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Hesperetin derivatives: Synthesis and anti-inflammatory activity

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ABSTRACT

Sixteen novel hesperetin derivatives containing Mannich base moiety were designed and synthesized and their anti-inflammatory activities were evaluated by inhibiting tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in mouse RAW264.7 macrophages. Compounds **3a**–**3k** showed better hydrophilic, while compounds **3l**–**3p** with aromatic groups was hydrophobic. The anti-inflammatory activity of title compounds was correlated with log*P* values, among them, compounds **3c**, **3e** and **3i** with minus log*P* values exhibited best anti-inflammatory activity through decreasing both IL-6 and TNF-α. Furthermore, the expression of LPS-induced notch1 and inos was reduced by compounds **3c**, **3e**, and **3i**, and compound **3e** attenuated LPS-induced inos protein levels in a dose-dependent manner.

Chinese traditional medicine citrus is the dried fruit skin of rutaceae plants orange (*Citrus reticulata* Blanco) and its cultivar. Hesperidin (hesperetin-7-*O*-rutinoside) and hesperitin (hesperetin-7-*O*-glycoside) are the main active constituents of citrus fruits which have diverse pharmacological activities, such as anti-inflammatory,[1] antioxidant,[2,3] antibacterial,[4,5] anti-cancer,[6,7] anti-hepatic fibrosis,[8] immunity adjustment.[9] However, hesperetin has a lower bioavailability than hesperidin because of the rutinoside moiety attached to the flavonoid[10] and orally ingested hesperidin is hydrolyzed to hesperetin in the gastrointestinal tract and conjugated during absorption.[11]

In previous studies, we found that 5,7,3′-triacetyl hesperetin could suppress adjuvant-induced arthritis (AA) in rats through modulating JAK2/STAT3 pathway, while 7,3′-dimethoxy hesperetin inhibited inflammation by inducing synovial apoptosis in AA rats.[12,13] Recently, the interactions between the Notch signaling pathway and angiogenesis have been described in rheumatoid arthritis (RA). Park[14] found that inhibition of Notch signaling could ameliorate experimental inflammatory arthritis. Thus we would explore whether hesperetin effects on Notch signaling. As is mentioned above, hesperetin's poor solubility in water and low bioavailability limits its further application. In order to improve its biological activity, it is therefore necessary to carry out structure modification of hesperetin to improve water-solubility.[15,16]

The presence of a Mannich base group in many active compounds could not only improve their biological activity, but also tailor their lipophilic properties.[17,18] Structural analysis indicates that the A ring of hesperetin is the most electron-rich with the C-6 and C-8 positions being the predominant site for Mannich reactions. High regioselectivity for the C-6 position can be achieved by optimizing the reaction temperatures and reaction times.[19] Herein, we synthesized a series of hesperetin derivatives with Mannich bases and evaluated their anti-inflammatory activities, and the preliminary mechanism of hesperetin derivatives was further studied.

Hesperetin derivatives (**3a**–**3p**) were obtained from two steps as described in Scheme 1. Hesperetin (**2**) was synthesized by sulfuric acid hydrolysis of hesperidin (**1**) in CH_3OH. The title compounds were synthesized by the classical Mannich reaction of hesperetin with formaldehyde in the presence of the primary amines or secondary amines in methanol. In our case, compound **2**, formaldehyde and amine were in 1:1:1.1 ratio, respectively, and stirred at 30 °C for overnight to afford the C6-aminomethylated hesperetin derivatives (**3a**–**3p**). The structures of title compounds were confirmed by NMR and mass spectra, and as shown in Figure 1, the C-6 substitution was further confirmed by X-ray crystallography.[20] The general synthetic procedure and spectral data of compounds **2** and **3** were recorded in detail.[22,23]

The structure of compound **3e** was determined by X-ray crystallography. Crystal data of **3e**: light orange crystals, 78% yield, mp: 279–280 °C; $C_{21}H_{25}NO_6$, $C_7H_7O_3S$, 1.5(H_2O), M = 585.63, Triclinic, space group $P\bar{1}$; a = 10.0751(8), b = 11.7584(13), c = 27.841(2) (Å); α = 95.527(8), β = 94.491(6), γ = 114.529(9), V = 2961.1(5) Å^3, T = 300.79(10) K, Z = 4, D_c = 1.314 g/cm^3, $F(000)$ = 1240, Reflections collected/independent reflections = 10,927/7565, Data/restraints/parameters = 10,927/114/866, Goodness of fit on F^2 = 1.036, Fine,

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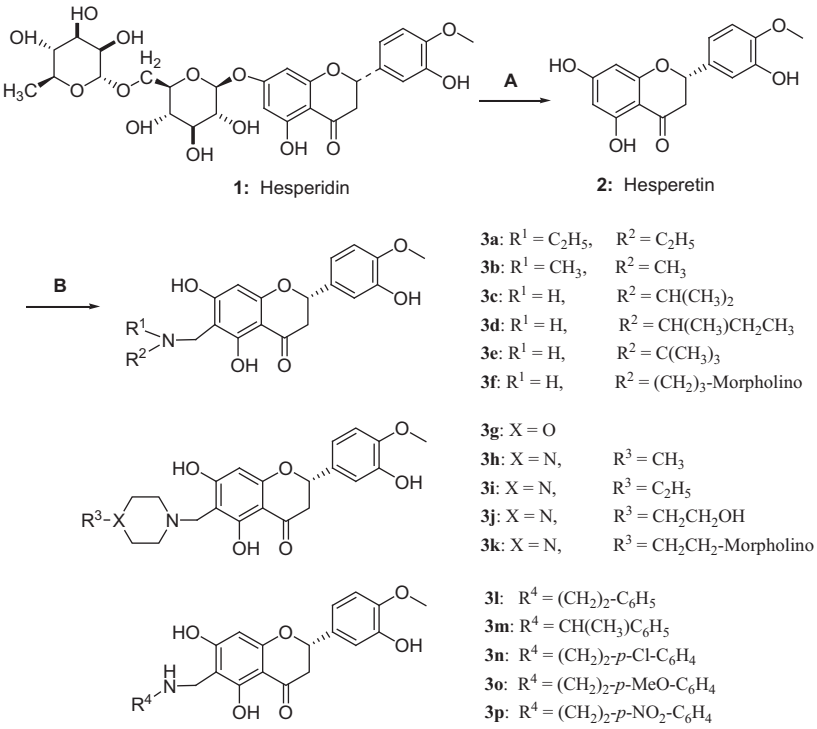

Scheme 1. Synthesis of title compounds **3a**–**3p**. Reagents and conditions: (A) 96% H_2SO_4, MeOH, 70 °C; (B) MeOH, formaldehyde (37%), amine, 30–60 °C.

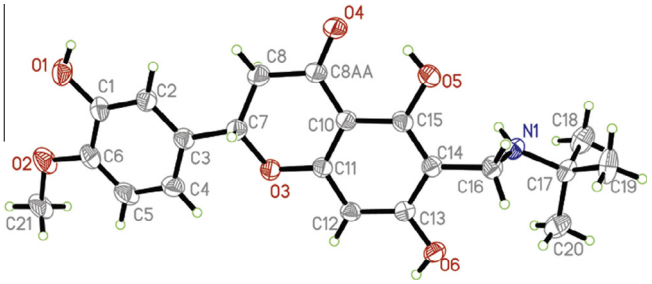

Figure 1. ORTEP drawing of compound **3e**.

R_1 = 0.0673, $wR(F^2)$ = 0.2089. CCDC 1418310 contains the supplementary crystallographic data for the structure. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk.[20]

The anti-inflammatory activity of hesperetin and its derivatives were evaluated by their inhibitory effect against LPS-induced TNF-α and IL-6 release in the mouse macrophages.[24–27] Firstly, MTT assay was used to screen the influences of hesperitin derivatives on the viability of LPS-activated RAW264.7 cells. As shown in Figure 2, the stimulator LPS (0.5–1 μg/mL) and all compounds (1–10 μM) were showed no toxic effects on RAW 264.7 cells. Even with the increase of the concentration, LPS (2 μg/mL) and compounds (**3e**, **3i**, **3l**, **3o** at 100 μM) showed much lower viability. Thus we selected the concentrations 10 μM and 1 μg/mL of LPS for the next experiments. Indomethacin was used as the positive control. As shown in Figures 3 and 4, hesperetin derivatives showed anti-inflammatory activity as hesperetin at the concentration 10 μM. Among them, Indomethacin, compounds **3c** and **3e** with aliphatic amine and Mannich bases **3g**–**3k** with alicyclic amine showed better inhibitory effect than hesperetin on LPS-induced TNF-α and IL-6, however, aromatic amine substituted ones (**3g**–**3k**) showed lower effect than hesperetin on TNF-α and IL-6, which may be due to the lower solubility. As shown in Figure 4, compounds **3c**, **3e** and **3i** with best anti-inflammatory activity showed an obvious dose–effect relationship through decreasing both IL-6 and TNF-α.

RAW264.7 cells were treated with LPS (0.5, 1, 2 μg/mL) and active compounds (L = 1 μM, M = 10 μM, H = 100 μM) for 24 h. Data are representative of at least three separate experiments. N = the cells without treatment. Statistical significance relative to the N group was indicated, $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001.

Macrophages were stimulated with LPS (1 μg/mL) for 22 h and then treated with vehicles, hesperetin or its derivatives (10 μM) for 24 h. IL-6 (A) and TNF-α (B) levels in the culture media were measured by ELISA and were normalized by the total protein. Values represent the mean ± SEM for five independent experiments and LPS is regarded as 100%. Statistical significance relative to the DMSO group was indicated, $^{\#}p$ <0.01. Statistical significance relative to the LPS group was indicated, $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001. In = Indomethacin (10 μM).

Macrophages were stimulated with LPS (1 μg/mL) for 22 h and then treated with compounds **3c** **3e** and **3i** at indicated concentrations (2.5, 5, 10 μM) for 24 h. TNF-α (A) and IL-6 (B) levels in the culture media were measured by ELISA and were normalized by the total protein. Values represent the mean ± SEM for three independent experiments and LPS is regarded as 100%. Statistical significance relative to the DMSO group was indicated, $^{\#}p$ <0.01. Statistical significance relative to the LPS group was indicated, $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001.

In order to know whether is the polarity of title compounds affects its anti-inflammatory activity in this study. Theoretical octanol/water partition coefficient (log$P$) of title compounds was measured by the shake-flask method.[28–30] As showed in Table 1, the anti-inflammatory activity of title compounds was associated with log$P$ values. Mannich bases of aliphatic amine series (**3a**–**3f**) and alicyclic amine series (**3g**–**3k**) showed better hydrophilic than hesperetin due to smaller or minus log$P$ values, while those of aromatic amine series (**3l**–**3p**) exhibited hydrophobic. Obviously, compounds **3c**, **3e** and **3i** with minus log$P$ values showed best