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Potent anti-melanogenic activity and favorable toxicity profile of selected 4-phenyl hydroxycoumarins in the zebrafish model and the computational molecular modeling studies

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ABSTRACT

7-Hydroxy-4-phenylcoumarin (7C) and 5,7-dihydroxy-4-phenylcoumarin (5,7C) have been evaluated as potential anti-melanogenic agents in the zebrafish (*Danio rerio*) model in comparison to commercially utilized depigmenting agents hydroquinone and kojic acid. 7C and 5,7C decreased the body pigmentation at 5 μg/mL, while did not affect the embryos development and survival at doses ≤50 μg/mL and ≤25 μg/mL. Unlike hydroquinone and kojic acid, 4-phenyl hydroxycoumarins were no melanocytotoxic, showed no cardiotoxic side effects, neither caused neutropenia in zebrafish embryos, suggesting these compounds may present novel skin-whitening agents with improved pharmacological properties. Inhibition of tyrosinase was identified as the possible mode of anti-melanogenic action. Molecular docking studies using the homology model of human tyrosinase as well as adenylate cyclase revealed excellent correlation with experimentally obtained results.

1. Introduction

Melanogenesis is physiological process in which melanocytes produce melanin, a biopolymer which contributes to skin pigmentation and hair color in mammals. There are several beneficiary functions of melanin in cell, especially the protection of skin against dangerous ultraviolet radiation and cancer development prevention.[1] However, an abnormal accumulation of melanin leads to numerous disorders such as freckles, chloasma, melasma, senile lentigines, ephelides and melanoderma. Further, melanin can induce inflammation such as allergic contact dermatitis, eczema and irritant contact dermatitis.[2–4] Dermatological diseases have visible nature and for that reason affected patients have considerable psychological consequences, especially because skin diseases have a strong impact on the physical appearance and emotional state of the patient and could result in a reduced quality of life.[5–7] For stated reasons treatment of these disorders, where the reduction of melanin production is an imperative, is an attractive target for the development on new depigmenting drugs with further application in pharmacological and cosmetic fields.

Melanin synthesis is driven by the tyrosinase, a copper-containing enzyme, which besides clear participation in the genesis of dermatological diseases is strongly associated with the appearance of certain neurodegenerative diseases and cancer types.[8,9] Tyrosinase has been recognized as a key target for the screening and discovery of novel bioactive agents for the therapy of dermatological pigmentary lesions.[10–12] Since a critical role in melanin production is determined by tyrosinase, its inhibition become increasingly important in the medicinal chemistry and in the cosmetic industry when related to hyperpigmentation disorders.[6]

The known tyrosinase inhibitors so far, such as hydroquinone, kojic acid, arbutin, and phenylthiourea have numerous adverse side effects such as poor skin penetration, low stability, permanent melanocyte loss, mutagenic and even cancerogenic potential,[13] hampering their long-term application. For these reasons, there is still a great demand in the skin pharmacotherapy for the development of novel non-toxic, effective and stabile tyrosinase inhibitory agents which could be applied in the treatments of dermatological disorders. Encouraged by such situation, the medicinal chemists have synthesized vast libraries of bioactive compounds over last decades, showing an excellent inhibitory activity against the mushroom tyrosinase, that is the most frequently used *in vitro* model for screening of the hypopigmenting agents in the

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development of skin-whitening substances. However, due to crucial difference between the mushroom and the human tyrosinase, such as the similarity of only 23% in amino-acid composition, different cell localization, and particularly affinity for L-DOPA, many of synthetic inhibitors with inhibitory activity against the mushroom tyrosinase remained weakly effective or even ineffective as inhibitors of the human tyrosinase.[13] In search for novel targets downregulating melanogenesis, the adenylate cyclase, an enzyme involved in the major signaling pathway leading to melanin synthesis, emerged as an interesting and promising target, which downregulation may result in the tyrosinase activity inhibition.[14] Given that *in vitro* testing on melanocytes and melanoma cell lines does not reflect real and complex metabolic conditions in living organisms, *in vivo* animal testing is of pivotal importance for discovery of novel, effective and safe tyrosinase inhibitors.

Owing to high correlation with humans in response to pharmaceuticals, the zebrafish (*Danio rerio*) model has been established as a versatile platform for drug discovery and toxicity assessment, simplifying the path to clinical trials in humans and reducing the failure of potential therapeutics at later stages of testing.[15–17] As the molecular determinants involved in the human melanogenesis are found in the zebrafish also, this model has successfully been used to study melanogenesis in human, and for the discovery of novel depigmenting agents, offering an exceptional opportunity for the evaluation of an antimelanogenic efficacy accompanied by toxicity.

Coumarins, an elite class of naturally occurring and synthetic compounds, have attracted a huge pharmacological interest over a last decade, due to their inherent therapeutic relevance. Owing to high diversity in their structural complexity, coumarins have exhibited a wide spectrum of biological activities including antioxidative, antimicrobial, anti-inflammatory, antidiabetes, anticoagulant, anticancer and enzymatic inhibition properties.[18,19] Recent findings have revealed that some coumarins (i.e. esculetin and umbelliferone) and their analogues can efficiently modulate tyrosinase activity,[20–22] making them interesting and valued therapeutic candidates for the treatment of skin disorders. Fais et al.[23] demonstrated the importance of the phenyl substituent in the coumarin's moiety for the tyrosinase inhibition activity, where the introduction of the 3-phenyl substituent to umbelliferone led to loss of inhibitory tyrosinase activity, while additional introduction of the hydroxyl to C5 position of the coumarin scaffold markedly increased its activity.[24] The effect of the introduction of the phenyl group in C4 position of umbelliferone has not previously been reported for the effect on tyrosinase activity.

In our previous study we found that two of 4-phenyl hydroxycoumarins, namely 7-hydroxy-4-phenylcoumarin (7C) and 5,7-dihydroxy-4-phenylcoumarin (5,7C), exerted cytotoxic activity against human A375 melanoma cells, and reduced the body pigmentation in the zebrafish embryo model.[25] Prompted by these findings and of their favorable pharmacokinetic properties according to Lipinski's the "Rule of five", we explored the anti-melanogenic efficacy of 7C and 5,7C *in vivo* using the zebrafish model, and addressed their cytotoxic activity towards zebrafish melanocytes and neutrophils, as important parameters for further consideration for medical application. In addition, the molecular docking study has been performed to explore possible structure binding interaction between selected 4-phenyl-hydroxycoumarins and the homology model of human tyrosinase and adenylate cyclase.

2. Materials and methods

2.1. Chemicals

7-Hydroxy-4-phenylcoumarin (7C), 5,7-dihydroxy-4-phenylcoumarin (5,7C), kojic acid, hydroquinone, α-melanocyte stimulating hormone (α-MSH) and dimethyl sulfoxide (DMSO) of cell tissue grade were obtained from Sigma (Sigma–Aldrich GmbH, Sternheim, Germany). Fish media components and other chemicals were purchased either from Sigma–Aldrich or Fisher Scientific.

2.2. In vivo zebrafish assays

All experiments involving zebrafish were performed in compliance with the European directive 2010/63/EU and the ethical guidelines of Guide for Care and Use of Laboratory Animals of Institute of Molecular Genetics and Genetic Engineering, University of Belgrade.

Adult wild type zebrafish (*Danio rerio*, wt) were obtained from a commercial supplier (Pet Center, Belgrade, Serbia), housed in a temperature- and light-controlled facility with 28 °C and standard 14:10-h light-dark photoperiod, and regularly fed with commercially dry flake food (TetraMin™ flakes; Tetra Melle, Germany) twice a day and *Artemia nauplii* once daily. Zebrafish embryos were produced by the pair-wise mating, collected and distributed into 24-well plates containing 10 embryos per well and 1 mL of the fish embryos water (2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM $NaHCO_3$, 0.07 mM KCl), and raised at 28 °C.

For evaluation of depigmenting activity of tested 4-phenyl hydroxycoumarins accompanied with developmental toxicity, wt embryos staged at 24 h post fertilization (hpf) were treated with five different concentrations (1, 5, 10, 25 and 50 μg/mL) of 7C and 5,7C, allowing sufficient time for melanocytes to develop, prior to treatment. DMSO (0.25%, v/v) and kojic acid (25 μg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 2.5 mg/mL) were used as controls. Experiments were repeated three times, using 120 embryos per concentration. After 48-h exposure (at the 72 hpf stage), a total of 100 zebrafish embryos was used for the determination of pigmentation, while remaining embryos have been allowed to develop up 96 hpf, for the developmental toxicity assessment. At 96 hpf, the embryos were anesthetized by addition of 0.1% (w/v) tricaine solution (Sigma-Aldrich, St. Louis, MO), photographed and killed by freezing at −20 °C for ≥24 h. Apical endopoints (Supplemental Table S1) used for the toxicity evaluation were recorded at 48, 72 and 96 hpf using an inverted microscope (CKX41; Olympus, Tokyo, Japan).

To determine whether achieved depigmenting effect by 7C and 5,7C could be due to their cytotoxic activity on the zebrafish melanocytes, wt embryos were exposed to tested compounds at the 6 hpf stage (when melanocytes are still not derived from their progenitors) and at the 24 hpf stage (melanocytes were differentiated and started to produce the pigment melanin). The melanocytes pattern (number, distribution, size, morphology and pigmentation) over the yolk sack was inspected, and compared between the 6 hpf- and the 24 hpf-treated groups, and with the untreated group. Reduced number of melanocytes, their changed morphology (loss of dendritic shape and an appearance of irregular, rounded or punctate cells) were used as indicator of the melanocytotoxic effect upon applied treatments, while a decrease of the cell pigmentation without effect on their number and morphology indicated the inhibitory effect on melanin synthesis.[26,27] DMSO (0.25%, v/v) was used as a negative control, while hydroquinone (5, 10, 12.5 and 25 μg/mL), a depigmenting agent with approved melanocytotoxic activity, was used as a control.

To evaluate the toxicity of 7C and 5,7C on the neutrophils development, transgenic zebrafish embryos *Tg*(*mpx:GFP*) expressing enhanced green fluorescent protein (EGFP) in neutrophils were used. Embryos of *Tg*(*mpx:GFP*) zebrafish were kindly provided by Dr. Ana Cvejic (Wellcome Trust Sanger Institute, Cambridge, UK) and raised in our zebrafish facility to adult stage under previously described life conditions. Transgenic embryos used in this assay were generated by natural spawning of *Tg*(*mpx:GFP*) adults and