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Coumarin derivatives, but not coumarin itself, cause skin irritation via topical delivery

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HIGHLIGHTS

- Skin toxicity caused by coumarins was derived from analogs but not from coumarin.
- The lipophilicity and structure are vital in controlling absorption of coumarins.
- Hair follicles seemed to be an important pathway for the passage of coumarins.
- A significant proliferation was detected by skin application of coumarins.

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ABSTRACT

Coumarin and its derivatives are widely employed as a fragrance in cosmetics and skin care products. The skin absorption level and possible disruption to the skin by topical application of coumarins were evaluated in this study. Percutaneous absorption of osthole, daphnoretin, coumarin, byakangelicin, and 7-hydroxycoumarin was assessed in vitro and in vivo. Skin physiology measurements and immunoblotting were utilized as methodologies for validating toxicity. The relationship between structures and permeation/toxicity of coumarins was elucidated. Both equimolar concentration and saturated solubility in 30% ethanol were used as the applied dose. Osthole with the most lipophilic characteristic demonstrated the greatest skin accumulation, followed by coumarin and 7-hydroxycoumarin. Coumarin was the permeant with the highest flux across the skin. The trend of in vivo deposition was consistent with that of the in vitro profiles. Skin uptake of osthole was 8-fold higher than that of coumarin. Hair follicles played a significant role as a pathway for transport of coumarin according to the examination of follicular accumulation. Osthole and 7-hydroxycoumarin slightly, but significantly, enhanced transepidermal water loss after a consecutive 5-day administration. The immunoblotting profiling verified the role of proliferation in skin damage induced by osthole, byakangelicin, and 7-hydroxycoumarin. The proliferation-related proteins examined in this work included glucose-regulated proteins, cytokeratin, and C-myc. Daphnoretin and coumarin showed a negligible alteration on protein biomarkers. The experimental results suggested that skin irritation caused by coumarins was mainly derived from the analogs but not from coumarin itself.
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1. Introduction

Coumarins are a class of naturally occurring benzo-α-pyrone found in a wide variety of microorganisms, plants, and animals. Among coumarins, coumarin (2H-1-benzopyran-2-one) is the parent compound mainly utilized as a fragrance ingredient

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in cosmetic products at concentrations of <0.5–6.4% (Felter et al., 2006). Coumarin was formerly used as a food additive, but it was withdrawn by the US FDA in 1957 due to data showing it to be toxic in animals including hepatotoxicity, renal dysfunction, and carcinogenesis in dogs, hamsters, and rats (Beckley-Kartey et al., 1997). Now it is extensively used in skin-care-related consumer products, including toilet soaps, bath oils, detergents, lotions, and hair preparations (Nelson and Yiannias, 2009). Such products may be used daily over a lifetime. The European Food Safety Authority (EFSA) demonstrated that exposure to coumarin from skin care products was twice as high as exposure from food (Mielke et al., 2011). The Federal Institute for Risk Assessment (BfR) of Germany also warned that cosmetics containing coumarins might cause skin allergies in sensitive individuals. The possibility of skin irritation/toxicity caused by coumarin and its derivatives remains controversial (Wisneski, 2001; Vocanson et al., 2007).

Since consumers frequently come into contact with cosmetic products containing coumarins, it is important to understand dermal/transdermal permeation of these compounds. Coumarins contain a large variety of chemicals. Percutaneous absorption of permeants greatly depends upon their physicochemical characteristics. Vocanson et al. (2006) suggest that pure coumarin did not reveal an irritant property to skin. In contrast, the other derivatives of coumarin are sensitizers to induce skin irritation. The absorption level of coumarin and its derivatives via the topical route has not been systemically examined. We aimed to establish permeation profiles of a series of coumarins in this study. Possible dermal irritation after exposure to coumarins was also evaluated by detecting functional proteins in the skin.

Some coumarin-related derivatives, including osthole, daphnoretin, coumarin, byakangelicin, and 7-hydroxycoumarin (Fig. 1), was selected to examine the influence of different characteristic groups on skin permeability. These five compounds were chosen due to their specific structure characters, which was beneficial to discuss the impact of specific moieties on skin permeation and toxicity. These include monosubstituted coumarins, disubstituted coumarins, and bis-coumarins (Borges et al., 2005). These compounds were abundant in plants such as *Ruta graveolens* and *Citrus sinensis* (Petit-Paly et al., 1989; Ziegler and Spiteller, 1992), which are often employed as the fragrance materials for commercial products. Dermal absorption within the skin and transdermal penetration across the skin was determined by the Franz cell skin permeation assay. Skin absorption of coumarins was also assessed in vivo by using the nude mouse as an animal model. Skin irritation caused by coumarins was explored using cultured keratinocytes and skin fibroblasts. We examined skin proteins with in vivo treatment of coumarins using the Western blotting assay. These proteins are biomarkers related to inflammation, proliferation, and differentiation.

2. Materials and methods

2.1. Materials

Osthole was purchased from Chemos GmbH (Regenstauf, Germany). Coumarin and 7-hydroxycoumarin were supplied by Sigma–Aldrich (St. Louis, MO, USA). Daphnoretin and byakangelicin were gifts from Natural Products Laboratory, Chang Gung University, which were purified from *Daphne genkwa* and *Angelica hirsutiflora*, separately. Their structures were elucidated by spectroscopic analysis and compared with literature data.

2.2. Solubility in ethanol/water

The saturated solubility of coumarins was measured in 30% ethanol/pH 7.4 citrate–phosphate buffer. The excess amount of each compound (12.3 mM for osthole, daphnoretin, and byakangelicin; 80 mM for coumarin; 20 mM for 7-hydroxycoumarin) was added to aqueous solution. The dispersion was shaken reciprocally at 37 °C for 24 h. The suspension was subsequently centrifuged at 10,000 rpm for 10 min and filtered through a polyvinylidene fluoride (PVDF) membrane (pose size = 0.45 μm). The compound concentration in filtrate was quantified by high performance liquid chromatography (HPLC).

2.3. HPLC setup

HPLC system was Hitachi 7-series (Tokyo, Japan). A C18 column was employed as stationary phase (LiChrosper®, Merck, Darmstadt, Germany). The mobile phase consisted of acetonitrile and pH 3 water adjusted by phosphoric acid. The ratio of acetonitrile and aqueous phase was 35:65, 45:55, 60:40, 60:40, and 70:30 for osthole, daphnoretin, coumarin, byakangelicin, and 7-hydroxycoumarin, respectively. Flow rate and wavelength was set to 1 ml/min and 323 nm. At the range 0–250 μg/ml, the concentration of coumarins was linearly proportional to their absorbance according to calibration curves. The limit of detection (LOD) of osthole, daphnoretin, coumarin, byakangelicin, and 7-hydroxycoumarin was determined to be 5, 10, 5, 20, and 10 ng/ml, respectively. The intra- and inter-assay precision and accuracy values were evaluated at the concentration range 0–250 μg/ml. The overall precision, defined by relative standard deviation (RSD), ranged from 0.5% to 3.6% on average. Analytical accuracy, expressed as the percentage difference between the mean of measured value and the known concentration, varied from −4.6% to 3.8%.

2.4. Animals

Female nude mice (eight weeks, BALB/cAnN.Cg-Foxnl^{nu}/Cr1Nar1) were supplied by National Laboratory Animal Center (Taipei, Taiwan). Specific pathogen-free (SPF) pigs (one week) were provided by Animal Technology Institute Taiwan (Miaoli, Taiwan). The animal experimental protocol was reviewed and approved by Institutional Animal Care and Use Committee of Chang Gung University. Ethical issues with animal experiments complied with Directive 86/609/EEC from European Commission. Full-thickness skin from dorsal area of nude mice and pigs was excised after sacrifice. The integrity of skin was evaluated by transepidermal water loss (TEWL) before sacrifice. The TEWL range between 5 and 15 g/m^2/h was recognized as intact skin.

2.5. In vitro percutaneous absorption

Skin penetration of coumarin and its derivatives was measured by a Franz diffusion cell. The skin was mounted between the compartments of donor and receptor immediately after sacrifice. The donor vehicle was 0.5 ml (637 μl/cm^2) pH 7.4 buffer containing 30% ethanol. Both equimolar concentration (12.3 mM) and saturated solubility were employed as the doses of the permeants. Receptor medium consisted of 30% ethanol in pH 7.4 buffer to maintain the sink condition. Effective diffusion area between compartments was 0.785 cm^2. The stirring rate and temperature were kept at 600 rpm and 37 °C, respectively. At appropriate intervals, 300 μl of receptor medium was taken and immediately supplied by an equal volume of fresh medium. The samples were assayed by HPLC. The accumulation of coumarins within skin was measured after a 24-h delivery. The skin was removed from Franz cell, then rinsed with water and blotted with tissue paper. The skin sample was weighed and minced by scissors, positioned in a glass homogenizer with 1 ml methanol, and ground for 5 min with an electric stirrer. The mixture was centrifuged at 10,000 rpm for 10 min. After filtration via PVDF membrane, the sample was detected by HPLC.

2.6. Hair follicle uptake

Differential stripping and cyanoacrylate skin surface casting were used to detect content of coumarins in follicles (Teichmann et al., 2005). Subsequent to stripping SC of skin removed from Franz cell, a follicular cast was prepared. A drop of superglue (ethyl cyanoacrylate 7004T, 3M, Taipei, Taiwan) was added on a glass slide, which was pressed onto the surface of SC-stripped skin. The cyanoacrylate polymerized, and the slide was expelled with one quick movement after 5 min. The superglue remaining on the slide was scraped off and positioned in a tube with 2 ml methanol. The tube was shaken for 3 h. The final product was vacuumed to evaporate methanol. The mobile phase was added to dissolve the residuals for HPLC assay.

2.7. In vivo percutaneous absorption

Nude mouse was employed as in vivo animal model. A glass cylinder with a hollow area of 0.785 cm^2 was attached to dorsal skin surface by superglue. An aliquot of 0.2 ml of 30% ethanol with the permeants (12.3 mM) was pipetted into cylinder. The application duration was 6 h. The animal was then sacrificed, and the treated skin area was excised. The extraction procedure of coumarins from skin was the same as in vitro percutaneous absorption.

2.8. Cell viability

Human-immortalized keratinocytes were a gift from Dr. Nan-Lin Wu (Department of Dermatology, Mackay Memorial Hospital, Taipei, Taiwan). Skin fibroblasts (Hs68) were purchased from American Type Culture Collection (Manassas, VA, USA). The condition for culturing both cell lines was described previously (Wu et al., 2006). Keratinocytes and skin fibroblasts were seeded in 96-well plates by appropriate culture medium for 24 h. Coumarins in DMSO were added to wells to make final

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