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Synthesis, characterization and inhibitory effects of crocetin derivative compounds in cancer and inflammation

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ABSTRACT

Crocetin is a natural product possessing extraordinary therapeutic effects for various diseases. However, its extremely low solubility limits its application greatly. Conjugation of organic compounds containing heteroatoms such as N to poor soluble molecules can help the synthesized derivative to form stable hydrogen bonds by lowering the salvation energy, which will improve the solubility of the synthesized compounds. Herein, crocetin was modified by conjugating with piperidyl, diethylin and benzylamine to improve their solubility and bioactivities. In the present study, the conjugation of crocetin with piperidyl, diethylin and benzylamine and their influence on the solubility and the pharmacological effects of crocetin were investigated. With the described strategy, crocetin derivatives were synthesized and their structures were elucidated by ^{1}H NMR, ^{13}C NMR and UPLC-MS spectroscopic analysis. The solubility of crocetin and its derivatives were identified. Upon that, the pharmacological effects of the crocetin derivatives on the tumor and inflammation treatment were investigated. It was shown that, in contrast to crocetin, of which, the solubility and pharmacological effects were low and limited, the synthesized compounds have significantly higher solubility and possess broad spectrum of anticancer effects in multiple tumor cell lines, including B16F10, MCF-7, A549 and SKOV3, as well as enhanced anti-inflammation efficacy in macrophage (RAW264.7) without causing cells damage. Conjugation of piperidyl, diethylin and benzylamine with the crocetin was demonstrated to be a highly efficient strategy to improve the solubility of crocetin. The synthesized crocetin derivatives were shown the promising therapeutics for the tumor and inflammation treatment with high safety.

1. Introduction

Plants are important resources to discover new drugs. Saffron, the dried stigma of the Crocus sativus L, is used as a food colorant and spice due to its color and taste [1]. More importantly, saffron is an oriental folk medicine and included in Traditional Chinese Medicine (TCM) formulations that has been widely used in the treatment of various diseases. Pharmacological studies showed that saffron may act as antioxidant, antidepressants, anxiolytics, memory enhancers, anticonvulsant, and antitussive. It also can improve male erectile dysfunction, hypotensive, and act as effective antinociceptive, anti-inflammatory and anticancer reagents [2]. As a main component and the major pharmaceutical effective constituent of saffron, crocetin has a wide resource including the fruit of Gardenia Jasminoides Ellis [3]. Crocetin, a kind of carotenoids, has a 20-carbon chain, six double bonds and two carboxylic acid groups and was demonstrated to have multiple

Abbreviations: ^{1}H NMR, 1H -nuclear magnetic resonance spectroscopy; ^{13}C NMR, 13C-nuclear magnetic resonance spectroscopy; TCM, traditional Chinese medicine; HRMS, high-resolution mass spectral; HOBt, 1-hydroxy, benzotriazole, hydrate; EDCI, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide; HCl, hydrochloride; $CHCl_3$, chloroform; DCM, dichloromethane; PE, petroleum ether; EtOAc, ethyl acetate; HCl, hydrochloric acid; MeOH, methanol; $NaHCO_3$, sodium bicarbonate; Et_3N, trimethylamine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MicroBCA, micro-bicinchoninic acid; MTT, methylthiazoletetrazolium; $CDCl_3$, deuterochloroform; DMSO, dimethyl Sulphoxide; ESI, electrospray ionization; UPLC-MS, ultra performance liquid chromatography–tandem mass spectrometer; LPS, lipopolysaccharides; NO, nitric oxide; BCA, bicinchoninic acid; SD, standard deviation; iNOS, inducible nitric oxide synthase

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pharmacological properties like anti-atherosclerosis [4], hyperlipidemia inhibition [4], anti-fatigue [5], cardioprotective [6], and neuroprotective effects [6]. Although there are many reports on the effects of saffron extract on cancer, there is still very limited information available for crocetin effects on cancer cell lines.

Moreover, due to the extreme low solubility of crocetin, researchers had to add a large amount of solubilizer to dissolve the compound completely and/or loading the compound in other drug delivery vehicles for investigation [7,8]. These pre-handling of the compounds caused much more complexity in elucidating the accurate effects of crocetin and the biosafety of the drug preparations. Thus, chemical modification might be a novel strategy to increase its solubility and beneficial to its efficacy and application.

Conjugation of organic compounds containing heteroatoms such as N to poor soluble molecules can help the synthesized derivative to form stable hydrogen bonds by lowering the salvation energy, which will improve the solubility of the modified compounds. Importantly, neutralization of the carboxyl group by amidation and adding lipophilic alkyl chain to amine group have been demonstrated to be able to increase the liposolubility and permeability of molecules [9,10]. However, until recently, the investigation of the chemical modification influence in the solubility and pharmacological effect of crocetin is scarcely reported. Therefore, in the present study, the conjugation of crocetin with piperidyl, diethylin and benzylamine were synthesized. The synthesized crocetin derivatives structures were elucidated by ^{1}H NMR, ^{13}C NMR and UPLC-MS spectroscopic analysis. Upon that, the anti-inflammatory activity and anticancer effects of the new compounds was investigated.

2. Materials and methods

2.1. Materials

HOBt, EDCI. HCl, $CHCl_3$, DCM, PE, EtOAc, HCl, MeOH, $NaHCO_3$, trimethylamine (Et_3N). DMEM, FBS, penicillin and streptomycin, trypsin were obtained from the Gibco BRL (Gaithersberg, MD). Total nitric oxide assay kit and MicroBCA protein assay kit were purchased from Beyotime Biotechnology Inc., Nantong, China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from AMRESCO (USA). The mouse macrophage cell line (RAW 264.7), human breast cancer cell line (MCF-7), human ovarian carcinoma cell line (SKOV3), human lung cancer cell line (A549), rat melanoma cell line (B16F10) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China).

2.2. Synthesis of crocetin derivatives

Crocetin was produced by Livzon Pharmaceutical Group Inc. Briefly, gardenia yellow was extracted from Gardenia jasminoides var. radicans Makino, and isolated by macroporous resin. Then, gardenia yellow was subjected to alkaline hydrolysis to afford crude crocetin, which was purified by recrystallization.

To a solution of crocetin (1.0 equiv) in dry DCM, HOBt (2.5 equiv) and EDCI (2.5 equiv) were added at 0 °C followed by Et_3N (2.5 equiv) and amines (2.5 equiv). The mixture was stirred at 0 °C for 4 h and overnight at room temperature. It was diluted with DCM and the organic layer was washed with 2% HCl (3 × 10 ml) and 5% $NaHCO_3$ (3 × 10 ml). The combined organic was evaporated in vacuo to obtain the products. The residue was purified by flash chromatography on silica gel, eluting with PE/EtOAc, DCM/MeOH, or DCM as indicated in each case. Crocetin derivatives, compounds A, B were synthesized by a previously reported method [11]. Compound C was prepared according to the general procedures for the synthesis of A and B. The materials used for preparing C contained crocetin (0.5 mmol, 164 mg), EDCI (1.25 mmol, 239 mg), HOBt (1.25 mmol, 169 mg), Et_3N (1.25 mmol, 172 µl) and benzylamine (1.1 mmol, 120 µl) in anhydrous DCM (200 ml). Briefly, HOBt (1.25 mmol, 169 mg) and EDCI (1.25 mmol, 239 mg) were added at 0 °C followed by Et_3N (2.5 equiv) and 4-fluorobenzylamine (1.1 mmol, 125 µl). The mixture was stirred at 0 °C for 4 h and overnight at room temperature. It was diluted with DCM and the organic layer was washed with 2% HCl (3 × 10 ml) and 5% $NaHCO_3$ (3 × 10 ml). The combined organic was evaporated in vacuo to obtain the product. The crude was purified by column chromatography to afford the compound C as a yellow powder.

2.3. Chemical characterization of compound C

^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker AV 600 spectrometer using TMS as an internal standard. Both ^{1}H and ^{13}C NMR experiments were measured on a Bruker Ascend® 600 NMR spectrometer (600 MHz for ^{1}H and 150.9 MHz for ^{13}C) with the solvent signal ($CDCl_3$, 7.26 ppm, ^{1}H; 77.16 ppm, ^{13}C; DMSO, 2.50 ppm, ^{1}H; 39.52 ppm, ^{13}C) as an internal reference. High Resolution Mass Spectra were performed by an Agilent 6230 ESI time-of-flight (TOF) mass spectrometer. All final compounds displayed ≥95% purity as determined by NMR and UPLC-MS analysis.

2.4. Solubility assays

Crocetin and its derivatives were dissolved in methanol followed by the measurement of their maximum absorption with ultraviolet spectrophotometer. Then the standard curve of crocetin in methanol was established. After that, an appropriate amount of drugs were dissolved in water and then ultrasounded for half an hour until the supersaturated solution was formed. The supersaturated solution was then centrifuged for 10 min at the speed of 12,000 rpm. 20 ml of the supernatant was collected and lyophilized, then re-dissolved with appropriate volume of methanol for the UV detection. The amount of the compounds in the methanol solution was calculated by the standard curve. Based on that, the water solubility of the tested compounds was calculated accordingly.

2.5. Cells culture

RAW 264.7, MCF-7, SKOV3, A549 and B16F10 cells were cultured in the DMEM medium with high glucose containing 10% FBS, 100 units/ml of penicillin and 100 µg/ml of streptomycin. The medium was changed every other day. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells reached 80–90% confluence were used in the further tests.

2.6. Proliferation inhibitory potential in different types of cancer cell lines

The different types of cancer cells were seeded at 5×10^3 cells/well in 96-well plates and incubated overnight, then treated with various concentrations of compounds. All the compounds were solubilized in DMSO, then diluted by DMEM. The concentration of DMSO in the culture medium was below 1%. The cells were incubated with the drug solutions for 24 h or 48 h, then moved to fresh medium (80 µl) followed by adding 20 µl of 5 mg/ml MTT. The incubation was continued for an additional 4 h at 37 °C. The medium was removed from cells and the remaining MTT-formazan production was dissolved in 150 µl of 100% DMSO. The OD value of each well was measured at 595 nm on a microplate reader. Proliferation inhibitory of the tested compounds in cells was calculated as the reduction of cell viability. Cells incubated with the same dose of DMSO solution was calculated as blank control group.

2.7. Nitric oxide (NO) inhibition efficacy in LPS activated macrophages

RAW 264.7 cells were seeded at 3×10^5 cells/ml in 24-well cell