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Competitive neutrophil elastase inhibitory isoflavones from the roots of *Flemingia philippinensis*

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ABSTRACT

Flemingia philippinensis has been used throughout history to cure rheumatism associated with neutrophil elastase (NE). In this study, we isolated sixteen NE inhibitory flavonoids (**1**–**16**), including the most potent and abundant prenyl isoflavones (**1**–**9**), from the *F. philippinensis* plant. These prenyl isoflavones (**2**, **3**, **5**, **7**, and **9**) competitively inhibited NE, with IC_{50} values of 1.3–12.0 μM. In addition, they were reversible, simple, slow-binding inhibitors according to their respective parameters. Representative compound **3** had an IC_{50} = 1.3 μM, k_3 = 0.04172 μM^{-1} min^{-1}, k_4 = 0.0064 min^{-1}, and K_i^{app} = 0.1534 μM. The K_{ik}/K_{iv} ratios (18.5 ~ 24.6) for compound **3** were consistent with typical competitive inhibitors. The prenyl functionality of isoflavones significantly affected inhibitory potencies and mechanistic behavior by shifting the competitive mode to a noncompetitive one. The remaining flavonoids (**10**–**16**) were confirmed as mixed type I inhibitors that preferred to bind free enzyme rather than the enzyme-substrate complex. Fluorescence quenching analyses indicated that the inhibitory potency (IC_{50}) closely followed the binding affinity (K_{SV}).

1. Introduction

Flemingia philippinensis, belonging to the legume family, is a polyphenol rich plant [1]. This species is well-known in folk medicine and has been used to cure rheumatism and to improve bone mineral density [2]. In southern China, *F. philippinensis* is cultivated on a large scale and widely consumed by local inhabitants as an important nutraceutical for nutritious and therapeutic purposes, especially against rheumatism and associated inflammatory ailments [3,4]. The main bioactive constituents of *F. philippinensis* include flavanones, chalcones, isoflavones, steroids, and triterpenes, many of which have been proven to possess anti-inflammatory, anti-estrogenic, immunosuppressive, and antioxidant activities [5–7]. The prenylated isoflavones in this plant have predominately been investigated for their bacterial neuraminidase inhibition activity [8]. Similarly, chalcones and flavonoids have shown strong inhibitory effects against tyrosinase [9]. Recently, chromenedione derivatives have been shown to have protein tyrosine phosphate 1B (PTP1B) inhibitory properties [10]. As these compounds are a valuable source of therapeutic material, further work is needed to elucidate their health-promoting effects. Despite the traditional usage of *F. philippinensis* to treat rheumatoid arthritis, the effects of these compounds on neutrophil elastase (NE) activity, which has been shown to be involved in rheumatoid arthritis, have not been thoroughly investigated.

Serine hydrolases are the most diverse and biologically vital enzymes in living organisms, as well as being one of the largest enzyme groups [11]. This class of enzymes has several members, including amidases, lipases, esterases, and proteases, which have been targeted by various clinically-approved drugs [12,13]; among them, neutrophil elastase (NE, EC 3.4.21.37) is an important enzyme belonging to the chymotrypsin family of serine proteases [14]. Biologically active NE is stored in azurophilic granules inside neutrophils and primarily cleaves the peptide bonds in proteins upon release, resulting in breakdown of extracellular matrix proteins such as collagen, fibronectin, elastin, and numerous plasma proteins [15]. Further, NE stimulates the release of inflammatory cytokines such as interleukin (IL)-6, IL-8, and other cytokines, which further exacerbates the inflammatory process [16]. The unconstrained proteolytic activity of NE results in various inflammatory ailments such as rheumatoid arthritis, reperfusion injury, chronic obstructive pulmonary disease, cystic fibrosis, atherosclerosis, and lung injury [17,18]. Especially, a deep correlation between rheumatoid arthritis (RA) and NE has been reported in several studies that showed higher levels of elastase-inhibitor complex (EIC)

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in active RA patients than in controls [19,20]. Similarly, an increase in NE activity has been measured in synovial fluid from RA patients [21]. Owing to these diverse effects, NE inhibition might be a beneficial approach to retard the negative health effects associated with RA and further strengthen the ongoing efforts against inflammatory diseases in general.

In this study, we investigated NE inhibitory isolates from *F. philippinensis*, with sixteen flavonoids isolated from the methanol extract of *F. philippinensis* root bark. Isolated compounds were analyzed for NE activity, and results showed that all compounds significantly inhibited the NE enzyme; however, the inhibitory potencies and mechanisms of inhibition differed according to their respective structures. We fully investigated inhibitory potencies, structure-activity relationships (SAR), and inhibitory mechanisms based on secondary plots using the Michaelis-Menten equation. Furthermore, the mutual affinity between inhibitor and enzyme was extensively examined using fluorescence quenching experiments.

2. Materials and methods

F. philippinensis plants were collected in August 2014 at a farm in Ninning, Guangxi province, China and identified by Dr. Yimmin Zhao. Voucher specimens (No. 530) were deposited in the Herbarium of Gaunxi Botanical Garden, China. Human recombinant neutrophil elastase (EC 3.4.21.37) and *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide were purchased from Enzo Life Sciences Inc. (New York, USA). Tris, oleanolic acid, chloroform-*d*, methanol-*d*, and acetone-d_6 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Silica gel (230–400 mesh), NP F254, RP-18 F254 thin-layer chromatography (TLC) plates, analytical grade water, acetonitrile, and methanol were obtained from Merck (Darmstadt, Germany). Spherical C18 100 Å reversed phase silica gels (particle size: 20–40 μm) were obtained from Silicycle (Quebec, Canada). Methanol, acetone, ethyl acetate, chloroform, and *n*-hexane were purchased from Duksan Co. (Gyenggi, Korea).

2.1. Instruments

1D and 2D NMR spectra (^{1}H, ^{13}C, DEPT-90, DEPT-135, COSY, HMQC, and HMBC) were recorded on a Bruker (AM 500 MHz) spectrometer using $CDCl_3$, CD_3OD, and acetone-d_6 as solvents and tetramethylsilane (TMS) as an internal standard. Electron ionization (EI) and EI-high resolution (HR) mass spectra were obtained on a JEOL JMS-700 instrument (Japan). Medium-pressure liquid chromatography (MPLC) analyses were performed using a LC-Forte/R 100 (YMC Co., Ltd., Kyoto, Japan) system equipped with a low-pressure gradient pump, column compartment, and a three-channel UV detector. Enzymatic assays were carried out on a SpectraMax M3 multi-mode microplate reader (Molecular Devices, USA).

2.2. Extraction and isolation of F. Philippinensis

Dried root bark from *F. philippinensis* (0.5 kg) was extracted with MeOH (10 L × 2) at room temperature to give a crude extract (56 g). The crude extract (2.5 g) was purified by means of MPLC (LC/Forte R 100, YMC) over reversed phase silica gels (350 g, 40 μm) and eluted using a H_2O/MeOH gradient (10–100%, 25 mL/min) to give 80 fractions. This MPLC procedure was repeated ten times using the same conditions before a final purification. Fractions 19–24 (6.3 g, 1.2 g × 5) were purified over an ODS-C18 column (200 g, 25 μm) by eluting with a H_2O/ MeOH gradient (0–100%, 15 mL/min) to give 80 fractions. Subfractions 23–26 (230 mg) were further purified by repeated MPLC with an ODS-C18 column (100 g, 25 μm) to give compounds **1** (12 mg), **3** (21 mg), and **16** (15 mg). Repeated MPLC of subfractions 27–31 (310 mg) yielded compounds **2** (26 mg), **4** (13 mg), **7** (16 mg), and **9** (11 mg). Fractions 25–31 (7.9 g, 1.2 g × 6) were purified over an ODS-C18 column (200 g, 25 μm) by eluting with a H_2O/MeOH gradient (0–100%, 15 mL/min) to give 80 subfractions. Subfractions 29–35 (510 mg) were further purified by repeated MPLC with an ODS-C18 column (200 g, 25 μm), resulting in compounds **5** (13 mg), **6** (19 mg), **10** (31 mg), and a mixture of **14** and **15**, which were separated via Sephadex LH-20 by eluting with 90% MeOH to afford compounds **14** (13 mg) and **15** (9 mg). Repeated MPLC of subfractions 36–41 (670 mg) on an ODS-C18 column (100 g, 25 μm) gave compounds **11** (14 mg) and **12** (7 mg), as well as a mixture of compounds **8** and **13**, which were purified via Sephadex LH-20 by eluting with 90% MeOH to afford compounds **8** (11 mg) and **13** (16 mg). All isolated compounds were identified via spectroscopic data and a comparison of previous studies [22–25]. Selected spectroscopic data are presented in the supplementary materials.

2.3. Measurement of neutrophil elastase activity

NE (EC 3.4.21.37) activity was assayed using standard procedures with slight modifications by measuring the formation of *p*-nitroaniline after N-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitro anilide hydrolysis at 405 nm [26]. Test samples were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM and used for the assay after diluting. The reaction mixture contained 130 μL 0.02 mM Tris-HCl buffer (pH 8.0), 10 μL test sample solution, 40 μL substrate (1.5 mM, MeOSuc-AAPV-*p*NA), and 20 μL human neutrophil elastase (0.2 unit/mL) and was placed in 96-well microplates. The reaction mixtures were mixed and incubated for 15 min at room temperature and then screened at 405 nm for 30 min every 30 s. Inhibitory activities were further characterized by determining the concentration required to inhibit 50% of the enzyme activity (IC_{50}), which was calculated using the following equation: Activity (%) = 100 $[1/(1 + ([I]/IC_{50}))]$, where [I] is the concentration of inhibitor.

2.4. Enzyme inhibition kinetics

The modality of enzyme inhibition was investigated in experiments using incremental concentrations (0.75 ~ 3.0 mM) of substrate by testing the compounds at different concentrations as indicated. The data were analyzed using the nonlinear regression program, Sigma Plot (SPCC Inc., Chicago, IL, USA), to find the individual parameters for each curve. The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were determined using a Lineweaver-Burk plot. K_I and K_{IS}, dissociation constants for inhibitor binding to either free enzyme or the enzyme–substrate complex, were derived from secondary plots of slopes of the straight lines and the vertical intercept ($1/V_{max}^{app}$) versus inhibitor concentrations [27]. Eqs. (1)–(3) are representative equations for deriving K_I and K_{IS}, respectively. The K_{ik} and K_{iv} inhibition constants were calculated according to Eqs. (4) and (5), as suggested by Yang et al. [28]. Linear regression analyses were performed with Excel.

$$1/V = K_m/V_{max}(1 + [I]/K_I)1/S + 1/V_{max} \quad (1)$$

$$\text{Slope} = K_m/K_I V_{max}[I] + K_m/V_{max} \quad (2)$$

$$\text{Intercept} = 1/K_{IS} V_{max}[I] + 1/V_{max} \quad (3)$$