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Resveratrol Oligomers Isolated from *Carex* Species Inhibit Growth of Human Colon Tumorigenic Cells Mediated by Cell Cycle Arrest

Antonio González-Sarrías,[†,||] Samantha Gromek,[§,||] Daniel Niesen,[§] Navindra P. Seeram,[*,†] and Geneive E. Henry[*,§]

[†]Bioactive Botanical Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States

[§]Department of Chemistry, Susquehanna University, Selinsgrove, Pennsylvania 17870, United States

ABSTRACT: Research has shown that members of the *Carex* genus produce biologically active stilbenoids including resveratrol oligomers. This is of great interest to the nutraceutical industry given that resveratrol, a constituent of grape and red wine, has attracted immense research attention due to its potential human health benefits. In the current study, five resveratrol oligomers (isolated from *Carex folliculata* and *Carex gynandra*), along with resveratrol, were evaluated for antiproliferative effects against human colon cancer (HCT-116, HT-29, Caco-2) and normal human colon (CCD-18Co) cells. The resveratrol oligomers included one dimer, two trimers, and two tetramers: pallidol (**1**); α-viniferin (**2**) and *trans*-miyabenol C (**3**); and kobophenols A (**4**) and B (**5**), respectively. Although not cytotoxic, the resveratrol oligomers (**1**−**5**), as well as resveratrol, inhibited growth of the human colon cancer cells. Among the six stilbenoids, α-viniferin (**2**) was most active against the colon cancer cells with IC_{50} values of 6−32 μM (>2-fold compared to normal colon cells). Moreover, α-viniferin (at 20 μM) did not induce apoptosis but arrested cell cycle (in the S-phase) for the colon cancer but not the normal colon cells. This study adds to the growing body of knowledge supporting the anticancer effects of resveratrol and its oligomers. Furthermore, *Carex* species should be investigated for their nutraceutical potential given that they produce biologically active stilbenoids such as α-viniferin.

KEYWORDS: *Carex follicullata*, *Carex gynandra*, stilbenes, resveratrol, α-viniferin, antiproliferative, cell cycle, colon cancer

INTRODUCTION

The *Carex* genus, which consists of grasses and sedges, is the largest genus in the Cyperaceae family and contains over 2000 species distributed worldwide. Plants of this genus are prevalent in the United States, with over 150 species growing in the state of Pennsylvania. Although these plants are abundant, only a few species have been studied, revealing the presence of resveratrol oligomers and other stilbene derivatives.[1−5] Thus, *Carex* spp. have attracted attention for their nutraceutical potential given that they produce bioactive plant compounds and are currently underutilized.[1]

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is found in grapes, red wine, peanuts, and some berries and has a wide range of biological effects including antioxidant,[6] antimicrobial,[7] anti-inflammatory,[8,9] and neuroprotective[10,11] properties. Resveratrol also suppresses the growth of a wide variety of tumor cells including breast, prostate, hepatic, skin, lung, colon, and pancreas cells.[12−17] Indeed, it has been suggested that resveratrol suppresses the growth of various cancer cell lines, partly by inhibition of DNA polymerase and ribonucleotide reductase and partly by inducing cell cycle arrest or apoptosis initiating pathways such as the caspase-8-dependent or caspase-9-dependent pathway.[16,18]

Like resveratrol, its oligomers containing two to eight resveratrol units exhibit a wide array of biological properties. The range of biological activities exhibited by these oligomers includes inhibition of matrix metalloproteinase-1 production,[19] antioxidant,[1,20] anti-inflammatory,[21,22] and anticancer[23−25] effects. Recent studies have focused on the mechanisms of action, such as apoptosis induction and/or arrest of cell cycle, related to the anticancer activities of resveratrol oligomers.[23,24]

Colon cancer is the third most common cancer in the world, with prevalence mainly in Western countries, and its incidence continues to rise every year.[26] Many chemopreventive agents act through antiproliferation, cell cycle arrest, and induction of apoptosis, which leads to inhibition of the carcinogenesis process. Extensive research has focused on the anticancer properties of polyphenols, reported in many in vitro and animal studies.[27]

In the current study, we report the isolation of five resveratrol oligomers (**1**−**5**) including one dimer, two trimers, and two tetramers from *Carex folliculata* and *Carex gynandra* and the evaluation of their antiproliferative activity against three human colon cancer cell lines (HT-29, HCT-116, and Caco-2) and one normal colon cell line (CCD-18Co), compared with resveratrol. Also, this is the first reported phytochemical investigation of *C. gynandra* and the evaluation of its secondary metabolites against this panel of human colon cells.

MATERIALS AND METHODS

General Experimental Procedures. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL ECP 400 MHz spectrometer or Bruker 300 MHz Biospin spectrometer using acetone-d_6 and

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methanol-d_4 as solvents and TMS as internal standard. Gradient HMQC and HMBC data were obtained using standard pulse programs. Mass spectral (MS) data were acquired on a Q-Star Elite (Applied Biosystems MDS) mass spectrometer equipped with a Turbo Ionspray source and were obtained by direct infusion of pure compounds. High-performance liquid chromatography (HPLC) was performed using a Waters Delta 600 system equipped with a Waters 2487 dual wavelength absorbance detector and a Shimadzu LC-10AS pump with a photodiode array (PDA) UV−vis detector. Column chromatography (CC) was performed using Fisher Scientific silica gel (230−400 mesh), and analytical TLC was performed using Sigma-Aldrich polyester backed plates precoated with silica gel UV_{254}. All solvents were of HPLC grade and were obtained from Fisher Scientific and Aldrich Chemical Co. The MTS salt [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-2*H*-tetrazolium salt] and etoposide standard were obtained from Sigma-Aldrich.

Plant Material. The aerial parts of *C. gynandra* Schwein were collected in Clearfield County, Pennsylvania, USA, in August 2009. The seeds of *C. folliculata* were collected in Westmoreland County, Pennsylvania, in September 2007. The plant materials were authenticated by Joseph Isaac of Civil and Environmental Consultants, Pittsburgh, PA, and voucher specimens (*C. gynandra* 20716; *C. folliculata* 19443) have been deposited at the Carnegie Museum Herbarium in Pittsburgh, PA.

Extraction, Isolation, and Structural Elucidation. Dried and ground *Carex gynandra* leaves (790.8 g) were extracted sequentially at room temperature with hexanes (3 × 2.0 L), acetone (3 × 2.0 L), and methanol (3 × 2.0 L). The combined extracts for each solvent were concentrated in vacuo to afford hexanes (8.25 g), acetone (21.6 g), and methanol (75.8 g) extracts, respectively. The acetone extract (20.7 g) was fractioned by silica gel CC, with an acetone/hexanes solvent gradient (1:9 to 1:0, v/v) to afford eight fractions, A1−A8. Silica gel CC of fraction A6 (5.34 g), eluting with ethyl acetate/hexanes (3:7 to 1:0, v/v) yielded nine fractions, B1−B9. Fraction B2 (1.72 g) was subjected to CC to afford six fractions, C1−C6, which were eluted with a solvent gradient of methanol/chloroform (1:9 to 1:3, v/v). Fraction C2 was obtained as a pure compound, **2** (198 mg). Fractions B3, B4, and C3 were subjected to reverse-phase HPLC purification using a Waters Atlantis dC18 column (19 × 150 mm, 5 μm, flow rate = 10 mL/min). Fraction C3 (117 mg) was purified using methanol/water (38:62, v/v) to afford compound **3** (31 mg). The retention time (RT) for compound **3** was 59 min. Purification of fraction B3 (141 mg), eluted with methanol/water (35:65, v/v), afforded compound **1** (5 mg), with a RT of 62 min. Purification of fraction B4 (425 mg) using methanol/water (4:6, v/v) afforded compound **5** (20 mg), with a RT of 82 min. Compound **4** was isolated from the seeds of *C. folliculata*, together with compound **1**, as described previously.[1] The NMR and mass spectral data for compounds **1**−**5** are consistent with those reported in the literature for pallidol (**1**),[1] α-viniferin (**2**),[22] *trans*-miyabenol C (**3**),[28] kobophenol A (**4**),[1,5] and kobophenol B (**5**).[4] The NMR data for compounds **1**−**5** are shown below.

*Pallidol (**1**):* ^{1}H NMR (CD_3OD, 400 MHz), δ 6.91 (4H, d, J = 8.8 Hz, H-2a,2b,6a,6b), 6.65 (4H, d, J = 8.8 Hz, H-3a,3b,5a,5b), 6.52 (2H, d, J = 2.2 Hz, H-14a,14b), 6.10 (2H, d, J = 2.2 Hz, H-12a,12b), 4.46 (2H, s, H-7a,7b), 3.72 (2H, s, H-8a,8b); ^{13}C NMR (CD_3OD, 100 MHz), δ 158.0 (C-13a,13b), 155.0 (C-4a,4b), 154.2 (C-11a,11b), 149.5 (C-9a,9b), 137.1 (C-1a,1b), 127.9 (C-2a,2b,6a,6b), 122.5 (C-10a,10b), 114.7 (C-3a,3b,5a,5b), 102.1 (C-14a,14b), 101.2 (C-12a,12b), 59.6 (C-8a,8b), 53.4 (C-7a,7b).

*α-Viniferin (**2**):* ^{1}H NMR [$(CD_3)_2CO$, 400 MHz], δ 7.22 (2H, d, J = 8.4 Hz, H-2a,6a), 7.06 (2H, d, J = 8.4 Hz, H-2b,6b), 7.03 (2H, d, J = 8.4 Hz, H-2c,6c), 6.79 (2H, d, J = 8.4 Hz, H-3b,5b), 6.77 (2H, d, J = 8.4 Hz, H-3a,5a), 6.73 (1H, d, J = 2.2 Hz, H-14c), 6.72 (2H, d, J = 8.4 Hz, H-3c,5c), 6.60 (1H, d, J = 1.8 Hz, H-14a), 6.26 (1H, d, J = 1.8 Hz, H-12a), 6.24 (1H, d, J = 2.2 Hz, H-12c), 6.22 (1H, d, J = 2.2 Hz, H-12b), 6.08 (1H, br s, H-7c), 6.00 (1H, d, J = 2.2 Hz, H-14b), 5.95 (1H, d, J = 9.9 Hz, H-7a), 4.91 (1H, d, J = 6.2 Hz, H-7b), 4.70 (1H, d, J = 9.9 Hz, H-8a), 4.62 (1H, d, J = 6.2 Hz, H-8b), 3.97 (1H, br s, H-8c); ^{13}C NMR [$(CD_3)_2CO$, 100 MHz], δ 161.0 (C-11a), 160.8 (C-11b), 160.2 (C-11c), 159.9 (C-13a), 158.7 (C-13c), 158.6 (C-13b), 157.7 (C-4b), 157.5 (C-4a), 157.1 (C-4c), 140.5 (C-9b), 139.0 (C-9c), 137.9 (C-9a), 131.8 (C-1b), 131.5 (C-1a), 131.2 (C-1c), 127.9 (C-2b,6b), 127.5 (C-2a,6a), 127.4 (C-2c,6c), 120.1 (C-10c), 118.9 (C-10a), 118.1 (C-10b), 115.3 (C-3a,3b,5a,5b), 115.0 (C-3c,5c), 107.8 (C-14b), 105.5 (C-14c), 105.0 (C-14a), 97.3 (C-12b), 96.2 (C-12a), 95.8 (C-12c), 94.9 (C-7b), 89.3 (C-7a), 85.6 (C-7c), 54.9 (C-8b), 52.1 (C-8a), 45.6 (C-8c).

*trans-Miyabenol C (**3**):* ^{1}H NMR [$(CD_3)_2CO$, 400 MHz], δ 7.14 (2H, d, J = 8.8 Hz, H-2a,6a), 7.09 (2H, d, J = 8.8 Hz, H-2c,6c), 6.87 (1H, d, J = 17.6 Hz, H-7c), 6.81 (2H, d, J = 8.8 Hz, H-3a,5a), 6.74 (2H, d, J = 8.8 Hz, H-3c,5c), 6.64 (1H, s, H-14c), 6.58 (1H, d, J = 17.6 Hz, H-8c), 6.54 (2H, d, J = 8.8 Hz, H-3b,5b), 6.48 (2H, d, J = 8.8 Hz, H-2b,6b), 6.33 (1H, s, H-12c), 6.27 (1H, s, H-12b), 6.20 (1H, s, H-12a), 6.15 (2H, s, H-10a,14a), 6.06 (1H, s, H-14b), 5.38 (1H, d, J = 5.9 Hz, H-7a), 5.18 (1H, s, H-7b), 4.62 (1H, s, H-8a), 4.29 (1H, s, H-8b); ^{13}C NMR [$(CCD_3)_2CO$, 100 MHz], δ 161.6 (C-11b), 161.3 (C-11c), 159.5 (C-11a,13a), 159.4 (C-13b), 158.8 (C-13c), 157.8 (C-4c), 157.6 (C-4a), 156.8 (C-4b), 146.9 (C-9a), 142.6 (C-9b), 135.2 (C-9c), 132.6 (C-1a), 132.5 (C-1b), 130.4 (C-7c), 128.4 (C-1c), 127.9 (C-2c,6c), 126.9 (C-2a,6a), 126.6 (C-2b,6b), 121.9 (C-8c), 120.6 (C-10c), 117.8 (C-10b), 115.9 (C-3c,5c), 115.7 (C-3a,5a), 114.8 (C-3b,5b), 106.7 (C-14b), 106.1 (C-10a,14a), 103.5 (C-14c), 101.7 (C-12a), 96.3 (C-12c), 95.5 (C-12b), 93.8 (C-7a), 91.4 (C-7b), 56.4 (C-8a), 50.4 (C-8b).

*Kobophenol A (**4**):* ^{1}H NMR (CD_3OD, 300 MHz), δ 7.29 (2H, d, J = 8.7 Hz, H-2a,6a), 7.02 (2H, d, J = 8.7 Hz, H-2d,6d), 6.82 (2H, d, J = 8.7 Hz, H-3a,5a), 6.72 (2H, d, J = 8.7 Hz, H-3d,5d), 6.58 (2H, d, J = 8.7 Hz, H-3c,5c), 6.49 (1H, d, J = 2.1 Hz, H-12b), 6.43 (2H, d, J = 8.7 Hz, H-2c,6c), 6.42 (2H, d, J = 8.7 Hz, H-3b,5b), 6.37 (1H, d, J = 2.1 Hz, H-14c), 6.15 (2H, d, J = 8.7 Hz, H-2b,6b), 6.04 (1H, d, J = 2.1 Hz, H-12c), 5.95 (1H, d, J = 2.1 Hz, H-14b), 5.93 (1H, t, J = 2.1 Hz, H-12d), 5.92 (2H, br s, H-10a,14a), 5.90 (1H, s, H-12a), 5.66 (2H, d, J = 2.1 Hz, H-10d,14d), 5.47 (1H, s, H-7a), 5.10 (1H, d, J = 10.5 Hz, H-7d), 5.01 (1H, d, J = 5.1 Hz, H-7c), 5.00 (1H, d, J = 3.9 Hz, H-7b), 4.22 (1H, s, H-8a), 3.38 (1H, d, J = 3.9 Hz, H-8b), 3.22 (1H, t, J = 5.1 Hz, H-8c), 2.90 (1H, dd, J = 10.5, 6.0 Hz, H-8d); ^{13}C NMR (CD_3OD, 75 MHz), δ 162.3 (C-11b), 161.2 (C-13b), 161.1 (C-11c), 159.6 (C-11a,13a), 158.6 (C-13c), 158.3 (C-4a,11d,13d), 157.9 (C-4d), 157.2 (C-4b), 156.1 (C-4c), 147.8 (C-9a), 144.8 (C-9b), 139.5 (C-1d), 136.6 (C-9d), 134.1 (C-9c), 134.0 (C-1b), 133.6 (C-1a), 131.9 (C-1c), 128.9 (C-2d,6d), 127.8 (C-2c,6c), 127.4 (C-2b,6b), 126.7 (C2a,6a), 124.6 (C-10c), 120.2 (C-10b), 116.6 (C-3a,5a), 116.1 (C-3b,5b), 116.0 (C-3d,5d), 115.5 (C-3c,5c), 110.9 (C-14c), 109.3 (C-10d,14d), 108.8 (C-14b), 106.9 (C-10a,14a), 103.4 (C-12d), 102.1 (C-12a), 96.3 (C-12b), 95.8 (C-12c), 94.3 (C-7b), 92.3 (C-7a), 85.9 (C-7d), 85.6 (C-7c), 62.3 (C-8d), 58.6 (C-8a), 52.9 (C-8b), 52.6 (C-8c).

*Kobophenol B (**5**):* ^{1}H NMR (CD_3OD, 400 MHz), δ 7.09 (2H, d, J = 8.8 Hz, H-2b,6b), 6.93 (1H, d, J = 2.2 Hz, H-14b), 6.78 (2H, d, J = 8.8 Hz, H-2d,6d), 6.77 (2H, d, J = 8.8 Hz, H-2a,6a), 6.71 (2H, d, J = 8.8 Hz, H-2c,6c), 6.69 (2H, d, J = 8.8 Hz, H-3b,5b), 6.65 (2H, d, J = 8.8 Hz, H-3c,5c), 6.63 (2H, d, J = 8.8 Hz, H-3a,5a), 6.56 (1H, s, H-12c), 6.55 (2H, d, J = 8.8 Hz, H-3d,5d), 6.20 (1H, t, J = 2.2 Hz, H-12d), 6.06 (1H, d, J = 2.2 Hz, H-12b), 5.77 (3H, br s, H-8b,10d,14d), 5.60 (1H, s, H-14a), 5.14 (1H, d, J = 10.2, H-7a), 5.08 (1H, s, H-7d), 4.25 (1H, t, J = 7.0 Hz, H-7c), 3.87 (1H, s, H-8d), 3.84 (1H, dd, J = 10.2, 1.8 Hz), 3.82 (1H, H-8c), 3.81 (1H, H-7b), 3.34 (1H, H-12a); ^{13}C NMR (CD_3OD, 100 MHz), δ 202.7 (C-11a), 195.4 (C-13a), 169.5 (C-9a), 160.8 (C-11c), 159.1 (C-11d,13d), 159.0 (C-11b), 158.9 (C-13b), 158.4 (C-4a), 156.9 (C-4d), 156.5 (C-4c), 156.1 (C-4b), 154.0 (C-13c), 148.1 (C-9d), 139.7 (C-9b), 133.0 (C-9c), 132.3 (C-1d), 131.2 (C-1b),

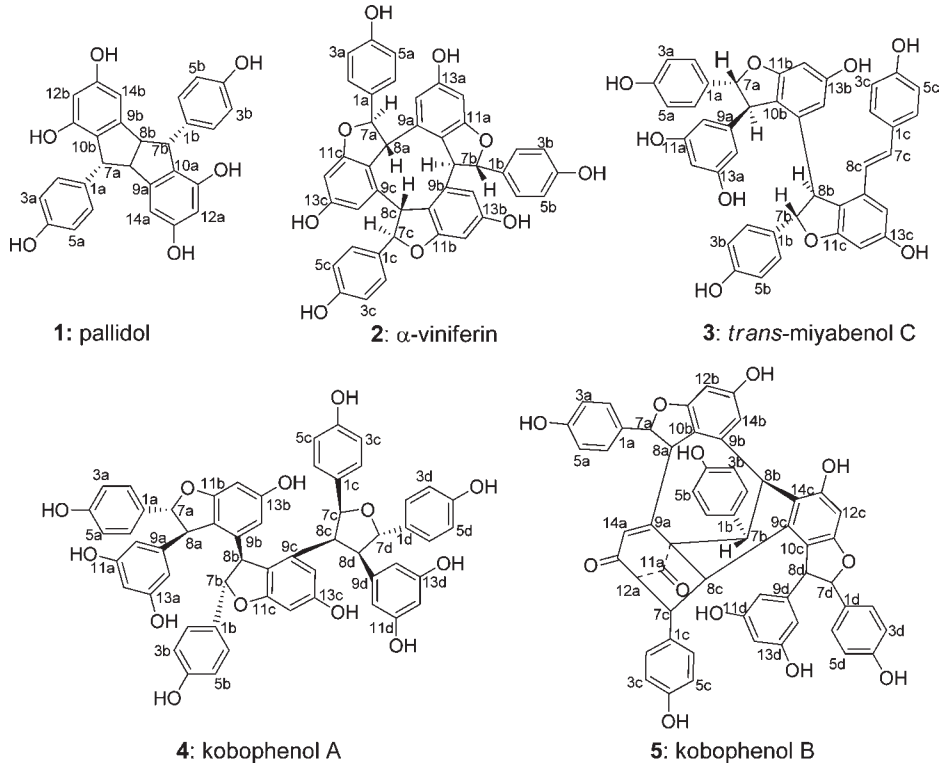

Figure 1. Structures of resveratrol oligomers isolated from *C. gynandra* and *C. folliculata*.

128.8 (C-2a,6a), 128.6 (C-1a), 128.5 (C-2c,6c), 128.3 (C-2b,6b), 128.2 (C-1c), 126.1 (C-2d,6d), 124.9 (C-14a), 124.6 (C-14c), 115.6 (C-10c), 115.33 (C-3a,5a), 115.27 (C-3b,5b), 115.24 (C-3c,5c), 114.9 (C-10b), 114.8 (C-3d,5d), 109.1 (C-14b), 104.6 (C-14d), 101.0 (C-12d), 96.5 (C-12c), 95.6 (C-12b), 91.3 (C-7d), 87.7 (C-7a), 68.4 (C-12a), 62.6 (C-10a), 54.8 (C-8d), 51.3 (C-8a), 46.3 (C-8c), 40.0 (C-7b), 39.0 (C-7c), 37.9 (C-8b).

Cell Lines and Cell Culture Conditions. Human colon cancer cell lines Caco-2 (adenocarcinoma), HT-29 (adenocarcinoma), and HCT-116 (carcinoma) and normal colon cells, CCD-18Co, were obtained from American Type Culture Collection (Rockville, MD). Caco-2 cells were grown in EMEM medium supplemented with 10% v/v fetal bovine serum, 1% v/v nonessential amino acids, 1% v/v L-glutamine, and 1% v/v antibiotic solution (Sigma). HT-29 and HCT-116 cells were grown in McCoy's 5a medium supplemented with 10% v/v fetal bovine serum, 1% v/v nonessential amino acids, 2% v/v HEPES, and 1% v/v antibiotic solution. CCD-18Co cells were grown in EMEM medium supplemented with 10% v/v fetal bovine serum, 1% v/v nonessential amino acids, 1% v/v L-glutamine, 1% pyruvate, and 1% v/v antibiotic solution and were used from PDL between 26 and 35 for all experiments. Cells were maintained at 37 °C in an incubator under a 5% CO_2/95% air atmosphere at constant humidity. The pH of the culture medium was determined using pH indicator paper (pHydrion Brilliant, pH 5.5−9.0, Micro Essential Laboratory, Brooklyn, NY) inside the incubator. Cells were counted using a hemocytometer and were plated at 3000−5000 cells per well, in a 96-well format for 24 or 48 h prior to sample treatment depending on the cell line. All of the test samples were solubilized in DMSO (<0.5% in the culture medium) by sonication and were filter sterilized (0.2 μm) prior to addition to the culture media. Control cells were also run in parallel and subjected to the same changes in medium with 0.5% DMSO.

Cell Proliferation and Viability Tests (Trypan Blue Exclusion and MTS Assays). At the end of either 48 or 72 h of sample treatment, trypsinized cells (2.5 g/L trypsin, 0.2 g/L EDTA) were suspended in cell culture medium and counted using a Neubauer hemocytometer (Bad Mergentheim, Germany); viability was measured using Trypan blue dye exclusion. Results of proliferation and viability in sample-treated cells are expressed as percentage of those values obtained for control (0.5% DMSO) cells. All experiments were performed in triplicate.

The MTS assay was carried out as described previously[1] with modifications. At the end of 48 or 72 h of treatment with serially diluted test samples (ranging from 1 to 200 μM concentrations), 20 μL of the MTS reagent, in combination with the electron coupling agent phenazine methosulfate, was added to the wells, and cells were incubated at 37 °C in a humidified incubator for 3 h. Absorbance at 490 nm (OD_{490}) was monitored with a spectrophotometer (SpectraMax M2, Molecular Devices Corp., operated by SoftmaxPro v.4.6 software, Sunnyvale, CA), to obtain the number of cells relative to control populations. Twenty microliters of 4 mg/mL etoposide (Sigma) was assayed as a positive control of inhibition of proliferation. The results are expressed as the concentration that inhibits growth of cells by 50% versus control cells (control medium used as negative control), IC_{50}. Data are presented as the mean ± SD of three separate experiments on each cell line (n = 2 plates per experiment; 2 wells per treatment per time point). Etoposide provided consistent IC_{50} values of 10−20 μM (HT-29, HCT-116, and Caco-2) and 30−40 μM for the CCD-18Co cells.

Flow Cytometry Analysis of Cell Cycle. Cells (2×10^5) were collected after the corresponding experimental periods, fixed in ice-cold ethanol/PBS (70:30) for 30 min at 4 °C, further resuspended in PBS with 100 μg/mL RNase and 40 μg/mL propidium iodide, and incubated at 37 °C for 30 min. DNA content (10000 cells) was analyzed using a FACS Calibur instrument equipped with FACStation running FACS Calibur software (BD Biosciences, San Diego, CA). The analyses of cell cycle distribution were performed in triplicate (n = 2 plates per experiment) for α-viniferin treatment (20 μM) for time points at 48 and 72 h. The coefficient of variation, according to the ModFit LT version 2 acquisition software package (Verity Software House, Topsham, ME), was always <5%.

Table 1. Antiproliferative Activities of Etoposide, Resveratrol, and Resveratrol Oligomers (1−5 on Human Colon Cell Lines at 48 and 72 h

	IC_{50}[a]							
	HCT-116[b]		HT-29[b]		Caco-2[b]		CCD-18Co[c]	
compound	48 h	72 h	48 h	72 h	48 h	7s h	48 h	72 h
resveratrol	159.5 ± 3.9	146.6 ± 2.6	164.7 ± 5.3	148.2 ± 4.5	130.6 ± 4.9	115.2 ± 3.9	189.7 ± 4.0	161.3 ± 4.2
pallidol (**1**)	167.2 ± 4.8	136.7 ± 4.4	170.5 ± 2.3	130.2 ± 2.2	159.7 ± 3.3	126.3 ± 3.4	165.2 ± 4.2	146.0 ± 6.5
α-viniferin (**2**)	16.1 ± 2.1	6.6 ± 0.6	46.1 ± 2.2	32.6 ± 2.1	38.4 ± 3.0	16.1 ± 1.7	89.9 ± 3.8	40.0 ± 4.3
trans-miyabenol C (**3**)	59.1 ± 4.2	50.2 ± 1.7	84.2 ± 3.5	58.4 ± 1.4	72.8 ± 2.9	58.2 ± 2.7	115.0 ± 1.7	86.2 ± 0.6
kobophenol A (**4**)	174.9 ± 5.1	157.5 ± 6.0	180.0 ± 2.8	151.4 ± 3.4	173.0 ± 3.7	159.0 ± 2.4	194.3 ± 3.2	164.1 ± 6.6
kobophenol B (**5**)	156.7 ± 2.7	135.9 ± 4.4	88.9 ± 4.0	54.5 ± 4.2	148.2 ± 3.0	132.1 ± 5.7	171.5 ± 3.7	143.8 ± 5.5
etoposide	25.1 ± 1.9	16.5 ± 1.2	18.1 ± 1.3	11.2 ± 1.7	19.4 ± 2.1	17.4 ± 1.1	48.2 ± 1.9	41.8 ± 2.3

[a] IC_{50} (in μM) is defined as the concentration required to achieve 50% inhibition over control cells (DMSO 0.5%); IC_{50} values are shown as the mean ± SD from three independent experiments. [b] Tumor cell lines. [c] Normal cell line.

Morphological Evaluation of Apoptosis. Cells (2.5×10^4/mL) were treated with α-viniferin (20 μM) for 48 and 72 h, fixed with methanol/acetic acid (3:1, v/v), and stained with 50 mg/mL Hoechst 33242 dye at 37 °C for 20 min. Afterward, the cells were examined under a Nikon Eclipse TE2000-E inverted microscope (Nikon, Melville, NY) with an X-Cite 120 Fluorescence Illumination System and using NIS-Elements AR 3.0 Imaging software to capture images. Etoposide (Sigma) 20 μM was assayed as a standard inducer of apoptosis. Morphological evaluation of apoptosis was carried out twice for each sample for time points at 48 and 72 h (n = 2 plates per experiment).

Statistical Analysis. A two-tailed unpaired Student's t test was used for statistical analysis of the data using Office Excel 2007 software. A p value of <0.05 was considered to be significant.

RESULTS AND DISCUSSION

Isolation of Resveratrol Oligomers. Five resveratrol oligomers (one dimer, two trimers, and two tetramers) were isolated from two *Carex* species, *C. gynandra* and *C. folliculata*. The dimer was identified as pallidol (**1**), the trimers were identified as α-viniferin (**2**) and *trans*-miyabenol C (**3**), and the tetramers were identified as kobophenol A (**4**) and kobophenol B (**5**) (Figure 1). In light of the strong biological activities associated with resveratrol and its oligomers,[1,6−25] we sought to compare the antiproliferative activity of resveratrol and the five *Carex* oligomers in three human colon tumor cell lines, in addition to one normal colon cell line.

Antiproliferative Activity of Resveratrol and Resveratrol Oligomers. Initially, the effects of resveratrol and its five oligomers on cell viability were examined. In all cases, cell viability was always >90% at tested doses (ranging from 1 to 200 μM), so the compounds were not considered to be cytotoxic. However, all of the resveratrol oligomers inhibited the proliferation of HCT-116, Caco-2, and HT-29 cell lines in a time-dependent manner. Table 1 shows IC_{50} values at 48 and 72 h for resveratrol and the resveratrol oligomers (**1**−**5**) on all of the cell lines.

After the sample treatments, the highest antiproliferative effects against the colon cancer cell lines were observed for the resveratrol trimers, α-viniferin (**2**) and *trans*-miyabenol C (**3**). The most effective compound was α-viniferin with IC_{50} values ranging from 6.6 to 32.6 μM at 72 h, followed by *trans*-miyabenol with IC_{50} values ranging from 50.2 to 58.2 μM. Notably, α-viniferin was as effective as the chemotherapy drug etoposide against Caco-2 cells and more effective than etoposide against HCT-116 cells. Except for kobophenol B (**5**), which showed activity comparable to that of *trans*-miyabenol C against HT-29 cells, resveratrol, the dimer pallidol (**1**), and tetramers kobophenol A and B (**4** and **5**) showed only moderate activity against all cell lines, with IC_{50} values ranging from 126.3 to 159.0 μM at 72 h. Also, α-viniferin and *trans*-miyabenol C showed selective antiproliferative activity for all three colon cancer cell lines when compared to normal cells, whereas kobophenol B (**5**) showed selective activity for the HT-29 cell line only.

In a previous study, pallidol and *trans*-miyabenol C were evaluated for cytotoxicity against a panel of colon and noncolon human tumor cell lines and showed no significant activity. However, the peracetylated derivative of pallidol showed strong activity against KB, CAKI-1, MCF-7, 1A9, and HCT-8 cell lines with ED_{50} values ranging from 1.6 to 8.0 μg/mL.[29] The strong activity of α-viniferin on colon cancer cells is in agreement with a prior paper showing 50−100% inhibition of SW480 colon cancer cells at 10 μM.[24] Interestingly, a previous study suggested that α-viniferin also showed selective antiproliferative activity against other types of cancer cell lines (submandibular gland carcinoma HSG and promyelocytic leukemia HL-60) but not normal human oral cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF).[30]

In addition to its antiproliferative activity, α-viniferin has been reported to possess in vitro inhibitory activity on acetylcholinesterase[31] and to suppress arthritic inflammation and bone destruction in rats.[32] The mechanism of the anti-inflammatory effect of α-viniferin has been delineated,[33] but the molecular mechanisms of action associated with its antiproliferative activity on human colon cancer cells have never been investigated. Although we did not probe into detailed mechanisms of action of α-viniferin, we sought to evaluate whether its antiproliferative effects were mediated through cell cycle arrest and/or by the induction of apoptosis (described below). It should be noted that for these experiments, α-viniferin was evaluated at a single concentration of 20 μM on the basis of its antiproliferative IC_{50} values. However, because the antiproliferative IC_{50} values of α-viniferin were different against the three colon cancer cell lines (see Table 1), a test concentration of 20 μM was selected as a "median IC_{50} value" for the cell cycle and apoptosis experiments.

Cell Cycle Distribution Analysis. Inhibition of proliferation of α-viniferin (at 20 μM) was further examined by measuring cell cycle distribution. At 48 h of the experiment, HCT-116, Caco-2, and HT-29 control cells were distributed with values as

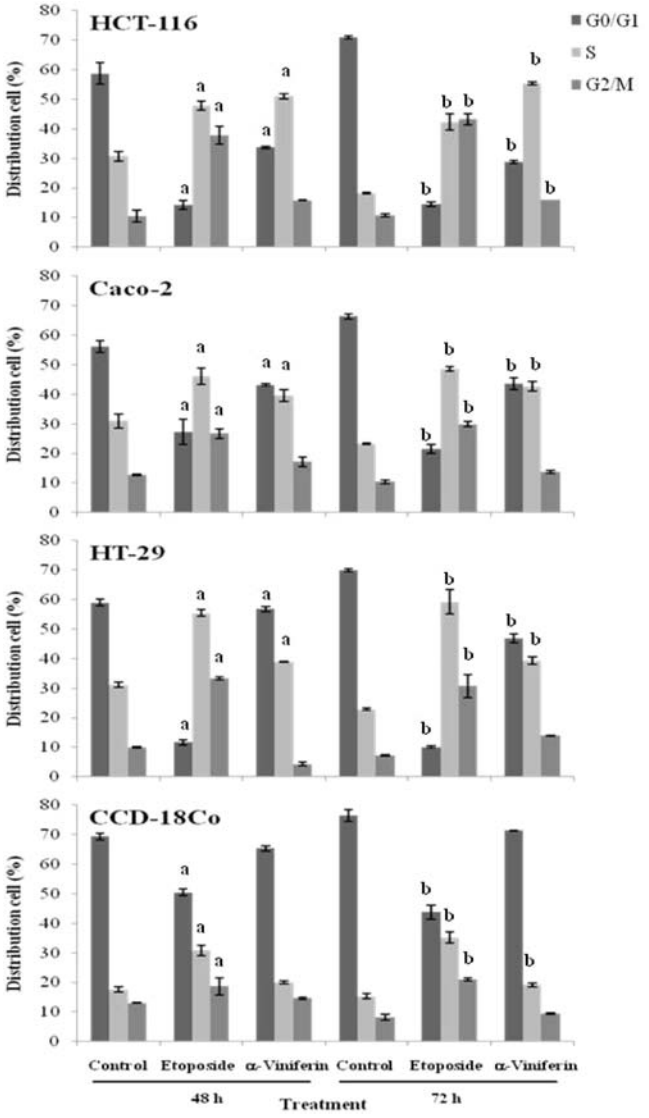

Figure 2. Analysis of cell cycle distribution of cell lines treated with α-viniferin (20 μM). Etoposide (20 μM) was used as a positive control. Distribution of cells in the G_0/G_1, S, and G_2/M phases at 48 and 72 h. Data are expressed as the mean value ± SD (n = 3). The letter "a", $p < 0.05$, atop a bar indicates a significant difference compared to untreated (control) cells at 48 h; the letter "b", $p < 0.05$, atop a bar indicates a significant difference compared to untreated cells at 72 h.

follows: 56.2−58.9% in G_0/G_1 phase, 30.8−31.1% in S phase, and 9.9−12.7% in G_2/M phase. At 72 h of the experiment, the proportion of these control cells in the G_0/G_1 phase increased to 66.9−70.5%, whereas cells in the S and G_2/M phases decreased to 18.2−23.3 and to 7.2−10.6%, respectively (Figure 2), indicating that there were no detectable effects of each cell line on cell cycle distribution.

At 48 h of treatment with α-viniferin, an increase of cancer cells in the S phase was observed ($p < 0.05$) (range 38.9−51.0%) concomitant with a decrease in G_0/G_1 ($p < 0.05$) (range 33.7−56.2%), whereas no significant changes of the G_2/M ratio were observed. This increase was maintained during the 72 h of treatment to 39.3−55.3% ($p < 0.05$), a ∼70% increase when compared to control cells, in the S phase accompanied by a decrease of cells in G_0/G_1 phase (range 28.7−46.8%) ($p < 0.05$), and a slight increase, even significant on HCT-116 cells, in the G_2/M phase was observed (Figure 2).

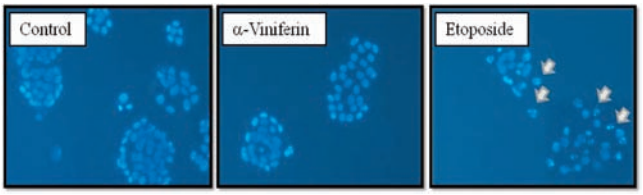

Figure 3. Morphological evaluation of apoptosis in HCT-116 cells with Hoechst 33258 dye upon treatment with α-viniferin (20 μM) and etoposide (positive control, 20 μM) at 72 h. The arrows designate typical apoptotic nuclei with condensed chromatin.

However, incubation of the normal colon CCD-18Co cells with α-viniferin for 48 and 72 h did not cause significant changes in cell cycle when compared with control cells, except a slight, but significant, increase for the S phase at 72 h (Figure 2). Finally, the incubation of etoposide (20 μM), used as a positive control, arrested S and G_2/M phases on all cell lines at 48 and 72 h.

Our findings are in accordance with previous studies that have demonstrated that resveratrol inhibits cell proliferation and favors an accumulation in the S phase of the cell cycle on colon cancer cell lines.[34] Notably, other resveratrol oligomers such as ε-viniferin, and its acetylated derivative, do not demonstrate any significant activity on the accumulation of HCT-116 and other human colon carcinoma cell lines in the S phase of the cell cycle.[23] In summary, our results indicate, for the first time, that the decrease of cell proliferation on colon cancer cells after treatment with α-viniferin is mediated by blocking the progression of cell cycle at the S phase.

Apoptosis Assessment. We evaluated whether α-viniferin (at 20 μM) induced apoptosis of the colon cells at 48 and 72 h by monitoring changes in nuclear chromatin distribution through staining with the DNA-binding fluorochrome Hoechst 33242 dye. Incubation of colon cancer and normal colon cells with α-viniferin mirrored the pattern followed by untreated cells, thus indicating the absence of apoptosis. Figure 3 shows the morphological evaluation of apoptosis in HCT-116 cells at 72 h. The other cell lines showed similar profiles (results not shown).

Previous studies have shown that resveratrol and some resveratrol oligomers, including *cis*-ε-viniferin, *trans*-ε-viniferin, gnetin H, and suffruticosols A and B, induce apoptosis of HL-60 cells at 25 μM concentrations[35] and of melanoma cells at 30 μM concentrations.[36] In addition, the resveratrol tetramer, vaticanol C, has also been shown to induce the apoptosis of SW480 colon cancer cells at 5 μM concentrations.[24] However, the present study is the first demonstration that α-viniferin does not induce apoptosis of any of the colon cancer cell lines studied here.

In summary, the inhibition of proliferation by α-viniferin on colon cancer cells is mediated by cell cycle arrest at the S phase, and not through apoptosis at the antiproliferative IC_{50} test concentration (20 μM) studied here. Furthermore, our results indicate a possible selectivity of some of these resveratrol oligomers toward colon cancer cells, suggesting that these compounds may have potential as colon cancer chemopreventive agents. However, it should be noted that in vitro findings are not translatable to the in vivo situation and fail to consider important physiological issues such as bioavailability and metabolism. In fact, several classes of dietary polyphenols are known to be extensively metabolized and converted by colon microbiota into other bioactive

forms.[37,38] Thus, whether the resveratrol oligomers evaluated in this study would be present in colonic tissue in their bioactive (intact and/or metabolized) forms to exert potential chemopreventive effects would require further studies.

AUTHOR INFORMATION

Corresponding Author

*(G.E.H.) E-mail: ghenry@susqu.edu. Phone/fax: (570) 372-4222/2752. (N.P.S.) E-mail: nseeram@mail.uri.edu. Phone/fax (401) 874-9367/5787.

Author Contributions

[||]These authors contributed equally to this study.

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