrosinase active site. *\( P \leq 0.05 \) versus kojic acid and **\( P \leq 0.05 \) versus PTU. All data were represented as the means ± SEM (standard error of mean).
As we expected, seven GLY (glycine) residues in tyrosinase chain A peptide were found outside the area of the purple line, which means that area is sterically disallowed for all amino acids except GLY (lacks a side chain). Fig. 2b shows the sequence view annotated by issues in geometry and electron density. Residues are color coded according to the number of geometric quality criteria for which they contain at least one outlier: red = 3, orange = 2, yellow = 1, green = 0 or more. A red dot indicates a poor fit to the electron density. Residues present in the sample, but not in the 3D model, are shown in grey. In our docking model, we found that p-coumaric acid binding site was located (X=8.148, Y=2.217, Z=8.555) far from the kojic acid binding site (X=9.158, Y=19.724, Z=6.980) (Fig. 2d). Kojic acid interacted with 3 amino acid residues (Pi-Alkyl interactions: PRO201 (distance=4.51Å), ARG209 (distance=5.38Å) / Carbon-Hydrogen bond: GLY200 (distance=3.44Å)) (Fig. 2f). Meanwhile, the PTU docking site (X=-12.036, Y=-14.514, Z=-5.679) was close to the kojic acid site (Fig. 2d) and the interacting amino acid residues were VAL218 (Pi-Sigma interaction, distance=3.64Å); ALA221 (Pi-Alkyl interaction, distance=5.05Å); GLY216 (conventional H-bonding, distance=2.35Å) HIS208 (Pi-Pi stacked interaction, distance=3.62Å). This indicates that p-coumaric acid and PTU interact to the totally different binding site of the tyrosinase, respectively. The molecular binding affinity (docking energy) scores on the tyrosinase also projected a similar trend with -6.00 and -5.53 kcal/mol for p-coumaric acid and PTU, respectively which infers p-coumaric acid binds tightly than PTU (Fig. 2h).

DISCUSSION

In a recent study, we could find that the p-coumaric acid affects the development of the zebrafish embryo, especially down-regulates the melanogenesis via tight docking into the tyrosinase enzyme. Tyrosinase, a copper-containing membrane-bound glycoprotein, is the rate-limiting enzyme that catalyzes the biosynthetic pathway of melanin (Orhan and Khan, 2014). For the past several decades, numerous tyrosinase enzyme blockers have been investigated for their use in cosmetics and pharmaceutical products to prevent the over production of melanin (Chang, 2009).

The kojic acid, a well-known tyrosinase ligand, that contains dermal cream was applied to the skin, it was slowly absorbed into the circulation from human skin (Kahn, 1995). Previous data supported that kojic acid at a concentration under 2% is safe in cosmetics. However, high concentrations (over 2%) or chronic application of kojic acid could induce adverse effects (Burnett et al., 2010). Since the kojic acid showed adverse side effects, poor skin penetration, low formulation stability and non-effective in vivo, their use is still limited. The government also strictly regulated the use of kojic acid in skin treatment due to its carcinogenicity (Shimizu et al., 2003). Thus, it is in great need of finding novel tyrosinase inhibitors from different sources. To be an ideal depigmenting agent, the p-coumaric acid must be highly safe and with no side effect. In order to evaluate the safety of the p-coumaric acid, the toxicity test on the zebrafish embryo was carried out. We performed several toxicity tests in zebrafish embryos. Firstly, we treated 4 hpf embryos, which are before gastrulation, with 50 μM p-coumaric acid at various concentrations to test early developmental toxicity. 20 hours after treatment, at 24 hpf, we could not detect any significant difference in their normal development between control DMSO and p-coumaric acid-treated embryos (Fig. 1g-1j). Secondly, we assessed the effects of p-coumaric acid on the normal heartbeats in zebrafish embryos. Up to 100 μM concentration, p-coumaric acid did not affect the normal heartbeats (approximately 200 per minute) in treated-embryos (Fig. 1k). Thirdly, we measured the growth of body length in developing embryos, which was normally measured to 3.5 mm at 48 hpf. Although the body length of the embryos treated with 100 μM p-coumaric acid is slightly shorter than the others, the difference is not significant (Fig. 1l). As shown in Fig. 1, p-coumaric acid treated zebrafish did not indicate any phenotypes of toxicity, and the overall phenotypical conditions were similar to that of the control ones. In cosmetic market, the demands for developing safe...
and novel skin whitening agents have been increasing. Abdel-Wahab M., et al. reported that p-coumaric acid protected doxorubicin induced oxidative stress (Abdel-Wahab et al., 2003). Pei et al. also suggested that p-coumaric acid showed low toxicity (LD$_{50}$=2850 mg/kg) from their animal test with mice (Pei et al., 2015). Recently Notified Guidelines by OECD (OECD Guidelines) recommended that the highest oral dose of test materials is 2,000 mg/kg. Thus, we can conclude that the p-coumaric acid is safe for pre-clinical animal study. Our data will be served as a proper reference of safety guideline for the cosmeceutical application and the human clinical study.

Previous studies proposed that the 3D crystal structure of tyrosinase has a large vacant space above the active center of the tyrosinase. These studies also suggested that residues ARG 209 and VAL 218, situated in a second shell of the residues surrounding the active site, play a role in substrate binding orientation based on their flexibility and position (Matoba et al., 2006; Sendovski et al., 2011). The PTU is commonly used to block embryo pigmentation and aid visualization of zebrafish development (Bohsack et al., 2011). The PTU inhibited thyroid signaling and follicle development in zebrafish and also blocked the synthesis of melanin from tyrosine as well as the intermediates required for catecholamine synthesis (Elsalini and Rohr, 2003). At the concentration of 200 μM, the PTU inhibits melanogenesis and reduces the eye size (Millott and Lynn, 1966; Whittaker, 1966; Karlsson et al., 2001; Li et al., 2012). Recently, Hall A., et al. found that the PTU induced the degradation of the tyrosinase following Golgi maturation (Hall and Orlow, 2005). The interaction of the PTU with the plant catechol oxidase containing a dicopper center and the 3D structural molecular interaction mechanisms were also reported (Klabunde et al., 1998). In another study on the PTU molecular docking analysis using SurfFlex-Dock in Sybyl v 8.1, thiourea unit of the PTU took the position in the narrow cavity near copper metal and had hydrogen bonds with GLU195 and ASN 205 of tyrosinase (Thanigaimalai et al., 2011). In our study using the PyRx Autodock 4 and the DS 4.0 revealed that the PTU located close to the kojic acid binding site (Fig 2d and Fig. 2g). On the other hand, the p-coumaric acid was bounded far from the kojic acid binding site (Fig. 2d) and the binding affinity of the p-coumaric acid was also significantly higher than that of the PTU to the tyrosinase (Fig. 2h). As shown in Fig. 1, the p-coumaric acid strongly reduced the concentration of the zebrafish embryo pigmentation in half compared to that of the PTU without any side effects. Therefore, the comparison study of the 2D and 3D molecular binding affinity of the p-coumaric acid on the tyrosinase with the PTU suggests the strong correlation with the in vivo zebrafish test results. Based on our docking model, the p-coumaric acid appeared as a strong binder due to its higher numbers of interacting character with 5 amino acid residues of the tyrosinase, and the short bond distance might be a critical factor for regulating tyrosinase activity.

Computer aided drug design (CADD) have several limitations and there is a long way to go. However, advanced approaches of CADD are essential in order to reduce the discovery costs, to speed up the process and to increase the success rate, and there have been increasing successful CADD cases. The CADD methods that are able to predict the binding affinity of small molecules to specific protein targets are of special interest because they can accelerate the discovery of new hit compounds. With the constant improvements in both computer power and algorithm design, the future of the CADD is promising. Our data strongly suggest that the CADD process using the PyRx and the DS tools is highly reliable and can be a good example for identifying the action mechanism between the tyrosinase and its interacting ligand. In conclusion, the discovery of the novel inhibitors for the tyrosinase are useful for reducing pigmentation disorders and other melanin-related health problems. Thus, the p-coumaric acid can be an alternative active compound to the PTU in zebrafish developmental research and be used as a safe pharmacological agent which is of strong potency in skin-whitening. In addition, our results strongly provide a deeper insight into the structural attributes and overall molecular interactions of the tyrosinase and its binding molecules.

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Conflict of interest
The authors have no conflict of interest.

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