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Synthesis and biological evaluation of hesperetin derivatives as agents inducing apoptosis

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ABSTRACT

A flavanone, hesperetin, has been known to exert antitumor activity by inducing apoptosis. To find hesperetin derivatives showing better antitumor activity, 12 derivatives were designed and synthesized. Their antitumor activities were measured using a long-term survival clonogenic assay. Among the compounds, **K-5b**, hesperetin-7-butyrate, showed the half-maximal cell growth inhibitory concentration three times as low as that of hesperetin. To compare the cytotoxicity of hesperetin and **K-5b**, the HCT116 human colon cancer cell line was treated with various concentrations of each compound. **K-5b** decreased the cell viability to a larger extent than hesperetin and triggered apoptosis more efficiently than hesperetin in an apoptosis detection assay using fluorescein isothiocyanate-labeled annexin V. Immunoblotting analysis showed that **K-5b** promoted caspase-mediated apoptosis more efficiently than hesperetin. Because hesperetin has been reported to induce apoptosis through the activation of the c-Jun N-terminal kinase (JNK) pathway, we tested whether **K-5b** activates JNKs. **K-5b** stimulated JNK1 and JNK2 more efficiently than hesperetin as shown by western blot analysis. In conclusion, hesperetin derivatives exerting better antitumor activity than hesperetin by inducing apoptosis were found.

1. Introduction

Flavonoids are generally stored in plants as glycosides because they are more stable than their aglycones. However, glycosides are hydrolyzed to aglycones to be used because of their poor bioavailability.[1] Flavonoid aglycones, including hesperetin, are more potent in their antiperoxidative action, such as nonenzymatic lipid peroxidation, than their corresponding glycosides.[2] Hesperetin-7-O-rutinoside, hesperidin, widely found in citrus fruits, including oranges, lemons, limes, and grapefruits, is metabolized to an aglycone, hesperetin, and a disaccharide, rutinose, by hesperidin-6-*O*-α-l-rhamnosyl-β-d-glucosidase.[3] Hesperetin, 3′,5,7-trihydroxy-4′-methoxyflavanone, reduces the intracellular replication of viruses, including herpes simplex virus type 1, poliovirus type 1, parainfluenza virus type 3, and respiratory syncytial virus,[4] and stimulates trypsin-activated phosphorylase kinase.[5] Besides, hesperetin inhibits receptor-mediated endocytosis of beta-hexosaminidase[6] and myeloperoxidase release.[7] Hesperetin itself has been reported to exert antitumor activity by inducing apoptosis in many cancer cells *in vitro* and *in vivo*.[8] Because hesperetin belongs to polyphenols, various functional groups can be substituted for its phenyl groups. However, its derivatives have rarely been reported, but it has been shown that 7,3′-dimethoxy hesperetin induces apoptosis.[9] In particular, the 5-hydroxy group forms a hydrogen bond with the ketone of the chroman-4-one moiety.[10] Therefore, we tried to prepare compounds derivatized at 4′- and 7-positions. There are many methods to screen for antitumor activity, among which clonogenic assays are long-term cell survival assays. While the assays require a long experimental time, such as seven days, they can discriminate between cancer cells with similar proliferation rates. Because the compounds synthesized in this study contain hesperetin as a common moiety, a clonogenic assay can give reliable results on cell proliferation. Colon cancer is the third most common type of cancer in the world.

Abbreviation: GI50, half-maximal cell growth inhibitory concentration.

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Hesperetin is known to have potent chemo-preventive and antitumor properties against intestinal carcinoma, probable due to direct exposure to transformed intestinal epithelium with much higher efficiency than other tissues.[11] As tumor suppressor p53 plays an important role in hesperetin-induced apoptosis,[12] we used HCT116 human colon carcinoma cells expressing wild-type p53. Half-maximal cell growth inhibitory concentration (GI_{50}) values for the compounds were obtained from the clonogenic assay, and the relationships between cytotoxicities of the derivatives and their structures were elucidated. For the derivative showing the best GI_{50} value, **K-5b**, further biological experiments were performed to confirm that it induces apoptosis. To understand the mechanism by which **K-5b** triggers apoptosis, we tested whether **K-5b** activates c-Jun N-terminal kinases (JNKs) because hesperetin has been reported to induce apoptosis through the activation of the JNK pathway.[13] The molecular binding modes among hesperetin, **K-5b**, JNK1, and JNK2 were elucidated using *in silico* docking. The goal of this study was to find hesperetin derivatives showing better biological activity than hesperetin itself. The title compound showed cytotoxicity three times as high as that of hesperetin. Analysis of the structures of the derivatives and their cytotoxicities may help us design a novel chemotherapeutic agent.

2. Materials and methods

2.1. General methods

To determine the structures of the hesperetin derivatives synthesized in this study, nuclear magnetic resonance (NMR) experiments were performed on a Bruker Avance 400 NMR spectrometer (9.4 T; Karlsruhe, Germany). All derivatives were dissolved in deuterated dimethyl sulfoxide (DMSO-d_6) and transferred into 2.5-mm NMR tubes. The concentrations of the samples were adjusted to approximately 100 mM. One-dimensional experiments, including ^{1}H NMR, ^{13}C NMR, and distortionless enhancement by polarization transfer, and two-dimensional experiments, including correlated spectroscopy, total correlated spectroscopy (TOCSY), nuclear Overhauser exchange spectroscopy (NOESY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bonded connectivities (HMBC), were carried out as described previously.[14] To confirm the results obtained in the NMR experiments, high resolution mass spectrometry (HR/MS) was performed on an ultraperformance liquid chromatography–hybrid quadrupole-time-of-flight mass spectrometer (Waters Corp., Milford, MA, USA) with the help of Prof. Choong Hwan Lee at Konkuk University, Korea.[15]

2.2. Preparation of hesperetin derivatives

Twelve hesperetin derivatives were synthesized containing substituents at the 3′- or/and 7-position (Table 1). Commercially available hesperetin (INDOFINE Chemical Company, Hillsborough Township, NJ, USA) was used with corresponding acid chlorides and triethylamine to synthesize the 12 derivatives. All of these 7- and 3′-*O*-acylated hesperetin derivatives, **K-1a** to **Y-4a**, were prepared by the following general procedure. A flame-dried round-bottom flask was charged under argon with hesperetin (1 equiv.) and acetone. To this solution, freshly distilled triethylamine (2 equiv.) was then added under argon. After the solution was stirred for 10 min at room temperature, the corresponding acid chloride (1.2–2.4 equiv.) was added quickly. The reaction mixture was stirred for 10 min at the same temperature and quenched with distilled water. This aqueous mixture was extracted with CH_2Cl_2, and combined organic extracts were dried over anhydrous $MgSO_4$. After removal of the solvent, the crude product was purified by flash column chromatography to get the desired products. The synthetic process is summarized in Scheme 1. All derivatives except **K-1b** are novel.

The spectral data of the 12 hesperetin derivatives obtained from the NMR and HR/MS experiments are summarized as follows.

2.2.1. Hesperetin-3′,7-dibenzoate (*K-1a*)

^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.96 (s, 1H, 5-OH), 8.13 (dd, 2H, H-2″, H-6″, J = 1.8, 7.8 Hz), 8.10 (dd, 2H, H-2‴, H-6‴, J = 1.8, 7.8 Hz), 7.76 (ddd, 2H, H-4″, H-4‴, J = 1.8, 7.8, 7.8 Hz), 7.61 (dd, 2H, H-3‴, H-5‴, J = 7.8, 7.8 Hz), 7.60 (dd, 2H, H-3″, H-5″, J = 7.8, 7.8 Hz), 7.479 (dd, 1H, H-6′, J = 2.3, 9.1 Hz), 7.475 (d, 1H, H-2′, J = 2.3 Hz), 7.25 (d, 1H, H-5′, J = 9.1 Hz), 6.57 (d, 1H, H-8, J = 2.1 Hz), 6.53 (d, 1H, H-6, J = 2.1 Hz), 5.71 (dd, 1H, H-2, J = 2.9, 12.9 Hz), 3.79 (s, 3H, 4′-OCH_3), 3.49 (dd, 1H, H-3, J = 12.9, 17.2 Hz), 2.92 (dd, 1H, H-3, J = 2.9, 17.2 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 197.9 (C-4), 164.0 (C-3′α), 163.5 (C-7α), 162.3 (C-5), 162.1 (C-9), 158.1 (C-7), 151.2 (C-4′), 139.3 (C-3′), 134.3 (C-4‴), 134.1 (C-4″), 130.7 (C-1′), 129.8 (C-2″, C-2‴, C-6″, C-6‴), 129.0 (C-3″, C-3‴, C-5″, C-5‴), 128.6 (C-1″), 128.5 (C-1‴), 125.8 (C-6′), 121.7 (C-2′), 112.9 (C-5′), 106.0 (C-10), 103.0 (C-6), 101.9 (C-8), 78.1 (C-2), 56.0 (4′-OCH_3), 42.2 (C-3). HRMS (m/z): calcd. for $(M+H)^+$: 511.1393; found: 511.1382.

2.2.2. Hesperetin-7-benzoate (*K-1b*)

^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.98 (s, 1H, 5-OH), 9.14 (s, 1H, 3′-OH), 8.10 (dd, 2H, H-2‴, H-6‴, J = 1.4, 7.4 Hz), 7.75 (ddd, 1H, H-4‴, J = 1.4, 7.4, 7.4 Hz), 7.60 (dd, 2H, H-3‴, H-5‴, J = 7.4, 7.4 Hz), 6.96 (d, 1H, H-2′, J = 1.9 Hz), 6.95 (d, 1H, H-5′, J = 8.3 Hz), 6.91 (dd, 1H, H-6′, J = 1.9, 8.3 Hz), 6.53 (d, 1H, H-8, J = 2.1 Hz), 6.52 (d, 1H, H-6, J = 2.1 Hz), 5.59 (dd, 1H, H-2, J = 3.0, 12.6 Hz), 3.78 (s, 3H, 4′-OCH_3), 3.39 (dd, 1H, H-3, J = 12.6, 17.2 Hz), 2.85 (dd, 1H, H-3, J = 3.0, 17.2 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 198.1 (C-4), 163.7 (C-7α), 162.3 (C-5), 162.2 (C-9), 158.1 (C-7), 148.1 (C-4′), 146.5 (C-3′), 134.3 (C-4‴), 130.7 (C-1′), 130.0 (C-2‴, C-6‴), 129.0 (C-3‴, C-5‴), 128.5 (C-1‴), 117.9 (C-6′), 114.2 (C-2′), 112.0 (C-5′), 106.0 (C-10), 102.8 (C-6), 101.9 (C-8), 78.7 (C-2), 55.7 (4′-OCH_3), 42.4 (C-3). HRMS (m/z): calcd. for $(M+H)^+$: 407.1131; found: 407.1136.

2.2.3. Hesperetin-3′,7-di-(4-methoxybenzoate) (*K-2*)

^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.96 (s, 1H, 5-OH), 8.07 (d, 2H, H-2″, H-6″, J = 9.0 Hz), 8.04 (d, 2H, H-2‴, H-6‴, J = 9.0 Hz), 7.45 (dd, 1H, H-6′, J = 2.1, 8.4 Hz), 7.43 (d, 1H, H-2′, J = 2.1 Hz), 7.22 (d, 1H, H-5′, J = 8.4 Hz), 7.11 (d, 2H, H-3″, H-5″, J = 9.0 Hz), 7.10 (d, 2H, H-3‴, H-5‴, J = 9.0 Hz), 6.53 (d, 1H, H-8, J = 2.1 Hz), 6.49 (d, 1H, H-6, J = 2.1 Hz), 5.69 (dd, 1H, H-2, J = 2.6, 13.0 Hz), 3.87 (s, 3H, 4″-OCH_3), 3.86 (s, 3H, 4‴-OCH_3), 3.78 (s, 3H, 4′-OCH_3), 3.48 (dd, 1H, H-3, J = 13.0, 17.1 Hz), 2.89 (dd, 1H, H-3, J = 2.6, 17.1 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 197.9 (C-4), 164.0 (C-7α), 163.8 (C-4‴), 163.7 (C-3′α), 163.3 (C-4″), 162.3 (C-5), 162.1 (C-9), 158.3 (C-7), 151.4 (C-4′), 139.4 (C-3′), 132.3 (C-2‴, C-6‴), 132.1 (C-2″, C-6″), 130.7 (C-1′), 125.8 (C-6′), 121.8 (C-2′), 120.7 (C-1‴), 120.5 (C-1″), 114.4 (C-3″, C-5″, C-3‴, C-5‴), 112.8 (C-5′), 105.9 (C-10), 103.0 (C-6), 102.0 (C-8), 78.2 (C-2), 56.0 (4′-OCH_3), 55.70 (4‴-OCH_3), 55.66 (4″-OCH_3), 42.2 (C-3). HRMS (m/z): calcd. for $(M+H)^+$: 571.1604; found: 571.1616.

2.2.4. Hesperetin-3′,7-di-(2-phenylacetate) (*K-3*)

^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.93 (s, 1H, 5-OH), 7.40 (dd, 1H, H-6′, J = 2.1, 8.6 Hz), 7.37 (d, 4H, H-2″, H-3″, H-5″, H-6″, J = 7.0 Hz), 7.36 (d, 4H, H-2‴, H-3‴, H-5‴, H-6‴, J = 7.0 Hz), 7.295 (dd, 2H, H-4″, H-4‴, J = 7.0, 7.0 Hz), 7.292 (d, 1H, H-2′, J = 2.1 Hz), 7.17 (d, 1H, H-5′, J = 8.6 Hz), 6.37 (d, 1H, H-8, J = 2.0 Hz), 6.35 (d, 1H, H-6, J = 2.0 Hz), 5.65 (dd, 1H, H-2, J = 2.9, 13.1 Hz), 3.958 (s, 2H, H-3′β), 3.955 (s, 2H, H-7β), 3.77 (s, 3H, 4′-OCH_3), 3.44 (dd,