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Synthesis of genistein derivatives and determination of their protective effects against vascular endothelial cell damages caused by hydrogen peroxide

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Abstract—A series of genistein derivatives, prepared by alkylation and difluoromethylation, were tested for their inhibitory effects on the hydrogen peroxide induced impairment in human umbilical vein endothelial (HUVE-12) cells in vitro. The HUVE-12 cells were pretreated with either the vehicle solvent (DMSO), genistein, or different amounts of the genistein derivatives for 30 min before exposed to 1 mM hydrogen peroxide for 24 h. Cell apoptosis was determined by flow cytometry with propidium iodide (PI) staining. Cellular injury was estimated by measuring the lactate dehydrogenase (LDH) release. Data suggested that the genistein derivatives possessed a protective effect on HUVE-12 cells from hydrogen peroxide induced apoptosis and reduced LDH release. Among these derivatives, 7-difluoromethyl-5,4′-dimethoxygenistein exhibited the strongest activity against hydrogen peroxide induced apoptosis of HUVE-12 cells.

Soybeans and soy products have been an important part of the oriental diet for centuries. In recent years, the therapeutic effects of certain compounds in the soy products have attracted increased attention among the science community. These compounds have been indicated to reduce the health risk of certain diseases, such as the cardiovascular ailments and various types of cancers.

Among these beneficiary compounds, phytochemicals were considered to play the major role. Phytochemicals are biologically active compounds in plants. Soybeans are a rich source of many phytochemicals including isoflavones, saponins, phytic acid, and phytosterols. Isoflavones are a class of phenolic compounds, which include daidzein, glycitein, and genistein. These compounds exist in soybeans either as glucosides or in free form (aglucons). Genistein is the major isoflavone in soy.

Endothelial cells are crucial in maintaining the physiological functions of the cardiovascular system and they are also involved in the development of a variety of human diseases.[1] The two leading cardiovascular diseases, hypertension and atherosclerosis, are complex processes that occur, at least in part, in response to the necrosis or apoptosis resulted vascular injury.[2,3] Increasing evidences suggested that oxidative-stress and free radicals produced during apoptosis were the major causes of endothelial damage. Therefore, pharmacological interventions targeting the endothelial remodeling have become a hot spot in the biomedical research.

Using natural products or extracts from plants for the treatment of oxidative stress-induced cell injury has been demonstrated by more and more researchers, for example, isoflavone significantly decreased the post menopause-related cardiovascular disease.[4] Genistein, an isoflavone compound, has been reported to inhibit the apoptosis in cultured endothelial cells and in animal models.[5,6] However, the low absorbance of flavonoids in intestines resulted in the low biological activities of this compound.[7,8] Therefore, designing new ready-to-absorb compounds using genistein as the lead compound is essential for the genistein bioactivity.

Keywords: Genistein derivatives; Protective effects; Vascular endothelial cell; 7-Difluoromethylyl-5,4′-dimethoxygenistein.
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Table 1. Characterization of the synthesized compounds

Compound	MS (EI, 70 eV)	IRυ_{max} (cm^{-1}, KBr)	^{1}H NMR (300 MHz, $CDCl_3$)	^{19}F NMR (282 MHz)
2	*m/z*: 332	1448, 1519, 1662 (C═O), 3319, 3528 (OH)	6.599 (1H, d, *J* = 2.4 Hz), 6.820 (1H, t, *J* = 12.0 Hz), 6.867 (1H, d, *J* = 2.4 Hz), 7.371 (2H, d, *J* = 12.0 Hz), 7.427 (1H, t, *J* = 72.9 Hz), 8.244 (1H, s), 9.550 (1H, s), 12.994 (1H, s)	−84.372 (d, *J* = 72.9 Hz)
3	*m/z*: 370	1430, 1448, 1498, 1653 (C═O), 3074 (OH)	6.651 (1H, t, *J* = 73.8 Hz), 6.590 (1H, d, *J* = 2.4 Hz), 6.638 (1H, t, *J* = 72.3 Hz), 6.684 (1H, d, *J* = 2.4 Hz), 7.229 (2H, d, *J* = 9.0 Hz), 7.551 (2H, d, *J* = 9.0 Hz), 7.975 (1H, s)	−80.953 (d, *J* = 73.8 Hz), −82.414 (d, *J* = 72.3 Hz)
4a	*m/z*: 348	1430, 1469, 1512, 1580, 1612, 1637 (C═O)	3.834 (3H, s),3.970 (3H, s), 6.540 (1H, d, *J* = 2.1 Hz), 6.642 (1H, t, *J* = 72.3 Hz), 6.724 (1H, d, *J* = 2.1 Hz), 6.940 (1H, d, *J* = 8.7 Hz), 7.472 (1H, d, *J* = 8.7 Hz), 7.811 (1H, s)	−82.150 (d, *J* = 72.3 Hz)
4b	*m/z*: 376	1476, 1581, 1613 (C═O), 1645, 2874, 2984	1.386–1.572 (6H, m), 4.023–4.181 (4H, m), 6.525 (1H, d, *J* = 2.4 Hz), 6.628 (1H, t, *J* = 72.6 Hz), 6.699 (1H, d, *J* = 2.4 Hz), 6.931 (2H, d, *J* = 8.4 Hz), 7.436 (2H, d, *J* = 8.4 Hz)	−82.085 (d, *J* = 72.6 Hz)
4c	*m/z*: 375	1469, 1513, 1581, 1614, 1651 (C═O), 2880, 2968	0.972–1.120 (4H, m), 1.157 (3H, t, *J* = 7.5 Hz), 1.223 (3H, t, *J* = 7.5 Hz), 3.987–4.164 (4H, m), 6.643 (1H, d, *J* = 2.4 Hz), 6.761 (1H, t, *J* = 72.6 Hz), 6.807 (1H, d, *J* = 2.4 Hz), 7.053 (2H, d, *J* = 9.0 Hz), 7.547 (2H, d, *J* = 9.0 Hz)	−82.142 (d, *J* = 72.6 Hz).
4d	*m/z*: 500	1454, 1513, 1579, 1614, 1647 (C═O), 2926	4.636 (4H, s), 7.314–7.342 (18H, m)	−82.161 (d, *J* = 72.2 Hz)
4e	*m/z*: 431, 517 (M^+)	1433, 1469, 1581, 1614, 1652 (C═O), 2859, 2956	0.906–2.016 (26H, m), 3.950 (2H, t, *J* = 6.6 Hz), 4.047 (2H, t, *J* = 6.6 Hz), 6.512 (1H, d, *J* = 2.1 Hz), 6.648 (1H, d, *J* = 2.1 Hz), 6.764 (1H, t, *J* = 72.9 Hz), 9.893 (2H, d, *J* = 9.0 Hz), 7.412 (2H, d, *J* = 9.0 Hz), 7.759 (1H, s)	−82.897 (d, *J* = 72.9 Hz)
4f	*m/z*: 544	1435, 1467, 1512, 1569, 1611, 1651 (C═O), 2858, 2929	0.860–1.218 (30H, m), 3.985–4.046 (4H, m), 6.378 (1H, d, *J* = 2.1 Hz), 6.423 (1H, d, *J* = 2.1 Hz), 6.531 (1H, t, *J* = 74.1 Hz), 7.155 (2H, d, *J* = 8.7 Hz), 7.543 (2H, d, *J* = 8.7 Hz), 7.748 (1H, s)	−80.555 (d, *J* = 74.1 Hz)
4g	*m/z*: 473, 600 (M^+)	1658 (C═O)	0.848–1.783 (38H, m), 3.966 (2H, t, *J* = 6.6 Hz), 4.040 (2H, t, *J* = 6.6 Hz), 6.513 (1H, d, *J* = 2.1 Hz), 6.627 (1H, t, *J* = 72.3 Hz), 6.674 (1H, d, *J* = 2.1 Hz), 6.925 (2H, d, *J* = 8.4 Hz), 7.423 (2H, d, *J* = 8.4 Hz), 7.753 (1H,s)	−82.052 (d, *J* = 72.3 Hz)
4h	*m/z*: 432	1472, 1513, 1579, 1628, 1650 (C═O), 2876, 2963	0.809–1.057 (12H, m), 2.176–2.198 (2H, m), 3.759 (2H, t, *J* = 6.6 Hz), 3.783 (2H, t, *J* = 6.6 Hz), 6.568 (1H, d, *J* = 2.4 Hz), 6.627 (1H, t, *J* = 72.9 Hz), 6.656 (1H, d, *J* = 2.4 Hz), 6.984 (2H, d, *J* = 9.0 Hz), 7.446 (2H, d, *J* = 9.0 Hz), 7.941 (1H, s)	−82.348 (d, *J* = 72.9 Hz)

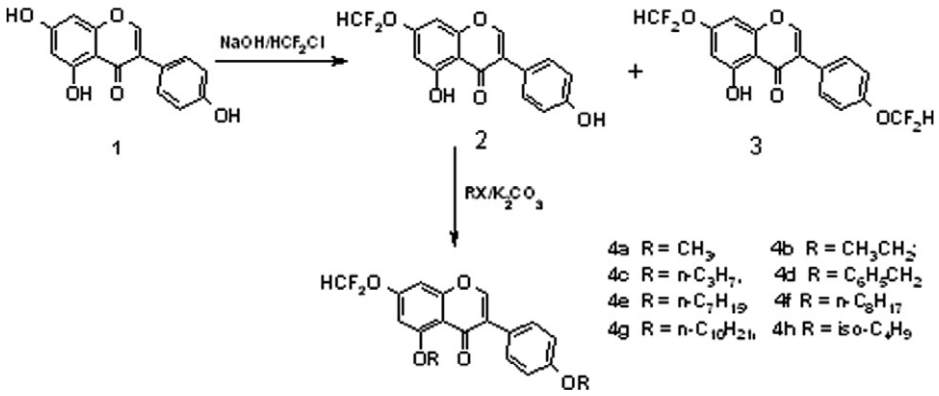

Figure 1. Synthetic scheme of the genistein derivatives.

It is well known that the introduction of the CHF_2 group into organic molecules can often dramatically change their physical and chemical properties.[9] In addition, the extension or the branching of hydrocarbon chain may affect their bioactivity.[10] Thus we designed, synthesized, and tested genistein derivatives that had 7-OH group substituted by $-OCHF_2$ and 5-OH, 4-OH groups substituted by alkoxylates. The activities of those derivatives were assayed with the established model of human umbilical vein endothelial (HUVE-12) cells cultured in vitro and apoptosis induced by oxidative stress, we discovered an active new chemical (7-difluoromethylyl-5,4′-dimethoxygenistein) that showed high protective activity against oxidative stress induced apoptosis of the vascular endothelial cells.

The readily available genistein (**1**) was used as the starting material for the preparation of the 7-difluoromethylated and 7,4′-gem-difluoromethylated genistein derivatives. Compound 1 (genistein) was mixed with HCF_2Cl/NaOH and incubated at 40 °C for 24 h. Ethyl acetate was then used for the extraction of the organic products and the extracted products were separated with column chromatography. A mixture of ethyl acetate and petroleum ether in different ratios was used for elution. The two compounds prepared were named 7-difluoromethyl-5,4′-dihydroxygenistein (**2**) and 5-hydroxyl-7,4′-gem-difluoromethylgenistein (**3**). Compound 2 was then mixed with K_2CO_3 and alkylating agents dissolved in acetone, and the mixture was allowed to react for 8–24 h at 40 °C. Eight new compounds (Compounds **4a**–**4h**, Fig. 1) were prepared. All the new compounds were characterized by detailed spectroscopic analysis of mass spectrometry (MS), infrared radiation (IR), and ^1H and ^{19}F nuclear magnetic resonance (NMR).

Human umbilical vein endothelial cell line (HUVE-12) was obtained from the China Center for Type Culture Collection (Wuhan, China). Cells were cultured in DMEM (Clonetics, Walkersville, USA) supplemented with 10% newborn calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technology, New York, USA) at 37 °C in a 5% CO_2 incubator. The cultured human umbilical vein endothelial cells (HUVE-12) were incubated for 30 min in vitro with either the vehicle solvent (0.02% DMSO), genistein or different concentrations of 7-difluoromethylyl-5,4′-di substituted alkoxyl genistein (0.1, 1.0, and 10.0 μM) before being challenged with 1 mM H_2O_2 for 24 h. HUVE-12 cells were also treated with various concentrations of dFMGEN, genistein (0.1, 0.3, 1.0, 3.0, and 10.0 μM) before being challenged with 1 mM H_2O_2 for 24 h. Cells were then harvested with 0.25% trypsin and washed with PBS. For the flow cytometry, cells at a density of 1×10^6/ml were fixed on ice with 70% ice-cold EtOH in PBS for 20 min, washed with PBS, incubated in propidium iodide (PI) solution (69 mM PI, 388 mM sodium citrate, 100 μg/ml RNase A) for 15 min at 37 °C, and immediately analyzed with the FAC Scan flow cytometer (Becton–Dickinson, San Jose, USA).[11] Lactate dehydrogenase (LDH) activity was determined using a colorimetric assay kit obtained from Sigma (St. Louis, MO). Before the cells were harvested, 100 μl of medium from each well was removed and used for LDH activity assay.

The new compounds synthesized during the reaction were characterized in detail by spectroscopic analysis (Table 1). Compounds **4a**–**4h** were identified as 7-difluoromethyl-5,4′-dimethoxygenistein (**4a**), 7-difluoromethyl-5,4′-diethyloxygenistin (**4b**), 7-difluoromethyl- 5, 4′-dipropeinoxygenistein (**4c**), 7-difluoromethyl-5,4′-dibenzyloxygenistein (**4d**), 7-difluoromethyl -5,4′-diheptaneoxygenistein (**4e**), 7-difluoromethyl -5,4′-dioctyloxygenistein (**4f**), 7-difluoromethyl-5,4′-dinonyloxygenistein (**4g**), and 7-difluoromethyl-5,4′-diisohexenylglutaconyloxygenistein (**4h**).

The flow cytometric analysis using propidium iodide (PI) staining indicated that the incubation of H_2O_2 for 24 h promoted the apoptosis of HUVE-12 cells. When cells were pretreated with 7-difluoromethylyl-5,4′-di-substituted alkoxyl isoflavones in different concentration, however, the rate of apoptosis decreased (Fig. 2A). The LDH activity (Fig. 2B) was induced by 1 mM H_2O_2 significantly. However, genistein and its derivatives reversed this effect. The derivative 7-difluoromethylyl-5,4′-dimethoxygenistein seemed to have a better protective effect against the endothelium injury from oxidative stress than the lead compound, genistein. The derivative 7-difluoromethylyl-5,4′-dimethoxygenistein was the most potent among all the derivatives.

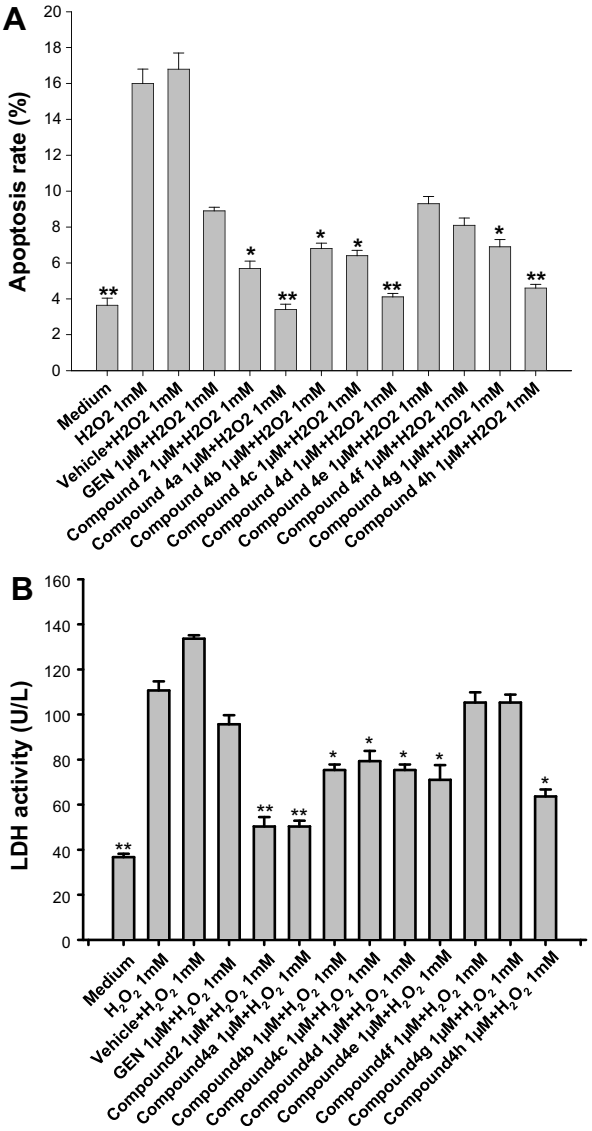

Figure 2. Protective effects of different genistein derivatives against vascular endothelial cell damages caused by hydrogen peroxide. (A) FCM has shown all genistein derivatives can decrease apoptosis rate of H_2O_2-induced HUVE-12 cells. Among them, 7-difluoromethylyl-5,4′-dimethoxygenistein was the most potent. Data shown here means from three independent experiments. * indicates $P < 0.05$ versus 1 mM H_2O_2, ** indicates $P < 0.01$ versus 1 mM H_2O_2; (B) HUVE-12 cells were seeded and treated as described in the materials and methods. The culture medium from each treatment was collected and the LDH activity was analyzed. Data shown here represent the average of three experiments. $^{*}P < 0.05$ versus 1 mM H_2O_2, $^{**}P < 0.01$ versus 1 mM H_2O_2.

Vascular endothelial cells have many physiological functions. They take part in blood clotting, immune response, metabolite transport, bioactive compound release, etc. Vascular endothelium damage is the pathological cause of many cardiovascular diseases. Under pathological conditions, such as atherosclerosis,[12] hypertension,[13] and myocardial infarction,[14] the structure and function of vascular endothelial cells are altered. Ross's 'damage reaction' theory[15] considers the vascular endothelial cell damage and cell function disorder as the initial pathological changes in atherosclerosis.

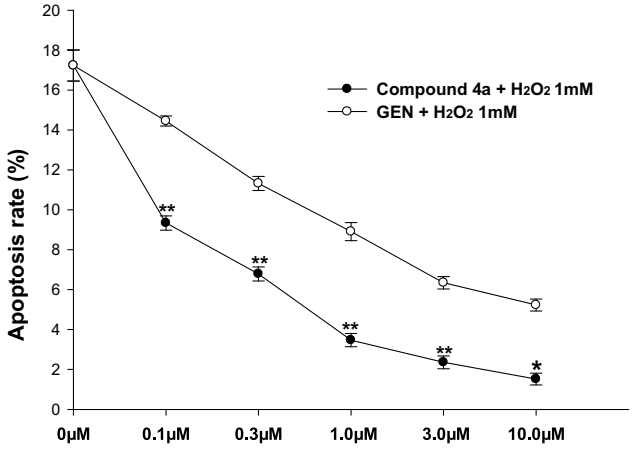

Figure 3. 7-Difluoromethylyl-5,4′-dimethoxygenistein suppressed H_2O_2-induced apoptosis of vascular endothelial cells in a concentration dependent manner. Data shown here means from three independent experiments. $^{*}P < 0.05$ versus genistein + 1 mM H_2O_2, $^{**}P < 0.01$ versus genistein + 1 mM H_2O_2.

LDH activity increase in the medium is a general marker for cell damage.[16] Results in our study suggested that the derivative of 7-difluoromethylyl-5,4′-dimethoxygenistein reduced the release of LDH H_2O_2-induced HUVE-12 cells. We also showed that this derivative suppressed H_2O_2-induced apoptosis of vascular endothelial cells in a concentration dependent manner (Fig. 3). All these data demonstrated that 7-difluoromethylyl-5,4′-dimethoxygenistein could provide protection against vascular endothelium injury caused by oxidative stress.

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