Rapid Communications

Ponicidin and Oridonin Are Responsible for the Antiangiogenic Activity of *Rabdosia rubescens*, a Constituent of the Herbal Supplement PC SPES

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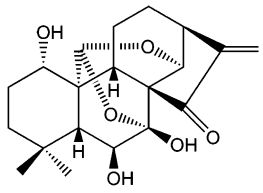
Abstract: Antiangiogenic activity has been identified in an aqueous EtOH extract of *Rabdosia rubescens*, a component of the dietary supplement PC SPES. Bioassay-guided fractionation using a novel in vitro human endothelial cell-based assay for angiogenesis afforded the diterpenoids ponicidin (**1**) and oridonin (**2**), with significant antiangiogenic activity at subcytotoxic concentrations, suggesting that these constituents may strongly contribute to the demonstrated clinical efficacy of PC SPES as a treatment for advanced prostate cancer.

PC SPES (PC for prostate cancer and SPES in Latin for "hope") is a dietary supplement consisting of extracts of one North American plant [*Serenoa repens* (Bartram) Small; saw palmetto] and seven Chinese herbs [*Dendranthema morifolium* (Ramat.) Kitam., *Ganoderma lucidum* Karst., *Glycyrrhiza glabra* L., *Isatis indigotica* Fort., *Panax pseudo-ginseng* Wall., *Rabdosia rubescens* (Hemsl.) Hara, and *Scutellaria baicalensis* Georgi], which has gained popularity as an alternative therapy for advanced prostate cancer (PC) due to its demonstrated clinical efficacy and improvement of quality of life for hormone-refractory PC patients.[1] A number of in vitro and animal studies have also confirmed the anti-PC activity of PC SPES,[2] and recent studies have suggested its potential in the treatment of colon[3] and pancreatic[4] cancers. We have demonstrated antiangiogenic activity for PC SPES, and this activity was subsequently shown to be due to one of its constituent plants, *Rabdosia rubescens* (Hemsl.) Hara (Lamiaceae). This finding is significant because PC, like all malignancies, depends on angiogenesis for growth and metastasis, and primary prostate tumors are often highly vascularized. A variety of in vitro and in vivo bioassays are available for evaluation of antiangiogenic activity, but the majority of these utilize techniques requiring special skills and/or bovine, murine, or human umbilical vein endothelial cells (HUVEC).[5] A recent review by Auerbach et al.[6] discusses the problems involved in interpreting results obtained in such in vitro antiangiogenesis assays that use primary nonhuman and large vessel cells. To address these problems, we have developed an antiangiogenic assay incorporating human dermal microvascular endothelial cells (HMEC-1) based upon the well-documented ability of human endothelial cells to migrate and form networks of capillary-like tubes when cultured in vitro on Matrigel,[7] a commercially available reconstituted basement membrane. Although several of the individual components of this in vitro model and the assay we have developed from it may be found in other reports,[8] we have been able to combine and modify these components to enhance the speed, flexibility, and economy of the resulting assay, making it suitable for drug discovery applications. In particular, the human microvascular cells used in this assay have been transfected with SV40 large T-antigen, resulting in an extended life span, and cloned, thus minimizing inter-assay variability. Herein we describe the application of this novel moderate-throughput assay for natural product-based drug discovery by demonstrating its utility in bioassay-guided fractionation of *R. rubescens* extract, resulting in the isolation and identification of two of its antiangiogenic constituents, ponicidin (**1**) and oridonin (**2**).



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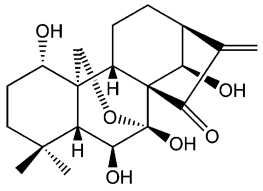

2

A cell-based assay developed from a novel in vitro model of the early stages of angiogenesis in human cells has been used to screen crude extracts and pure compounds for their ability to inhibit formation of capillary-like networks. This in vitro assay has several features that make it particularly suitable for identification of therapeutically relevant inhibitors of angiogenesis and defining their mechanisms of action. It makes use of human microvascular endothelial cells rather than bovine, murine, or human terminally differentiated large vessel cells (HUVEC). These cells are similar to those that are responsible for tumor angiogenesis in vivo and are assayed while in contact with a physiologically relevant matrix. The culture medium is defined except for its supplementation with a highly purified serum, minimizing variability between assays. The custom-fabricated Petri dishes allow direct observation of the living cells during migration and formation of anastomoses and capillary-like structures. The two-dimensional structures formed are easily visible, and results can be obtained within 18 h. Since it is a cell-based assay, multiple potential

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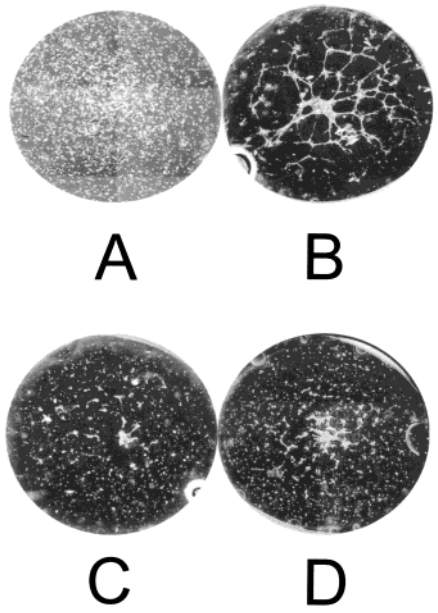

Figure 1. Results of antiangiogenesis assay showing inhibition of network formation by ponicidin (**1**) and oridonin (**2**). (A) Attached HMEC-1 cells prior to migration; (B) cells after 18 h in the presence of DMSO control; (C) cells after 18 h with 1.5 μg/mL of **1**; and (D) cells after 18 h with 3.0 μg/mL of **2**.

molecular targets exist, enabling detection of either enhancement or inhibition by modulators with diverse mechanisms of action. The protocol can be scaled up or down to provide variable throughput, a distinct advantage in a natural product-based drug discovery program.

A 70% aqueous EtOH extract of PC SPES was found to be active in the above antiangiogenesis assay at a concentration of 250 μg/mL. Evaluation of 70% aqueous EtOH extracts derived from all seven constituent herbs (see above) of PC SPES suggested that the extract of *R. rubescens* strongly contributed to the observed antiangiogenic activity, inhibiting network formation of HMEC-1 at 45 μg/mL. Fractionation of this extract as described in the Experimental Section resulted in the isolation of ponicidin (**1**) and oridonin (**2**) with significant antiangiogenic activities at 1.5 and 2.5 μg/mL, respectively. Results of a typical antiangiogenesis assay are shown in Figure 1. To determine whether the observed activities were a consequence of cytotoxicity or of a more specific effect on cell migration and network formation, the cytotoxicities of ponicidin (**1**) and oridonin (**2**) on HMEC-1 cells were determined by the resazurin assay,[9] and both compounds were shown to have an IC_{50} greater than 4 μg/mL (data not shown). Since the observed inhibition of network formation occurs at subcytotoxic concentrations of both these compounds, it can be concluded that the observed effect is not due solely to their cytotoxicity to HMEC-1 cells. The antiangiogenic activity of ponicidin was found to be greater than that of oridonin at similar concentrations. Although anticancer activity for ponicidin (**1**) has been described,[10] there are no previous reports on antiangiogenic activity for this compound or oridonin (**2**). Studies are currently in progress to define the mechanism(s) underlying the antiangiogenic activity of this class of compounds.

Experimental Section

Cells and Culture Conditions. Experiments were performed with the SV-40 large T antigen-immortalized human dermal microvascular endothelial cell line, HMEC-1, which was developed by the Biological Products Branch at the Centers for Disease Control and Prevention (Atlanta, GA) in collaboration with the Department of Dermatology at Emory University (Atlanta, GA).[11] Cells were cultured in complete medium: MCDB 131 supplemented with 10 mM Glutamax-1 (Gibco BRL-Life Technologies, Grand Island, NY), 10% defined fetal bovine serum (Hyclone Laboratories, Logan, UT), 10 ng/mL human recombinant epidermal growth factor (BD Biosciences, Bedford, MA), and 1 μg/mL hydrocortisone (Sigma, St. Louis, MO). Cells were maintained in a humidified water-jacketed incubator at 5% CO_2 and 37 °C. Cells used were at passages between 18 and 23.

Assay Dish and Matrix Preparation. Holes (4.4 mm diameter) were cut in the bottom half of 35 mm × 10 mm polystyrene Petri dishes (Corning Inc., Corning, NY). Glass coverslips were glued to the bottom of the dish, creating a well. The dishes were cured overnight, rinsed twice with distilled water, air-dried, and sterilized by UV irradiation. Chilled growth factor reduced, phenol red-free Matrigel (BD Biosciences, Bedford, MA) was diluted to a concentration of 7.5 mg protein/mL in Dulbecco's cation-free phosphate-buffered saline (DCF-PBS). Matrigel (14 μL) was aliquoted into each assay dish well, and the dishes were incubated at 37 °C for 1 h.

In Vitro Assay. Cell monolayers were detached with trypsin-EDTA, neutralized with complete medium, counted, and pelleted. After resuspension in serum-free medium, 13 000 cells/dish were seeded onto gelled Matrigel and the assay dishes were incubated at 37 °C until attachment. After attachment, 0.6 mL of complete medium containing twice the concentration of the drug was added. The cells were incubated for 18 h and then evaluated for the extent of network formation.[12] The conditioned medium was removed and saved for further analysis, and the networks were fixed in situ with 2.5% glutaraldehyde at room temperature for 3 h, followed by mounting and microscopic evaluation. Phase contrast images at 4× magnification were captured with an Olympus IMT-2 inverted microscope equipped with a Hammamatsu Orca100 CCD camera and a Ludl XY motorized stage connected to a Pentium class PC running Windows NT. Montage composites were generated with simple PCI (C Imaging Incorporated, Pittsburgh, PA) software.

Extraction and Isolation. *Rabdosia rubescens* was collected from Ji Yuan City in Henan Province, People's Republic of China, in October 2002, and was authenticated by Prof. Song Xuehua, Director of the Medicinal Plant Herbarium of the China Pharmaceutical University (CPU). A voucher specimen (UA-RR-01) is on deposit at the CPU Medicinal Plant Herbarium. The dried and powdered whole plant (10 kg) was extracted (×3) with 70% aqueous EtOH (EtOH−water, 70:30, 30 L) and evaporated to yield a dark green semisolid (1.33 kg, 13.3%), which was found to be active in our antiangiogenesis bioassay. Bioactivity-guided fractionation of this extract (40.0 g) by solvent−solvent partition (involving hexane−80% aqueous MeOH and $CHCl_3$−60% aqueous MeOH)[13] afforded the bioactive $CHCl_3$ fraction (5.50 g), a portion (5.0 g) of which was subjected to Sephadex LH-20 gel permeation chromatography[14] and repeated silica gel column chromatography to afford **1** (4.9 mg) and **2** (5.7 mg) as off-white solids with spectral data (NMR and MS) identical with those reported for ponicidin (rubescensine B)[15] and oridonin (rubescensine A),[16] respectively. Comparison with authentic samples further confirmed their identities.

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