Skin permeation and comparative evaluation of gentisic acid ester derivatives as skin-lightening agents

Nan Shun Ma[1], Marion Abraham[2], Myeong Jun Choi[3], Jung Sun Kim[4]*

[1]College of Pharmacy, Pusan National University, Busan 609-735, South Korea
[2]Department of Marine Chemistry, Leibniz Institute of Baltic Sea Research, 18119 Rostock, Germany
[3]Phytos Co., Gyeonggi University, Suwon, Gyeonggi-do 443-760, South Korea
[4]Division of Health Sciences, Dongseo University, Busan 617-716, South Korea
*Correspondence: jsk@gdsu.dongseo.ac.kr

Gentisic acid (GA) derivatives are known to exhibit inhibitory activity against tyrosinase. Skin permeation of five GA derivatives have been evaluated and correlated with their tyrosinase inhibition and cytotoxicity. All compounds tested were stable at 37 °C up to 12 h. Skin permeation study in mice showed the highest permeation rate for the methyl derivative, MG (232.0033 µg/cm²/h) over GA (172.0852 µg/cm²/h), followed by the ethyl derivative, EG (182.9242 µg/cm²/h). Although all the esters tested were more cytotoxic to melanocytes than GA, MG was the least cytotoxic among the five esters. MG and EG also showed similar mushroom tyrosinase inhibition to gentisic acid (IC_{50} 176 µg/mL). MG showed the highest skin permeation rate among the GA derivatives tested, and was considered a promising candidate together with EG based on the tyrosinase inhibition and melanocyte cytotoxicity.

Key words: Gentisic acid – Methyl gentisate – Ethyl gentisate – Skin permeation – Tyrosinase inhibition – Melanocyte.

Melanin pigments are widespread in the animal kingdom. In humans, they are responsible for the color of skin, hair and iris of the eyes. They are produced by melanocytes that are located in the bottom of the epidermis. Melanin is synthesized when induced by UV radiation to prevent damages from environmental influences. However, it can be the cause of pigmentary disorders as in the case of freckles, melasma or senile lentigines which are considered serious aesthetic problems [1]. Genetic variants within the genes involved in skin pigmentation are reported to be determinants of several skin cancers [2]. For these reasons, developing drugs that can down regulate the synthesis of melanin is necessary.

Tyrosinase is the key enzyme of melanogenisis and could be an effective target enzyme for developing skin-lightening agents [3]. Tyrosinase catalyzes the oxidation of the amino acid L-tyrosine to L-3-(3,4-dihydroxyphenyl)-L-alanine (L-dopa) and subsequently to L-dopaquinone. In the absence of cysteine, L-dopaquinone is converted to cyclodopa and subsequently to eumelanin, giving rise to brown and black pigmentation. In the presence of cysteine, L-dopaquinone is converted to 5-S-cysteinyldopa and finally to pheomelanin that yields in amber and red pigments. The ratio of eumelanin to pheomelanin depends on the availability of cysteine in the melanocytes [1]. Tyrosinase is a copper metalloenzyme and it is assumed that tyrosinase inhibiting compounds chelate the copper and therefore block the active site of this enzyme [3, 4].

There are many compounds that have been identified to inhibit tyrosinase from natural products [5]. Hydroquinone, ascorbic acid, kojic acid, arbutin, gentisic acid and its esters have been reported as skin-lightening cosmetic products [6-10]. The vast search for potential tyrosinase inhibitors have accumulated into a huge number of compounds for use in food and cosmetics derived from natural and synthetic sources [11]. Among these, hydroquinones tend to exhibit very efficient tyrosinase inhibiting activities although they could be highly cytotoxic or mutagenic. However, gentisic acid, a natural product from the root of Gentiana, is known to be a safe and mild agent for treating cutaneous hyperpigmentation disorders including melasma and UV-induced ephelides [12]. The alkyl esters of gentisic acid were reported to be good inhibitors of melanogenesis, showing tyrosinase inhibiting activities and mutagenicity to some extent, among which the methyl gentisate was reported as a promising candidate as a skin-lightening agent [10].

Although skin-whitening agents are applied to and target the skin, studies on these agents are limited to *in vitro* chemical studies, not placing much emphasis on how these compounds could actually penetrate and deposit into the skin. Bian *et al*. has reported the development of a matrix-type formulation for topical delivery of GA into melanocytes where the effect of drug concentrations, enhancers and various adhesives on the permeation rate and skin deposition of GA were investigated [13]. Because melanocytes locate to the bottom layer of the epidermis, a skin-lightening effective agent has to be able to permeate through the skin in order to exert its inhibiting effect within the melanocytes. The potential as a skin-lightening topical drug does not solely rely on the compound's tyrosinase inhibiting activity, but also on its cytotoxicity and structural parameters that are predominantly lipophilicity for sufficient skin permeation and water solubility for its formulation.

In the current study alkyl side chains were added to GA with anticipation of enhancement of skin permeation efficiency. Herein we report on a skin permeation analysis of five gentisic acid esters harboring alkyl side chains of different length in comparison to the gentisic acid as parent compound. Furthermore, tyrosinase inhibition and cytotoxicity to Melan A cells have been studied and correlated to structural parameters in a structure-activity-relationship analysis.

I. MATERIALS AND METHODS

1. Materials

All chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, United States) and were reagent grade or better. Gibco RPMI media for maintenance of Murin Melan-A cells were purchased from Invitrogen (Seoul, South Korea). Silica gel plates (Silicage l60F254) used for thin layer chromatography and silica gel (Merck 60) for column chromatography were purchased from Merck Co. (Darmstadt, Germany). Melting points were determined with the Buchi M-530 capillary melting point apparatus. Infrared spectral data (IR) were obtained on a Mattson 3000 Fourier Transform spectrometer and are reported in cm^{-1}. Proton (^1H NMR) nuclear magnetic resonances were recorded on a Varian Gemini-300 Fourier Transform spectrometer. The NMR spectra (300 MHz) were recorded in the deuterated solvent indicated with chemical shifts reported in δ units downfield from tetramethylsilane (TMS). Coupling constants are reported in hertz (Hz). Ultraviolet spectra were recorded on an

Optizen 2120 UV/Vis spectrometer. HPLC analyses were performed with a Young-lin ACME HPLC equipped with a Young-lin S930D pump and Young-lin UV 730D detector.

2. Synthesis of gentisic acid esters

The gentisic acid esters were synthesized as described by Curto *et al*. [10]. The methyl, ethyl, *n*-propyl, *i*-propyl and *n*-butyl esters of gentisic acid were prepared by refluxing gentisic acid in an excess (1 g, 6.49 mmol) of the corresponding alcohols for 1-2 days using *p*-toluenesulfonic acid as a catalyst. The course of the reaction was followed by thin layer chromatography (TLC). The products were purified by evaporation of the solvent, extraction with ethyl acetate, water and small aliquots of aqueous sodium bicarbonate until no gentisic acid was visible in the organic phase by TLC. The organic layer was dried over sodium sulfate and evaporated in vacuum. The structures of the gentisic acid analogues were verified by melting point (m.p.) determination, ^{1}H NMR as well as IR spectroscopy.

3. HPLC analysis and quantification

For chromatographic separation, 20 μL of the gentisic acid esters were separated on an RP-18 column (Lichrospher 125 × 4 mm, 5 μm particle size, Merck, Germany) at ambient temperature at a flow rate of 1 mL/min. Acetonitrile and water containing 2 % (v/v) phosphoric acid have been used as mobile phase in different ratios for separation of the gentisic acid esters (gentisic acid (GA) 10:90, gentisic acid methyl ester (MG) 25:75, gentisic acid ethyl ester (EG) 30:70, gentisic acid propyl ester (PG) and gentisic acid isopropyl ester (IPG) 40:60, gentisic acid butyl ester (BG) 45:55). The mobile phase was filtered through a membrane filter (47 mm, 0.2 μm, Satorius Co., Germany) and degassed in an 8510 Branson ultrasonicator prior use. For detection the variable wavelength detector was set at 239 nm. For quantification different concentrations of each gentisic acid ester was injected and separated as described above. The peak area of each gentisic acid ester was plotted against the respective concentrations to obtain the following calibration curves: GA, $y = 9.895x + 0.621$, $r^2 = 0.999661$; MG, $y = 51.609x + 22.044$, $r^2 = 0.999688$; EG, $y = 46.741x - 30.272$, $r^2 = 0.998451$; PG, $y = 41.55x - 15.706$, $r^2 = 0.999968$; IPG, $y = 42.073x - 8.697$, $r^2 = 0.999995$; BG, $y = 76.194x - 9.574$, $r^2 = 0.999971$.

4. Determination of capacity factors

Gentisic acid esters were dissolved in methanol to a final concentration of 20 μg/mL and separated by HPLC as described above. The capacity factor k' of each gentisic acid ester was calculated as $k' = (t_R - t_0)/t_0$, with t_R as the compound's retention time and t_0 the retention time of the mobile phase. The capacity factor analysis was done in triplicates.

5. Determination of water solubility

To determine the water solubility of the gentisic acid esters, excess amounts of each compound was added to 1 mL of water. The solution was placed in a shaking water bath for 24 h at 37 °C to reach equilibrium. After filtering through a Minisart KC4 filter (0.45 μm, Satorius, Germany), the filtrate was appropriately diluted with methanol and analyzed by HPLC as described above.

6. Temperature stability analysis

Solutions containing 2.5 μg/mL of GA, MG, EG, IPG, PG and 1.25 μg/mL of BG were placed in a shaking incubator at 37 °C. At predetermined time intervals, 100 μL samples were drawn and the concentration of the gentisic acid esters were determined by HPLC as described above. The relative percentage of the remaining compound was calculated with the initial concentration considered as 100 %.

7. Preparation of hairless mouse skin

The animals used for the skin permeation studies were five week old male ICR hairless mice obtained from Orient (Kyounggi, Korea). The animals received standard laboratory chow and had free access to water before the experiment. They were sacrificed by ether right before the experiment, and full-thickness skin was surgically removed. The skin specimen were cut into appropriate sizes after carefully removing subcutaneous fat and washed with normal saline.

8. *In vitro* skin permeation analysis

In vitro mouse skin permeation studies of the gentisic acid esters were conducted using Franz diffusion cells at 37 °C. Freshly excised mouse skin was mounted between the donor and receptor cells with the stratum corneum side facing the donor cell. The area of diffusion for all experiments was 2.14 cm^2. The receptor cells were filled with 12 mL of 40 % (v/v) propylene glycol in buffered saline (PBS) to maintain sink conditions. The donor cells contained super-saturated solutions of the respective gentisic acid ester in water and were covered with parafilm. At each predetermined time interval, 1 mL of sample was taken from the receptor cells and refilled with the same volume of fresh receptor solution. Samples were stored at -21 °C until analyzed by HPLC as described above.

9. Determination of mushroom tyrosinase inhibiting activity

To analyze the inhibiting effect of the different gentisic acid esters with respect to mushroom tyrosinase, different concentrations of each compound were tested. Stock solutions containing 1 mg/mL of the respective gentisic acid ester in 10 % (v/v) DMSO were diluted to concentrations of 300, 200, 150, 100, 50 and 10 μg/mL. The test was performed in a 96-well microplate and each test consisted of 20 μL of the respective gentisic acid ester, 80 μL PBS buffer, 50 μL of a 4 mM L-tyrosine solution and 50 μL of a solution containing 100 Units/mL mushroom tyrosinase. The following samples served as control samples: a blank sample without mushroom tyrosinase, a positive control sample without any gentisic acid ester and a negative control sample omitting gentisic acid esters and mushroom tyrosinase. The omitted substances were replaced by appropriate volumes of PBS buffer. The samples were incubated for 1 h at 37 °C and absorbances were determined at 490 nm. The tyrosinase inhibiting activity was calculated as $[\% \text{ inhibition}] = [1-(S-B)/(P-N)] \times 100 \%$ (S: sample, B: blank, P:positive control, N: negative control). For each gentisic acid ester, the concentration reducing the mushroom tyrosinase activity to 50 % (IC_{50} [μg/mL]) was calculated.

10. Analysis of the cytotoxicity of gentisic acid esters on Murin Melan-A cells

Murin Melan-A cells were maintained according to Bennett *et al*. [14] and cultured on RPMI medium. Cell growth and viability were assayed using the neutral red assay that is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind the supravital dye Neutral Red.

Initially, Murin Melan-A cells were cultured until they approached confluency. Cells were trypsinized and seeded into a fresh culture dish until a cell density of 10^5 cell/mL was reached. Into each well of a 96-well microplate, 150 μL cell suspension were transferred. Cells were further allowed to grow for 24 h after which the growth medium was changed. Fifteen microlitres of the gentisic acid ester solutions was added to the cell suspensions and incubated for four days at darkness. Murin Melan-A cells were washed twice with PBS buffer and dissolved in 1 N NaOH / 10 % (v/v) DMSO. The absorbance was measured spectrophotometrically using a microplate reader at a wavelength of 490 nm. Cytotoxicity of the gentisic acid esters was expressed as IC_{50} that describes the concentration of the gentisic acid esters at which the cell viability was 50 % as compared to non-treated cells.

Download English Version:

https://daneshyari.com/en/article/2483391

Download Persian Version:

https://daneshyari.com/article/2483391

Daneshyari.com