Notes

Activities of Plant-Derived Phenols in a Fibroblast Cell Culture Model

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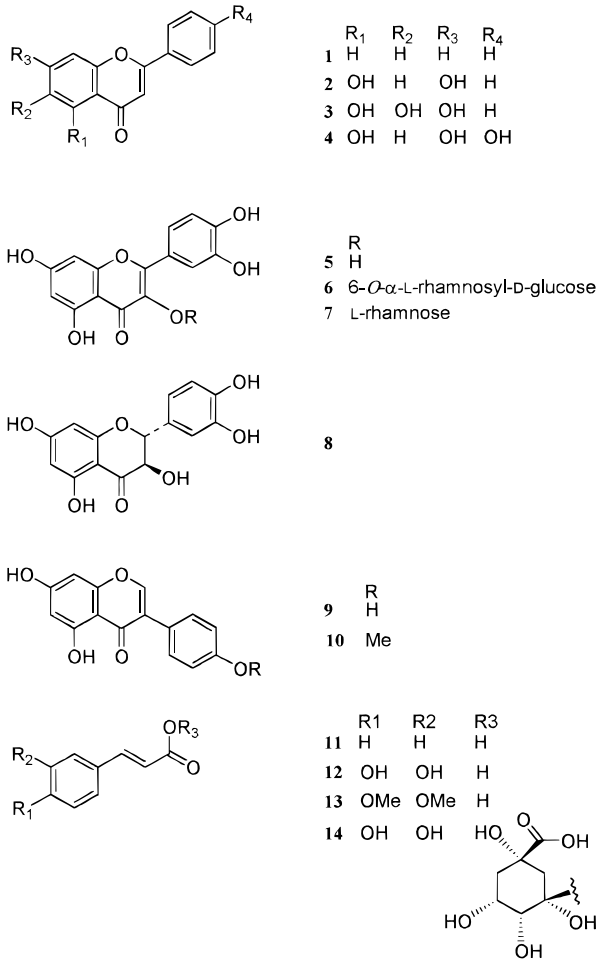
Received April 17, 1998

Fourteen plant phenols of five structural groups (flavones, flavonols, flavan-3-ol, isoflavones, and phenylpropanoids) demonstrated concentration-dependent inhibitory or modulatory effects in a fibroblast cell culture model. The most potent inhibitory activity in this investigation was exhibited by apigenin (**4**), with flavone (**1**), chrysin (**2**), and genistein (**9**) being of somewhat lesser potency. These findings help to provide a better understanding of the action of these plant phenols in inflammatory/immune responses.

Phenolic substances from plants have a wide spectrum of biological activities, including activities in various cell culture models (e.g., lymphocytes, human umbilical vein endothelial cells, and osteoclasts).[1−3] Fibroblasts have been used as a standard cell line for many cell biological studies. A member of the connective-tissue cell family, fibroblasts are responsible for the synthesis and metabolism of most connective-tissue components and also play an active role in the body's general inflammatory/immune responses. During inflammation, fibroblasts are key cells in granulation tissue and scar formation.[4−6] Furthermore, there are strong interactions between components of the immune system and fibroblasts. Specifically, certain cytokines (IL-1, IL-6, TNF) not only activate B-cells, T-cells, and NK-cells and facilitate the proliferation of plasma cells but also stimulate the proliferation of fibroblasts.[5−7] So, modulation of the immune response and inflammatory processes appears to overlap.[8]

In this paper, we describe the activities exhibited in a fibroblast cell culture model of 14 plant phenols representing flavones [flavone (**1**), chrysin (**2**), baicalein (**3**), and apigenin (**4**)], flavonols [quercetin (**5**), rutin (**6**), and quercitrin (**7**)], a flavan-3-ol [catechin (**8**)], isoflavones [genistein (**9**) and biochanin A (**10**)], and phenylpropanoids [*trans*-cinnamic acid (**11**), caffeic acid (**12**), 3,4-dimethoxycinnamic acid (**13**), and chlorogenic acid (**14**)]. We are not aware of any literature in which plant phenols have been screened previously for their effects on fibroblast cell cultures.

The inhibitory effects of compounds **1**−**14** on fibroblast growth and viability were concentration-dependent (Table 1). Only apigenin (**4**) showed significant inhibition of fibroblast growth at all tested concentrations (0.01−100 μg/mL) ($0.0001 < p < 0.05$). Flavone (**1**), chrysin (**2**), and genistein (**9**) were significantly cytotoxic in the concentration range 1−100 μg/mL ($0.0001 < p < 0.04$). Baicalein (**3**), quercetin (**5**), catechin (**8**), biochanin A (**10**), *trans*-cinnamic acid (**11**), caffeic acid (**12**), and 3,4-dimethoxycinnamic acid (**13**) demonstrated significant cell growth inhibition in the concentration range 10−100 μg/mL ($0.0001 < p < 0.05$), while chlorogenic acid (**14**) and quercitrin (**7**) significantly suppressed cell growth only at the highest concentration used (100 μg/mL) ($0.0002 < p < 0.01$). Rutin (**6**) did not inhibit significantly cell growth up to 100 μg/mL ($p < 0.13$).



The inhibition of fibroblast growth by plant phenols evaluated in this study correlated with a decrease in cell viability. Pearson's correlations between cell yields and viability were in the range 0.869−0.981 (Table 1). On the basis of Student's *t*-tests for evaluation of differences among

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10.1021/np9801559 CCC: $18.00
Published on Web 02/19/1999

Table 1. Inhibitory Effects of Compounds **1**−**14** in a Fibroblast Cell Culture[a]

compound	inhibitory effect
flavone (**1**)	287.1 ± 57.4[b] (0.961[c])
chrysin (**2**)	248.2 ± 42.2 (0.955)
baicalein (**3**)	342.3 ± 31.8 (0.968)
apigenin (**4**)	141.4 ± 28.3 (0.869)
quercetin (**5**)	217.0 ± 21.7 (0.969)
rutin (**6**)	>164.0 (0.912)
quercitrin (**7**)	>223.0 (0.988)
catechin (**8**)	>344.0 (0.961)
genistein (**9**)	183.9 ± 36.8 (0.962)
biochanin A (**10**)	219.5 ± 11.2 (0.981)
trans-cinnamic acid (**11**)	671.4 ± 60.4 (0.998)
caffeic acid (**12**)	553.8 ± 52.0 (0.988)
3,4-dimethoxycinnamic acid (**13**)	>480.3 (0.976)
chlorogenic acid (**14**)	>280.0 (0.927)

[a] Data are expressed as mean ± SE of eight experiments. [b] IC_{50} value (μM). [c] Correlation between fibroblast yield and viability.

the IC_{50} values, the order of decreasing inhibition potency of the plant phenols was found to be: **4** = **9** > **5** = **10** > **2** = **1** > **3** > **12** > **11**. It was not possible to determine the IC_{50} values for compounds **6**−**8**, **13**, and **14** because their effects did not exceed 50% of fibroblast growth inhibition.

Our results suggest that phenylpropanoids were less cytotoxic for the fibroblast cell culture than the flavones, isoflavones, and flavonols. Quercetin (**5**) was a strong fibroblast growth inhibitor, while its corresponding glycosides **6** and **7** did not possess this activity. Hydroxylation at position C-4' enhanced the inhibition activity of the flavone derivatives, while the presence of an unsubstituted hydroxyl group at position C-4' enhanced cytotoxicity of the isoflavone derivatives. Hydroxylation at C-3 and C-4 increased the inhibition activity of the phenylpropanoids; methoxylation at C-3 and C-4 decreased activity in this compound class.

Five compounds representing the flavonols (**5**−**7**), an isoflavone (**9**), and a phenylpropanoid (**14**), along with their inhibitory effects on fibroblast growth at high concentrations, also demonstrated stimulation of cell growth at lower concentrations. Quercetin (**5**), genistein (**9**), and chlorogenic acid (**14**) exhibited stimulatory effects (22.8−5.3%) at the concentration levels 0.01−0.1 μg/mL ($0.002 < p < 0.049$), with quercitrin (**7**) showing stimulation of cell growth (18.2−4.9%) ($0.01 < p < 0.05$) in the concentration range 0.01−1.0 μg/mL. Rutin (**6**) stimulated cell proliferation at the concentrations of 0.01−10 μg/mL ($0.0001 < p < 0.05$) and demonstrated maximum activity (30.4%) at a concentration of 0.1 μg/mL. The positive control (epidermal growth factor; EGF) showed 70% stimulation of fibroblast growth ($p < 0.0001$) at the same concentration (0.1 μg/mL). The flavonol glycosides displayed their stimulation properties in relatively wider concentration ranges than the corresponding aglycon. The effects of these compounds (**5**−**7**, **9**, and **14**) differ from the action of nonsteroid antiinflammatory drugs and glucocorticoids, which suppress fibroblast growth at all concentrations and do not show stimulatory effects.[5,6]

Not all results obtained from cultivated fibroblasts can be applied to cells in vivo. However, fibroblasts show many morphological and biochemical similarities in vitro and in vivo, suggesting that there are also functional analogies between them.[4] We have explored these analogies to make the following speculations. Of interest to humans is that the average diet in Europe and North America contains approximately 1 g/day of flavonoids[9] and only a few milligrams of isoflavones.[10] The diet in East Asia, on the other hand, is enriched with isoflavones, indicating a daily intake up to 100 mg.[10] On the basis of this information, we assume that in a normal human diet the maximum achievable concentration (on a body weight basis of 70 kg) is 14.3 μg/mL for flavonoids and 1.4 μg/mL for isoflavones. Our data showed that the IC_{50} values of flavonoids and isoflavones for the fibroblast model are in the range 38.2−99.8 μg/mL, values that are not likely to be reached in the human under normal circumstances. Therefore, this study suggests that normal intake of flavonoids and isoflavones will not affect fibroblast growth. However, with pharmacological intervention using a specific plant phenol a higher concentration could be reached[1] at which fibroblast proliferation may be suppressed.

The data collected in this study may be considered to be consistent with previous findings in the scientific literature. Rutin (**6**) was active in the treatment of inflammation or defective T-lymphocyte function,[11] chlorogenic acid (**14**) demonstrated the protection of gastric mucosa against irritation,[12] and catechin (**8**) was effective for the treatment of osteoarthritis.[13] In addition, it has been reported that certain plant phenols are suitable for the treatment of burns.[14] Inhibitory and modulatory effects of plant phenols on fibroblast growth might be beneficial for the treatment of skin injuries in general, because most of the pathology in wound healing is due to either insufficient or excessive fibroblast activities.[4] Our findings also may be important for a better understanding of the action of plant phenols in the inflammatory/immune responses.

Experimental Section

Test Compounds. Compounds **1**−**14** were obtained from Sigma Chemical Co. (St. Louis, MO).

Assay Materials. Low glucose Dulbecco's modified Eagle's medium (DMEM) with 4.0 mM l-glutamine and 110 mg/L of sodium pyruvate, Dulbecco's phosphate buffered saline (DPBS) without calcium or magnesium, calf serum, and gentamicin were obtained from GIBCO (Gaithersburg, MD). The trypsin-EDTA solution, amphotericin B, penicillin-streptomycin, epidermal growth factor (EGF), nigrosin, and poly(ethylene glycol) 400 (PEG 400) were obtained from Sigma. A hemocytometer was used for the counting of cells. The 25 mL flasks having a 0.2 μm vented cap and 96-well Microtest III tissue culture plates were obtained from Becton-Dickinson Labware (Franklin Lakes, NJ).

Fibroblast Cell Culture Assay. A cell culture of ATCC CCL-163, BALB/3T3 clone A31 fibroblasts (BALB/c, embryo, mouse) was cultivated according to a published procedure[15] in DMEM medium supplemented with 10% calf serum plus amphotericin B (2.5 μg/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were grown in the flasks in a CO_2 incubator supplied with 10% CO_2 and 90% humid air at 37 °C. Flasks were seeded with 3×10^5 cells/flask. Medium renewal was carried out two times a week until confluence was reached. Cells were subcultured once in 7−10 days using 0.25% trypsin−EDTA for the harvesting of cells. The protocols used in this investigation were in accordance with published procedures,[2,16] with minor modifications.

The plant phenols (**1**−**14**) used in this study were dissolved initially in PEG 400 in order to obtain 1% stock solutions (freshly prepared for each assay); each was then diluted with cell culture medium. The test compounds and a positive control (EGF) were tested in the 0.01−100 μg/mL concentration range. Cell growth in a medium containing PEG 400 at its maximum concentration of 1% was 100% as compared with cell growth in a control medium ($p < 0.92$). Cell suspensions (1×10^4 cells/well) in log phase were added to each well of 96-well plates. Cells were placed in 0.2 mL of the medium containing the test compounds for 48 h and incubated in a CO_2 incubator at 37 °C; the medium was then removed, the cells washed with DPBS, and the medium replaced. Cells were grown for an additional 24 h. Counts of cells and determinations of cell

viability were conducted by microscopic observation after a total of 72 h of cultivation. Cell yield was calculated using the mean amount of cells per well as a function of the amount in control, which was defined as 100%. Viability of cells was determined using a nigrosin assay according to Kaltenbach et al.[17] Viability was calculated as the percentage of unstained cells.

Statistical Analysis. The MINITAB Statistical Software[18] was used for analysis of results. Data were analyzed using Pearson's correlation and Student's *t*-test to evaluate significant differences. The IC_{50} values were calculated by a simple linear regression in the range of 20−80% inhibition of cell growth.

Acknowledgment. We are grateful to William R. Schenk and Dr. Daniel Kagan for their help in the preparation of this paper. We thank Ray Figueroa for valuable discussions and Patricia E. Eisenhardt for editorial assistance.

References and Notes

(1) Namgoong, S. Y.; Son, K. H.; Chang, H. W.; Kang, S. S.; Kim, H. P. *Life Sci.* **1994**, *54*, 313−320.
(2) Pieters, L.; de Bruyne, T.; Claeys, M.; Vlietinck, A. J. *J. Nat. Prod.* **1993**, *56*, 899−906.
(3) Barnes, S.; Blair, H. U.S. Patent No. 5,506,211, 1996; Apr 9.
(4) Gabbiani, G.; Rugger-Brandle, E. In *Handbook of Inflammation*; Glynn, L. E., Ed.; Elsevier/North-Holland Biomedical Press: Amsterdam, 1981; Vol. 3, Chapter 1, pp 1−50.
(5) Insel, P. A. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th ed.; Hardman, J. G., Limbird, L. E., Molinoff, P. B., Ruddon, R. W., Gilman, A. G., Eds.; McGraw-Hill: New York, 1995; Chapter 27, pp 617−657.
(6) Schimmer, B. P.; Parker, K. L. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th ed.; Hardman, J. G., Limbird, L. E., Molinoff, P. B., Ruddon, R. W., Gilman, A. G., Eds.; McGraw-Hill: New York, 1995; Chapter 59, pp 1459−1485.
(7) Diasio, R. B.; LoBuglio, A. F. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th ed.; Hardman, J. G., Limbird, L. E., Molinoff, P. B., Ruddon, R. W., Gilman, A. G., Eds.; McGraw-Hill: New York, 1995; Chapter 52, pp 1291−1308.
(8) Labadie, R. P. In *Bioactive Natural Products. Detection, Isolation and Structural Determination*; Colegate, S. M., Molyneux, R. J., Eds.; CRC Press: Boca Raton, FL, 1993; Chapter 14, pp 279−317.
(9) Middleton, E.; Kandaswami, C. *Biochem. Pharmacol.* **1992**, *43*, 1167−1179.
(10) Coward, L.; Barnes, N. C.; Setchell, K. D. R.; Barnes, S. *J. Agric. Food Chem.* **1993**, *41*, 1961−1967.
(11) Horrobin, D. F. U.S. Patent No. 4,888,326, 1989; Dec 19.
(12) Gosswein, C. U.S. Patent No. 4,865,847, 1989; Sep 12.
(13) Niebes, P. J.; Vincze, A. B.; Roba, J. L.; Lambelin, G. E.; Matagne, D. M.; Hanon, E. T.; Franz, M. R. U.S. Patent No. 4,268,517, 1981; May 19.
(14) Szijjarto (née Auber), E.; Raskai, M. U.S. Patent No. 4,466,961, 1984; Aug 21.
(15) Aaronson, S. A.; Todaro, G. J. *J. Cell. Physiol.* **1968**, *72*, 141−148.
(16) Yagi, A.; Akita, K.; Ueda, T.; Okamura, N.; Itoh, H. *Planta Med.* **1994**, *60*, 171−174.
(17) Kaltenbach, J. P.; Kaltenbach, M. H.; Lyons, W. B. *Exp. Cell Res.* **1958**, *15*, 112−117.
(18) MINITAB Statistical Software. Release 10 *Xtra*. Basic Statistics, Regression Analysis; MINITAB, Inc.: State College, 1995.

NP9801559