In Vitro Screening for Angiostatic Potential of Herbal Chemicals

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PURPOSE. Herbal medicine has long been used in traditional medicinal systems. The authors carried out a first-line screening of four herbal chemicals with reported antioxidative properties and capabilities to suppress endothelial cell growth and migration. These herbal chemicals were isoliquiritigenin (ISL) from licorice, epigallocatechin gallate (EGCG) from green tea, resveratrol (Rst) from grapes, and gambogic acid (GA) from the resin of Garcinia hanburyi.

METHODS. Cytotoxicity was studied by MTT cell viability/proliferation assay on human retinal pigment epithelial cells (ARPE19). Effects on vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation and migration were investigated by a scratch-wound model using human umbilical vein endothelial cells (HUVECs). The effects on VEGF signaling in HUVECs were analyzed by Western blotting.

RESULTS. At sub-cytotoxic levels, ISL (10 μM), EGCG (50 μM), and Rst (10 μM) suppressed HUVEC proliferation and migration under VEGF (20 ng/mL) stimulation in our scratch-wound model. HUVEC migration was reduced more by ISL and EGCG than bevacizumab, a humanized monoclonal antibody against VEGF. The efficiency of Rst was similar to that of bevacizumab. GA, however, was toxic to cells even at nanomolar concentrations. Western blot analysis showed that these chemicals affected focal adhesion kinase activation and expression of pigment epithelial growth factor.

CONCLUSIONS. ISL, EGCG, and Rst are highly effective and efficient in suppressing endothelial cell proliferation and migration, with low cytotoxicity on ARPE19 and HUVEC lines. They are potentially useful for further investigation to develop anti-angiogenic therapies by virtue of their small molecular sizes for easy penetration through tissue cells and their low effective dosages. (Invest Ophthalmol Vis Sci. 2010;51:6658–6664) DOI:10.1167/iovs.10-5524

Herbal drugs are known to play important roles in traditional and even modern medicine in different cultures. Herbal medicine, or herbalism or phytotherapy, refers to the medicinal use of different parts of herbs. Often a cocktail composed of assorted herbal substances mixed at different ratios and prepared by discrete protocols is tailor-made for individual patients. In China the use of herbs can be dated back to the third century BCE. Since then, more than 7000 species have been reported in the Chinese literature. Among them, 150 are commonly used nowadays as food supplements or for medicinal purpose. In the United States, according to the National Health Interview Survey 2002, approximately 13% of the elderly (65–69 years old) consumed herbal supplements. In a 2004 report, as many as 25% of US adults admitted using herbal compounds or extracts to treat medical illness. Popularity in the use of herbal materials is similarly popular with Europeans. It is even increasing in developing countries, where the traditional medicinal products constitute the major part of disease intervention. Chemical compositions of herbal medicine are complex. Usually extracts of multiple herbs are involved. Therefore, the exact mechanisms of actions and the rationale of preparatory procedures are often partially explained. In this study, we attempted to study the cytotoxicity and capability to suppress endothelial cell growth and migration of selected herbal chemicals with potent antiangiogenic therapeutic properties.

Angiogenesis is the process of formation of new blood vessels. During embryonic development, angiogenesis is regulated for development and differentiation of the embryonic vasculature. It is also essential for various tissue processes in organ development and wound healing. In pathologic angiogenesis, the fragility and poor organization of newly formed capillaries leading to leakage and fibrous proliferation cause the onset of tissue edema and hemorrhage. Physiological balance between vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) is crucial to maintain normal vasculature. Disruption of this balance may lead to angiogenic diseases. Various drugs, including angiostatins and endostatin, can be used to halt the angiogenic process, with variable efficacies and delivery efficiencies. The recent availability of the monoclonal humanized anti-VEGF antibodies pegaptanib, ranibizumab and bevacizumab has allowed greater control over deregulated angiogenesis, in particular tumor development and ocular neovascularization. However, especially in the case of ocular neovascularization, repeated intravitreal injection, risk for endophthalmitis, and high treatment cost are the major setbacks.

In this study, we carried out a systematic first-line examination to investigate the antiangiogenic potential of selected herbal chemicals with defined molecular structures, including isoliquiritigenin (ISL) from licorice, epigallocatechin gallate (EGCG) from green tea extract, resveratrol (Rst) from grape skin and seed, and gambogic acid (GA) from the resin of the Garcinia hanburyi tree (Fig. 1). By time-course analysis using a scratch-wound model, we found that subtoxic doses of ISL, EGCG, and Rst were potent in suppressing endothelial cell growth and migration under VEGF stimulation. GA was cytotoxic to cells even at low nanomolar ranges and should be monitored closely for safe use.

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MATERIALS AND METHODS

Chemicals
Isoliquiritigenin (ISL; (E)-1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one or 4,2',4'-tri-hydroxychalcone; \( \text{C}_{15}\text{H}_{12}\text{O}_4 \); CAS No.: 961-29-5) was purchased from Tauto Biotech (Shanghai, China). Gambogic acid (GA; \( \text{C}_{38}\text{H}_{44}\text{O}_8 \)); CAS No.: 2752-65-0) was from Gaia Chemicals (Gaylordsville, CT). Epigallocatechin gallate (EGCG; 2R,3R)-2-(3,4,5-trihydroxy-trans-stilbene or 5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol; \( \text{C}_{22}\text{H}_{18}\text{O}_{11} \); CAS No.: 989-51-5), resveratrol (Rst; 3,4',5-trihydroxy-benzoate; \( \text{C}_{22}\text{H}_{18}\text{O}_{11} \); CAS No.: 961-29-5), and 4-(2',4'-trihydroxychalcone; \( \text{C}_{15}\text{H}_{12}\text{O}_4 \); CAS No.: 961-29-5) was purchased from Tauto Biotech (Shanghai, China). (diluted to appropriate concentrations in culture media, and the final concentration was 50 mM, aliquotted, and stored at -20°C until use. They were then diluted to appropriate concentrations in culture media, and the final DMSO concentration was below 0.1%.

Cell Culture and Drug Preparation
The human retinal pigment epithelial cell line ARPE19 (CRL-2302; American Type Culture Collection [ATCC], Rockville, MD) was maintained in DMEM/F12 medium supplemented with 10% FBS and antibiotics. The human umbilical vein endothelial cell (HUVEC) line (CRL-173A; ATCC) was propagated in complete endothelial cell growth medium with 10% FBS and antibiotics on gelatin-coated surfaces. All herbal chemicals were dissolved in DMSO to a stock concentration of 50 mM, aliquotted, and stored at -20°C until use. They were then diluted to appropriate concentrations in culture media, and the final DMSO concentration was below 0.1%.

VEGF Signaling Analysis
After treatment for 24 hours, HUVECs were lysed in radioimmunoprecipitation assay buffer freshly added with phosphatase inhibitors (phoSTOP; Roche), protease inhibitor cocktail (Complete; Roche), and 1 mM phenylmethylsulfonyl fluoride, and soluble protein was subjected to Western blot analysis with rabbit polyclonal antibodies against human phospho-FAK (Y576/577) or mouse monoclonal antibody against phospho-FAK (Y576/577), total FAK, or mouse monoclonal antibody against human PEDF, respectively, followed by enhanced chemiluminescence. Band intensity was analyzed with imaging software (Quantity One Imaging; Bio-Rad, Hercules, CA).

RESULTS
Validation of HUVEC Growth and Migration Assay and Controls
The assays were performed with controls—bevacizumab (Avastin [Roche] 312 µg/mL) with or without VEGF (20 ng/mL) and PP2 (10 µM)—to block Src kinase activity in VEGF signaling cascade. Figure 2 showed a time-dependent increase in the number of HUVECs in the denuded area. At 24 hours, untreated HUVECs with 14.1 ± 5.7 cells/mm² were observed. In the presence of VEGF, more HUVECs migrated to the area (35 ± 9.3 cells/mm²). Treatment with bevacizumab (Avastin; Roche) reduced VEGF-stimulated migration, and the cell density was 18.4 ± 4.2 per mm². Application of PP2 in the presence of VEGF further reduced HUVEC migration to 15 ± 5 cells/mm². Our result validated the in vitro scratch-wound assay to HUVEC growth and migration.
Effect of ISL on HUVEC Migration

We tested the cytotoxicity of ISL in the range of 5 to 50 μM by MTT cell proliferation/viability assay. At lower dosages (5 and 10 μM), ISL promoted ARPE19 cell growth (untreated cells at day 5: 5 μM, 153%; 10 μM, 145%; Fig. 3A). At concentrations higher than 15 μM, ISL inhibited cell proliferation to levels lower than untreated control at all time points of assay. This indicated the cytotoxic threshold level of ISL was approximately 10 μM.

To test for the effects of ISL in VEGF-induced HUVEC growth and migration, three doses (5, 10, and 15 μM) were used. Compared with VEGF control, ISL showed a dose-dependent decrease of HUVEC migration (Fig. 3B). With reference to the untreated control at day 5, 10 and 20 μM ISL resulted in 18.2 ± 2.7 cells/mm² wound area, which was similar to control (18.4 ± 4.2 cells/mm²).

Effect of EGCG on HUVEC Migration

EGCG, in the range from 10 to 100 μM, exhibited a dose-dependent effect on ARPE19 cell proliferation (Fig. 3C). At dosages below 50 μM, EGCG promoted cell proliferation at all time points of assay (untreated cells at day 5: 10 μM, 163%; 50 μM, 121%; Fig. 3C). For 100 μM EGCG treatment, the rate was reduced to 17% of untreated control at day 5. Hence, the cytotoxic threshold level of EGCG was approximately 50 μM.

Three doses (10, 20, and 50 μM) of EGCG were selected for HUVEC migration experiments. Compared with VEGF control, EGCG showed a dose-dependent suppression of HUVEC migration (Fig. 3D). Among the three doses, 50 μM EGCG demonstrated the best result. At 24-hour treatment, cell density in the denuded area was 9.4 ± 2.7 cells/mm² for 50 μM EGCG, approximately half that of control (18.4 ± 4.2/mm²; Fig. 3D).

Cell migration density after 50 μM EGCG treatment was significantly lower than in VEGF control (35 ± 9.3 cells/mm²; P < 0.05, Student’s t-test). On the other hand, less inhibition was seen for 10 μM (30.9 ± 8.1 cells/mm²) and 20 μM EGCG (20 ± 3.2 cells/mm²).

Effect of Rst on HUVEC Migration

Rst at all the tested concentrations (2.5–50 μM) did not stimulate ARPE19 cell proliferation (Fig. 3E). At 5-day treatment with Rst below 20 μM, the rate did not vary much from that of untreated control (2.5 μM, lower by 2%; 10 μM, lower by 12%; 20 μM, lower by 17.7%). For 25 and 50 μM Rst treatments, the rates were reduced to 62% and 31.4% of untreated control, respectively. We selected 5 to 20 μM Rst for VEGF-induced HUVEC growth and migration assay. Compared with VEGF control, Rst at 10 and 15 μM reduced HUVEC migration (Fig. 3F), whereas 5 μM Rst had negligible effect. Treatment with 10 μM Rst resulted in 18.2 ± 6.5 cells/mm² wound area, which was similar to control (18.4 ± 4.2 cells/mm²).

GA Was Cytotoxic

GA at doses below 25 nM suppressed HUVEC proliferation (Fig. 3G). With reference to the untreated control at day 5, 10 nM GA reduced the cells by 16% and 25 nM GA reduced the cells by approximately 40%. GA from 50 to 200 nM inhibited cell survival; most cells died after 5 days (Fig. 3G). For HUVEC migration study, GA (10–100 nM) treatment greatly reduced the number of cells in the denuded area (10 nM GA, 8.7 ± 1.4 cells/mm²; 50 nM GA, 5.8 ± 2.9 cells/mm²; 100 nM GA, 9.2 ± 6.7 cells/mm²), possibly because of extensive cell death (Fig. 3H).

ISL, EGCG, and Rst Suppressed HUVEC Migration Independently of VEGF

We investigated whether the chemical effects of ISL (10 μM), EGCG (50 μM), and Rst (10 μM) were mediated through VEGF signaling. The scratch-wound HUVEC migration was conducted in the presence of herbal chemicals with or without VEGF (20 ng/mL). At all points of examination, there was no difference in the migration result caused by VEGF (Fig. 4), indicating that these chemicals could exert their effect to suppress HUVEC growth and migration in a VEGF-independent manner.

Protein Expression

Starved HUVECs were treated with bevacizumab (Avastin [Roche], 312 μg/mL) or the herbal chemicals (50 μM EGCG, 10 μM ISL, and 10 μM Rst) in the presence of 20 ng/mL VEGF for 24 hours. Western blot analysis of soluble cell lysates showed very low expression of FAK, phospho-FAK, and PEDF in starved cells before treatment (Fig. 5A). In cells incubated with VEGF, FAK was stimulated more than 10-fold compared with starved cells (Fig. 5B). The expression of phospho-FAK (Y576/577) was approximately 20% that of total FAK. This level was unchanged in bevacizumab (Avastin; Roche) or EGCG-treated cells. Treatment with ISL mildly increased total FAK expression as well as the proportion of phospho-FAK in total FAK (~50%). However, Rst substantially reduced FAK expression by approximately 30% compared with VEGF-only cells, and approximately 20% were phosphorylated.

PEDF expression was increased in cells treated with VEGF and bevacizumab (Avastin; Roche; ~2.0-fold when compared
with VEGF-only sample; Figs. 5A, 5B). PEDF was maintained at a slightly higher level (\(1.5\)-fold as VEGF control) for cells treated with EGCG or ISL but not for cells with Rst treatment (reduced to 32% as VEGF control).

**DISCUSSION**

Proliferation and migration of capillary endothelial cells are critical for blood vessel spreading and infiltration to tissues or organs and are regulated by proangiogenic and anti-angiogenic agents. Among them, VEGF plays an important role in stimulating endothelial cell proliferation and migration. Use of the recombinant humanized monoclonal antibody bevacizumab (Avastin; Roche) chelates VEGF-165 and prevents its binding to its cognate receptor in the endothelial cells to initiate cascades of growth and migration responses. In this study, we used a VEGF-induced HUVEC migration assay as an in vitro screening method for the selected herbal chemicals. The rate and extent of endothelial cell migration at one site could be monitored microscopically at various time points. This two-dimensional assay represents an in vitro aspect of wound healing such that the confluent endothelial monolayer is wounded by scraping, and the cells are stimulated and migrate to reform the monolayer. Hence, this model serves to investigate the multistep process of endothelial cell spreading, proliferation, and migration in one system. This is crucial for the study of chemotactic responses initiated by chemoattractants or chemorepellents. Other methods are also available in which to study the influence of angiogenesis regulatory molecules on endothelial cell migration. A commonly used one is the membrane-based Boyden chamber model, which primarily evaluates the linear migration of cells in response to chemoattractant molecules. In this study, we used the scratch wound assay because it could investigate the multistep process of endothelial cell spreading, proliferation, and migration, which are typical events in the wound-healing process. It could perform high throughput screening in 96- or 384-well plates. We could also perform time-lapse monitoring of the distance of cell migration and the rate of proliferation in the denuded area. The Boyden chamber assay, however, measures cells that are completely migrated to the detection chamber. A disadvantage of the scratch wound assay is that assessment of results must be made carefully. In our experiment, HUVECs were starved to induce a quiescent state, which is critical for the genuine response of proangiogenic VEGF because endothelial cells in vivo are usually quiescent. To avoid apoptosis, we treated the cells by

**Figure 3.** ARPE19 cell viability after treatment with (A) ISL, (C) EGCG, (E) Rst, and (G) GA. Changes of HUVEC proliferation and migration by a scratch-wound model after treatment with (B) ISL, (D) EGCG, (F) Rst, and (H) GA. (A, C, E, G) Control cell viability was set as 100% at all examined time points.
low-serum (0.2% FBS) for not more than 4 hours. Similar assays have been reported in a previous study.

Various herbal extracts, some of which have long been used in traditional medicine, are known for their antiangiogenic activities based on results of laboratory assays.\textsuperscript{25,26} In our experiment, we selected four herbal chemicals (ISL, EGCG, Rst, and GA) to study their effects on endothelial cell growth and migration. Among the criteria used for selecting these chemicals were that they must be from herbal sources, they must have defined molecular structures, they must have small molecular sizes, their parental plants must have been reported to affect blood vessel development, and they must have shown to affect tumorigenesis in cell or animal models. Our results showed that ISL, EGCG, and Rst, when applied at subtoxic doses, exhibited potent efficiency in suppressing HUVEC proliferation and migration. GA was found to be highly cytotoxic even when the application dose was as low as nanomolar in concentrations.

ISL (molecular formula, C\textsubscript{15}H\textsubscript{12}O\textsubscript{4}), isolated from licorice (\textit{Glycyrrhiza uralensis}), belongs to the family of favoid (Fig. 1). It acts against inflammation,\textsuperscript{27} oxidative stress,\textsuperscript{28} carcinogenesis,\textsuperscript{39,30} and platelet aggregation\textsuperscript{31} and as an inhibitor to aldose reductase.\textsuperscript{32} It has anticarcinogenic activity as mediated by cell cycle arrest, induction of apoptosis,\textsuperscript{33,34} and suppression of lipoygenase.\textsuperscript{35} Its angiostatic effect is the strongest among known chemical molecules extracted from licorice.\textsuperscript{36} It has been shown to block JNK- or p38/MAPK-responsive pathways to suppress matrix metalloproteinases and to increase the level of tissue inhibitor of matrix metalloproteinases.\textsuperscript{37,38} We hypothesized that the extracellular matrix stabilization could reduce endothelial cell motility. In this study, we consistently detected reduced HUVEC proliferation and migration by ISL at the subtoxic dose of 10 \textmu M. The effect was even stronger than that of bevacizumab within 24 hours of examination. Indeed, when comparing different selected herbal chemicals, ISL at 10 \textmu M demonstrated the best efficacy in reducing HUVEC migration. This effect was not mediated by suppressing VEGF binding to its cognate receptor in endothelial cells or VEGF signaling because similar inhibition was found for conditions without VEGF supplementation. Although FAK was activated by ISL treatment, the elevated PEDF could be associated with its inhibitory effect on HUVEC growth and migration.\textsuperscript{39}

We also observed that EGCG at subtoxic doses (20 and 50 \textmu M) suppressed HUVEC proliferation and migration. This effect (in particular, 50 \textmu M EGCG) was more robust than that of bevacizumab. Both bevacizumab and EGCG stimulated PEDF expression in HUVECs, indicating a possible link to antiangiogenesis. Hence, EGCG could be another herbal molecule that deserves a detailed investigation for anti-angiogenic action.

EGCG (molecular formula, C\textsubscript{22}H\textsubscript{18}O\textsubscript{11}) is one of the polyphenols in abundant quantity in green tea (leaves of \textit{Camellia sinensis}; Fig. 1). Green tea consumption has been shown to protect humans from the occurrence or progression of cancers of the skin, breast, prostate, lung, colon, liver, and stomach, among others.\textsuperscript{40} It has antioxidative, anti-inflammatory, anti-arteriosclerotic, and antibacterial activities.\textsuperscript{41,42} Its angiostatic potential was suggested by its suppression of blood vessel formation and development in a chick chorioallantoic membrane model.\textsuperscript{43} When green tea extract was given in drinking water, corneal neovascularization was significantly attenuated in mice,\textsuperscript{44} and oxygen-induced retinal neovascularization was suppressed in rats.\textsuperscript{45} Its biological action was hypothesized to mediate through a reduced complex formation among VEGF...
receptor 2, β-catenin, VE-cadherin, and phosphoinositol-3-kinase, which is critical for endothelial cell migration and vessel formation. Based on our protein analysis, it did not affect FAK but did elevate PEDF expression, which might link to its antiangiogenesis capability. Yet further characterization is needed for a better understanding of how this green tea molecule affects endothelial cells.

The effect of Rst on HUVEC proliferation and migration was subsidiary to that of ISL and EGCG. Rst at a dose of 10 μM exhibited an effect comparable to that of bevacizumab. However, Rst has much smaller molecular size than does bevacizumab (Rst, 228 Da; bevacizumab, ~150,000 Da), giving it an advantage of higher penetration efficiency in tissues, and a lower effective dosage is therefore possible.

Rst (molecular formula, C14H12O3) is a phytoalexin commonly found in various plants and plant foods, including grapes, peanuts, pine trees, and berries (Fig. 1). Based on botanical studies, it acts as a stress protein responsive to exposure to ozone, heavy metals, climate change, infection by botanical studies, it acts as a stress protein responsive to exposure to ozone, heavy metals, climate change, infection by

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