Differentiation Inducing Activities of Isocoumarins from Hydrangea Dulcis Folium

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In the course of searching for differentiation inducers against leukemic cells from plants, we have recognized the differentiation inducing activities of the methanolic extract of Hydrangea Dulcis Folium. Activity guided separation of the extract was carried out using M1 cells, and seven isocoumarins were isolated as active substances. These isocoumarins showed the activities at the concentration of 100 μM and non-cytotoxic effects even at 300 μM.

Key words differentiation; Hydrangea Dulcis Folium; M1 cell; phagocytosis

Differentiation inducers are of potential interest for the treatment of human cancers promoting the terminal differentiation of certain human tumor cells. We have reported the differentiation inducing activities of triterpenes, flavones, lignans, and steroids.[1)] These compounds differentiated mouse myeloid leukemia (M1) cells into phagocytic cells, and some of them also induced the differentiation of human acute promyelocytic leukemia (HL-60) cells. In the course of searching for differentiation inducers against leukemic cells, the methanolic extract of Hydrangea Dulcis Folium was recognized to have differentiation inducing activities. The active constituents of the extract were identified.

A suspension of the methanolic extract of Hydrangea Dulcis Folium in water was extracted with ethyl acetate. The ethyl acetate layer showing the differentiation inducing activity against M1 cells was subjected to repeated chromatography on silica-gel and high performance liquid chromatography (HPLC) to afford seven active components (**1** (3*R*-phyllodulcin),[2a)] **3** (thunberginol C),[2b,c)] **4** (thunberginol D),[2b,c)] **5** (thunberginol G),[2b,c)] **6** (thunberginol A),[2d)] **7** (thunberginol B)[2d)] and **8** (thunberginol F)[2d)]) with non active components (**2** (hydrangenol),[2a)] **9** (3*R*-phyllodulcin 3′-glucoside),[2e)] **10** (3*R*-phyllodulcin 8-glucoside)[2a)] and **11** ((−)-hydrangenol 4′-glucoside)[2a)]) (Chart 1). The absolute configurations at C-3 of phyllodulcin derivatives (**1**, **9**, **10**) and hydrangenol glucoside (**11**) were identified by comparing their circular dichroism (CD) spectra with previously reported values.[2a)] The other dihydroisocoumarins (**2**—**5**) were known to epimerize at C-3, and they were subjected to bioassay in racemates.[2c)]

Differentiation inducing activities of isocoumarins (**1**—**11**) were tested using mouse myeloid M1 cell line (Fig. 1). Dihydroisocoumarins (**1**—**5**) and isocoumarins (**6**—**8**) showed these activities at a concentration of greater than 200 μM against M1 cells. In the case of dihydroisocoumarins, their activities were altered by the number or attached position of hydroxyl groups. Compounds **4** and **5**, having an *o*-diphenol group, showed higher activities than **3** and **2**, respectively. Compounds **3** and **4**, having a 6-hydroxy group in their structures, induced a larger amount of M1 cells into phagocytic cells compared with **2** and **5**, respectively. Highly oxygenated isocoumarins seemed to have higher activities.

Dihydroisocoumarin glucosides (**9**—**11**) were all inactive in the induction of the differentiation of M1 cells (data not shown). They showed hardly any antiproliferative activities, and a more than 70% of growth ratio was found in 300 μM of these compound treated groups. Glucosides were good pro-inducers.

Isocoumarins (**6**—**8**) were recongnized to have higher activities than dihydroisocoumarins (**1**—**5**). Phagocytic activities were observed when M1 cells were treated with 100 μM of isocoumarins (**6**—**8**) and the activities were nearly equal to 300 μM treated groups of dihydroisocoumarins (**1**—**5**). Compounds **6** and **7** have three hydroxyl groups at the same positions as **5** and **3**, respectively. These two isocoumarins induced M1 cells into phagocytic cells at a high ratio. Immunomodulatory activities of thunberginol A (**6**) and related compounds on lymphocyte proliferation were reported,[3)] and isocoumarins (**6**—**8**) were shown to have more potent antiallergic activity than dihydoisocoumarins (**1**, **2**).[3)] The similarity of the structure requirement for the differentiation activity and the antiallergic activity is interesting.

As **1** showed higher activity than **5** although both have a 8,3′,4′-tri *O*-function, structure–activity relationships of isocoumarins (**1**, **3**, **6**) were investigated. Some isocoumarins were methylated, and their differentiation inducing activities against M1 cells were evaluated. Compound **1**, which has a 4′-methoxyl group in its structure, exhibited the activity at

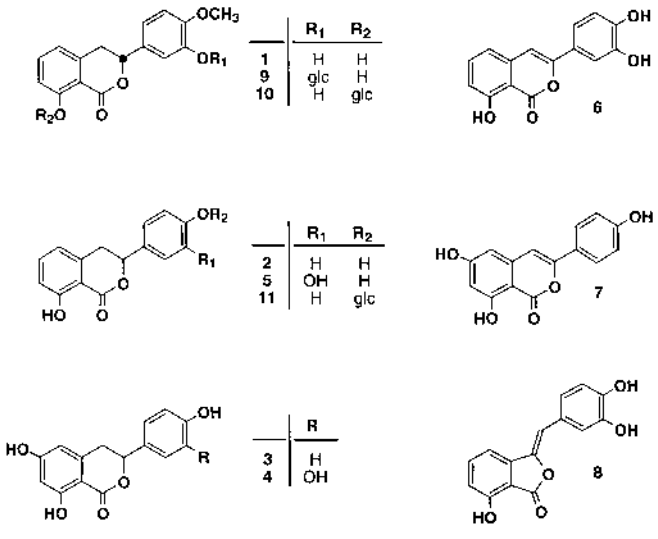

Chart 1

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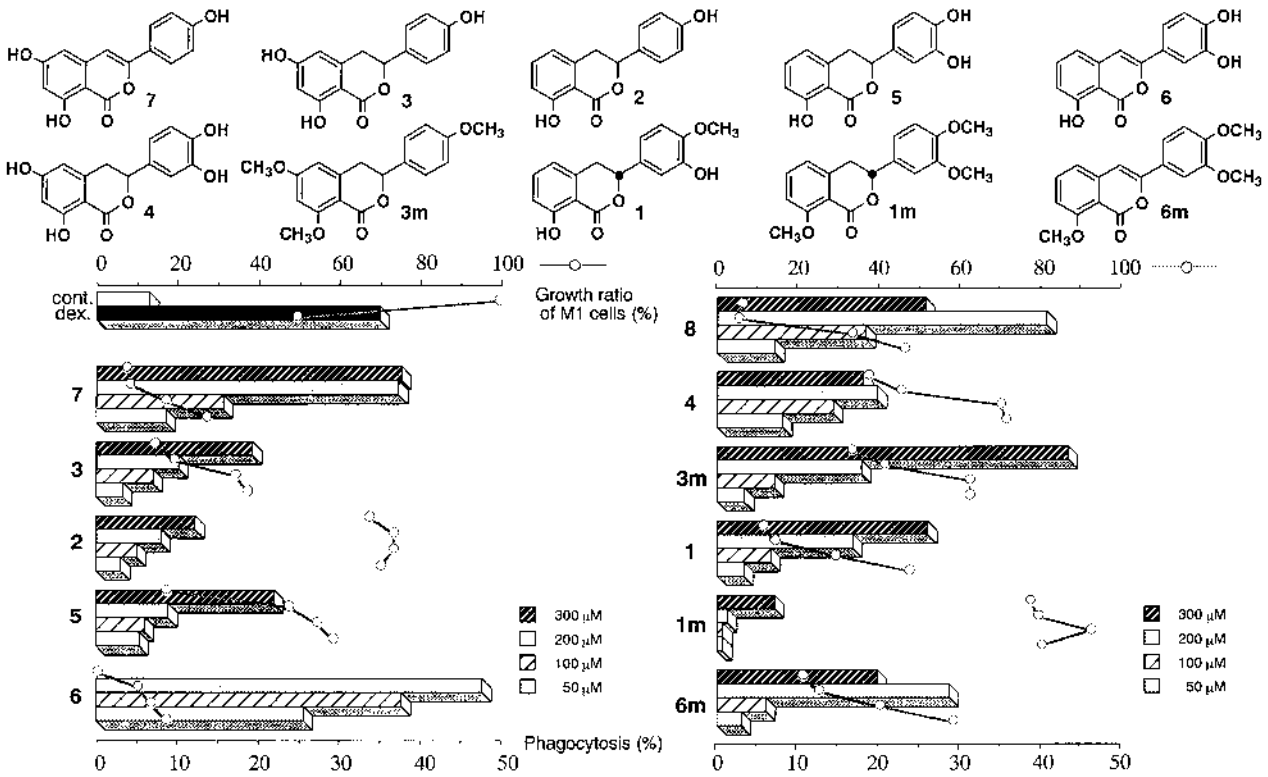

Fig. 1. Cell Growth and Phagocytic Activity f M1 Cells Treated with Isocoumarins

the concentration of 200 μM. But compounds **1m**, **3m** and **6m**, having trimethoxyl groups, were observed to have weak activities compared with their parental compounds. Compound **1m** had lost most of the activities, and the importance of the hydroxyl groups was recognized.

Phyllodulcin (**1**) has differentiation inducing activity against M1 cells and has less cytotoxicity. As it is one of the main components of Hydrangeae Dulcis Folium and present in a high proportion, this crude drug could be valuable as a differentiation inducer source.

Experimental

General Procedure Mass spectra (MS) were obtained using a JEOL JMS-SX 102 mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on JEOL JNM-GSX 270, JNM-GSX 500 and JNM-A 400 spectrometers (270.05 and 67.8 MHz, 500.00 and 125.65 MHz, 400.00 and 100.4 MHz, respectively), and chemical shifts were given in δ (ppm) with tetramethylsilane (TMS) as an internal standard. HPLC was carried out on a JASCO model 800 series instrument using D-ODS-7 YMC columns.

Isolation Commercially available Hydrangea Dulcis Folium (1 kg from Niiya in Shimizu, Shizuoka prefecture) was extracted with hot MeOH under reflux. The extract was concentrated under reduced pressure and then partitioned between ethyl acetate and water. The ethyl acetate layer was passed through a Mitsubishi Diaion CHP-20 column, and absorbed material was eluted with MeOH and ethyl acetate successively. From the MeOH eluate, **1** (3*R*-phyllodulcin, 21.5 g), **2** (hydrangenol, 8.4 g), **3** (thunberginol C, 70 mg), **4** (thunberginol D, 800 mg), **5** (thunberginol G, 30 mg), **6** (thunberginol A, 1.9 g), **7** (thunberginol B, 10 mg), **8** (thunberginol F, 50 mg), **9** (3*R*-phyllodulcin 3′-glucoside, 10 mg), **10** (3*R*-phyllodulcin 8-glucoside, 30 mg) and **11** ((−)-hydrangenol 4′-glucoside, 50 mg) were isolated by subsequent chromatography on silica gel and HPLC.

Methylation of 1 Compound **1** (10 mg) was methylated with diazomethane in the usual manner to give **1m** (10 mg). Methylation of **3** and **6** were performed in the same manner. Compounds **1m** and **6m** were identified by comparison of spectral data with reported values.[2d,e] **3m**: Amorphous powder. FAB-MS *m/z*: 315 $[MH]^+$. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.38 (1H, d, J=8 Hz), 6.92 (1H, d, J=8 Hz), 6.44 (1H, d, J=2 Hz), 6.34 (1H, d, J=2 Hz), 5.35 (1H, dd, J=12, 3 Hz), 3.25 (1H, dd, J=16, 12 Hz), 2.97 (1H, dd, J=16, 3 Hz), 3.94, 3.87, 3.82 (3H each, s).

Cell Culture M1 cells were grown in Eagle's mimimum essential medium (MEM) medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml kanamycin and 2 mmol/l L-glutamine in 10% heat-inactivated calf serum (CS) over the range of 1×10^5 /ml to 2×10^6 /ml in a 5% CO_2 humidified atmosphere at 37 °C.

Materials Eagle's MEM, Eagle's amino acids and vitamins medium were purchased from Nissui Pharmaceutical Co., Ltd. CS was from Gibco. Antibiotics were from Meiji Seika Kaisha, Ltd. L-Glutamine was from Wako Pure Chemical Industries, Ltd. Dexamethasone was from Nakalai Chemicals, Ltd. and polystyrene latex particles were from The Dow Chemical Company.

Measurement of Phagocytosis Phagocytic activity was assayed as reported previously.[1)]

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