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Resveratrol Directly Targets COX-2 to Inhibit Carcinogenesis

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Abstract

Targeted molecular cancer therapies can potentially deliver treatment directly to a specific protein or gene to optimize efficacy and reduce adverse side effects often associated with traditional chemotherapy. Key oncoprotein and oncogene targets are rapidly being identified based on their expression, pathogenesis and clinical outcome. One such protein target is cyclooxygenase-2 (COX-2), which is highly expressed in various cancers. Research findings suggest that resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) demonstrates non-selective COX-2 inhibition. We report herein that resveratrol (RSVL) directly binds with COX-2 and this binding is absolutely required for RSVL's inhibition of the ability of human colon adenocarcinoma HT-29 cells to form colonies in soft agar. Binding of COX-2 with RSVL was compared with two RSVL analogues, 3,3',4',5'5'-pentahydroxy-*trans*-stilbene (RSVL-2) or 3,4',5-trimethoxy-*trans*-stilbene (RSVL-3). The results indicated that COX-2 binds with RSVL-2 more strongly than with RSVL, but does not bind with RSVL-3. RSVL or RSVL-2, but not RSVL-3, inhibited COX-2-mediated PGE₂ production *in vitro* and *ex vivo*. HT-29 human colon adenocarcinoma cells express high levels of COX-2 and either RSVL or RSVL-2, but not RSVL-3, suppressed anchorage independent growth of these cells in soft agar. RSVL or RSVL-2 (not RSVL-3) suppressed growth of COX-2^{+/+} cells by 60 or 80%, respectively. Notably, cells deficient in COX-2 were unresponsive to RSVL or RSVL-2. These data suggest that the anticancer effects of RSVL or RSVL-2 might be mediated directly through COX-2.

Keywords

cell transformation; fluorescence; resveratrol analogs

INTRODUCTION

Resveratrol (RSVL), a phytoalexin found in grape skins, red wine and peanuts, has been presented as a promising chemopreventive agent against cancer [1-5]. The presence of resveratrol in red wine may contribute to this beverage's proposed cancer preventive effects [6]. The anticancer activities of resveratrol were first appreciated when Jang *et al.* [7] demonstrated in a two-stage mouse skin cancer model that resveratrol possesses cancer chemopreventive activity against all three major stages of carcinogenesis comprising initiation, promotion and progression. Induction of apoptosis by resveratrol has been reported to be at least partially responsible for its chemopreventive activity [8,9]. Recently, we showed that resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway [10]. ERKs and p38 were reported to mediate resveratrol-induced activation of p53 and apoptosis through the induction of phosphorylation of p53 at Ser¹⁵ [11] and JNKs

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activation [12]. Based on the structure of resveratrol, we synthesized two resveratrol derivatives, which differed only in the number of hydroxyl groups on the B ring, and demonstrated that these analogues had differential effects on cell transformation and apoptosis in JB6 Cl41 cells [13]. In contrast to resveratrol, neither derivative induced p53 activation or apoptosis. Instead the derivative containing three hydroxyl groups on the B ring markedly inhibited epidermal growth factor (EGF)-induced phosphatidylinositol-3 kinase (PI-3K) and Akt activation [13].

The nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, aspirin, piroxicam, sulindac, and celecoxib, all of which inhibit the cyclooxygenase (COX-1 and COX-2) enzymes [14-16], have been suggested to possess potent anticancer activities. The inhibition of COX-2 activity is relevant to cancer chemoprevention because COX catalyzes the conversion of arachidonic acid to pro-inflammatory substances such as prostaglandins (PGs). The enhanced synthesis of PGs can favor the growth of malignant cells by increasing cell proliferation [17]. The anti-inflammatory activity of resveratrol seems to be mainly associated with the suppression of COX-1 and COX-2 activities, although results have been inconsistent. For example, Jang *et al.* [7] reported that resveratrol had no effect on the cyclooxygenase activity of COX-2. In contrast, Subbaramaiah *et al.* [18] clearly showed that resveratrol suppressed the synthesis of prostaglandin E₂ (PGE₂) by inhibiting COX-2 enzyme activity. Murias *et al.* [19] demonstrated the ability of a series of methoxylated and hydroxylated resveratrol derivatives to inhibit COX-1 and COX-2 isoenzymes *in vitro*. The effect of structural parameters on COX-2 inhibition was evaluated by quantitative structure-activity relationship (QSAR) analysis [19] and showed that the binding mode of hydroxylated resveratrol analogues involved hydrogen bonding with amino acid residues Arg120, Ser530, and Tyr385. Docking studies on COX-2 protein structure revealed that hydroxylated, but not methoxylated resveratrol analogs, were able to bind at the previously identified binding sites of the enzyme [19]. Resveratrol was reported to inhibit PMA-induced COX-2 expression by blocking activation of ERKs and p38 MAPK in mouse skin *ex vivo* [20]. TPA (12-*O*-tetradecanoylphorbol-13-acetate) is known to induce COX-2 expression in mouse skin through the activation of nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1) [1,21]. Resveratrol was reported to reduce PMA-induced PGE₂ production by down-regulating COX-2 gene transcription in human mammary and oral epithelial cells indirectly through the modulation of protein kinase C (PKC), ERKs, and AP-1 activities [18]. In the present study, resveratrol was shown to directly bind with the COX-2 protein with a K_d of 58 μM. Resveratrol decreased COX-2-mediated PGE₂ production, inhibited the ability of HT-29 colon cancer cells to form colonies in soft agar, and suppressed the growth of COX-2^{+/+} murine embryonic fibroblasts. The structural analogue, RSVL-2, exhibited a more potent inhibitory effect compared to RSVL in the same concentration range. In contrast, another structural analogue, RSVL-3, had no effect.

MATERIALS AND METHODS

Cells and Reagents

Recombinant cyclooxygenase 2 (COX-2) and resveratrol were purchased from Sigma (St Louis, MO) and dimethylsulfoxide (DMSO) was from Pierce (Rockville, IL). Stock solutions of resveratrol (40 mM) in DMSO were stored at -20 °C. The analogues of resveratrol (RSVL), 3,3',4',5',5-pentahydroxy-*trans*-stilbene (RSVL-2) and 3,4',5-threemethoxy-*trans*-stilbene (RSVL-3) were gifts from Dr. Chi-Tang Ho, Department of Food Science, Rutgers University, New Brunswick, NJ [13,22]. The COX-2 antibody and the secondary bovine anti-goat IgG-AP for detecting COX-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CNBr-activated Sepharose 4B (glutathione-Sepharose 4B) was obtained from Amersham Biosciences (Piscataway, NJ). The Chemiluminescent COX (ovine) Inhibitor Screening Assay

kit (Catalog No 760101) was obtained from Cayman Chemical (Ann Arbor, MI). The HCT116 and HT-29 human colon adenocarcinoma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). COX2^{+/+} or COX-2^{-/-} murine embryonic fibroblasts (MEFs) were generously provided by Dr. J. Reese (Department of Pediatrics, University of Kansas, Medical City, KS) [23]. HT-29, COX2^{+/+}, or COX2^{-/-} cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Grand Island, NY) and HCT116 cells were cultured in McCoy's 5A medium (Mediatech Inc, Herndon, VA). Both media contained 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 µM/ml gentamicin and cells were grown in monolayers at 37 °C, under 5% CO₂.

Affinity Chromatography and *In Vitro* Pull-Down Assay

Resveratrol (6 mg) was first coupled to the CNBr activated Sepharose 4B matrix-beads (0.3 g) in 0.5 M NaCl and 35% DMSO (pH 8.3) overnight, at 4 °C, according to the manufacturer's instructions. The binding between the matrix and resveratrol was examined by color reaction with FeCl₃ (10 µl sample + 490 µl H₂O + 500 µl 0.01% FeCl₃ in 0.01 N HCl, OD₅₁₀). A calibration curve of different concentrations of resveratrol with FeCl₃ was used to determine that 94.5 % of the added resveratrol binds with the matrix. HT-29 colon cancer cells were added to lysis buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, and 0.02 mM PMSF, 1× protease inhibitor mixture), sonicated and centrifuged at 15,000 rpm for 30 min at 4 °C. The recombinant COX-2 (100 ng) or the supernatant fraction prepared from HT-29 cells (600 µg protein) was incubated with resveratrol-Sepharose 4B (or Sepharose 4B only, used as a control) beads (50 µl) in 5 ml of reaction buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 2 µg/ml bovine serum albumin, 0.02 mM PMSF, 1× protease inhibitor mixture). After incubation with gentle rocking overnight at 4 °C, the beads were vigorously washed with 50 ml of 0.1% Tween, 0.5% Tween or 1% Tween in PBS and then 3 times with PBS followed by centrifugation at 15,000 rpm for 15 min, and proteins bound to the beads were analyzed by immunoblotting with a COX-2 antibody. For recombinant COX-2, the supernatant fraction (2 µl) mixed with 38 µl H₂O and 10 µl 5× SDS buffer was heated at 95 °C for 5 min, cooled on ice, and centrifuged at 14,000 rpm for 15 min and 6 µl was loaded into a 10% polyacrylamide gel for electrophoresis (SDS-PAGE). For lysate prepared from HT-29 cells, 20 µl 5× SDS buffer were added directly to the beads and heated at 95 °C for 5 min, cooled on ice, and centrifuged at 14,000 rpm for 15 min and 15 µl were resolved by 10% SDS-PAGE. Proteins were subsequently transferred onto an Immobilon-P membrane (Millipore, Chelmsford, MA). Antibody-bound COX-2 was detected by chemiluminescence (ECF Western blotting kit; Amersham Pharmacia Biotech) and analyzed using the Storm 840 Scanner (Molecular Dynamics, Sunnyvale, CA).

Determination of the Dissociation Constant (K_d) of the Resveratrol and COX-2 Interaction

Fluorescence studies were performed using a JASCO fluorescence spectrophotometer. A quartz cuvette of 500 µL path length was used and the band pass for excitation and emission monochromators was 10 nm. Emission spectra were recorded over a range of 340–500 nm using 320 nm as the excitation wavelength and a fluorescence intensity of 389 nm was used to calculate the K_d (free resveratrol has a fluorescence maximum at 389 nm). Fluorescence intensity increased after the interaction of COX-2 with resveratrol. For determination of K_d, COX-2 (0.2 µM) was mixed with different concentrations of resveratrol (0–120 µM) in buffer (100 mM NaCl, 50 mM NaH₂PO₄, 0.1 mM EDTA pH 6.5) and incubated for 10 min at room temperature prior to recording emission spectra at 25 °C. Buffer blanks containing only different concentrations of resveratrol were used to subtract background. The K_d was calculated by plotting 1/[bound resveratrol] with 1/[free resveratrol] and determining the slope as previously reported [24,25].

Surface Plasmon Resonance (SPR)

The affinity of the interaction between COX-2 and resveratrol or its analogues was measured using the BIAcore-X biosensor (Uppsala, Sweden) and the dextran matrix of a sensor chip containing nickel-nitrilotriacetic acid to immobilize a His-tagged COX-2 as the target. All steps in the immobilization process were carried out at a flow rate of 5 μ l/min. All experiments were performed at 25 °C in running buffer (0.01 M mM HEPES pH 7.4, 0.15 M NaCl, 50 μ M EDTA, 0.005% Tween 20) using a flow rate of 30 μ l/min. The nickel solution contained 500 μ M NiCl₂ in running buffer. Resveratrol or its analogues (each at 300 μ M) were added during the binding phase (0.1 μ g His-COX-2). For regeneration of the sensor chip, running buffer containing 300 μ M imidazole/0.5 NaCl, 0.35 M EDTA, and 100 μ M NaOH was used.

Inhibition of COX-2 Enzyme Activity by Resveratrol

The effect of resveratrol, its analogues, or celecoxib (CEL) on COX-2-mediated PGE₂ production was measured using the Chemiluminescent COX (ovine) Inhibitor Screening Assay kit from Cayman Chemical. COX activity was determined by measuring the synthesis of PGE₂ according to the instructions provided with the kit. A standard curve with PGE₂ was generated at the same time and from the same plate and was used to quantify PGE₂ levels produced in the presence of test samples.

Soft Agar Colony Formation Assay

The anchorage independent growth assay is commonly used to assess the ability of cells to become transformed to cancer cells (visualized by the formation of colonies in soft agar) in the presence of a tumor promoter such as epidermal growth factor or 12-*O*-tetradecanoylphorbol-13-acetate. Cancer cells are already transformed and therefore have the ability to form colonies in soft agar without stimulation by a tumor promoter and this is typically referred to as “expression of their phenotype”. In the current studies, the effect of resveratrol and its analogues on the ability of HT-29 colon cancer cells to form colonies (i.e. express their phenotype) was assessed by the soft agar colony formation assay. The assay was performed in 6-well plates and in each well, HT-29 cells (2×10^3 /ml) were cultured, with or without resveratrol or its analogues (10–20 μ M), in 1 ml of 0.33% BME (basal medium Eagle's) agar containing 10% FBS and overlaid with 3.5 ml of 0.5% BME agar containing 10% FBS. The cultures were maintained in a 37 °C, 5% CO₂ incubator. After 10 days, the number of colonies was scored using the LEICA DM IRB inverted research microscope (Leica Mikroskopie und Systeme GmbH, Germany) and the Image Pro Plus software program (v.4; Media Cybernetics, Silver spring, MD).

Cell Growth

HT-29 human colon cancer cells, COX-2^{+/+} or COX-2^{-/-} (1×10^4) murine embryonic fibroblasts (MEFs) were cultured in 6-well plates with or without resveratrol, its analogues, or CEL (10 μ M). Cells were treated with trypsin, harvested, and counted each day over a 6-day period using the Coulter Z cell counter (Coulter Corporation, Miami, Florida).

Immunoblotting

To determine expression of the COX-2 protein, equal numbers of HCT116 or HT-29 human colon cancer cells, or COX-2^{+/+} or COX-2^{-/-} MEFs (8×10^5) were cultured in 10% FBS/DMEM for 12–15 h in 10-cm diameter dishes. After 70–80% confluence was reached, cells were harvested by washing once with ice-cold PBS and disrupted in 200 μ l of RIPA buffer (1 $\times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na₃VO₄, 1 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The samples were sonicated and centrifuged at 14,000 rpm for 15 min. The quantity of protein was measured by the Bradford method [26] and normalized to controls. The samples (30–50 μ g protein) were diluted with 5 $\times$

SDS and heated to 95 °C for 5 min, cooled on ice, and centrifuged at 14,000 rpm for 5 min. Then the samples were loaded into each lane of a 10% SDS polyacrylamide gel for electrophoresis (SDS-PAGE) and subsequently transferred onto an Immobilon-P transfer membrane (Millipore, Chelmsford, MA). Some transfer membranes were washed with stripping buffer (7 M guanidine hydrochloride, 50 mM glycine pH 10.8, 0.05 mM EDTA, 0.1 M KCl, and 20 mM β -mercaptoethanol) and reprobed with anti- β -actin. The COX-2 protein was detected by chemiluminescence (ECF Western blotting kit; Amersham Pharmacia Biotech) and analyzed using the Storm 840 Scanner (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Resveratrol Interacts With COX-2 *In Vitro* and *Ex Vivo*

Cell lysates from HT-29 or HCT116 colon cancer cells were examined for expression of COX-2 (Figure 2A) and results indicated that COX-2 is highly expressed in HT-29 cells compared to HCT116 cells. HT-29 cells were therefore utilized to study the interaction of COX-2 and resveratrol (RSVL, Figure 1A). The interaction of RSVL with a recombinant COX-2 protein was assessed by resveratrol-Sepharose 4B affinity chromatography *in vitro* (Figure 2B) and *ex vivo* (Figure 2C). Results indicated that COX-2 interacted with the resveratrol-Sepharose 4B beads but not with the Sepharose 4B beads both *in vitro* and *ex vivo* (Figure 2B and 2C). To confirm this result, we used the BIAcore-X to assess the kinetics of the association and dissociation of a His-tagged COX-2 protein with RSVL and two analogues, RSVL-2 and RSVL-3. Typical sensograms showed quantitative analysis of the binding of His-COX-2 with RSVL (Figure 2D). Moreover, the hydroxylated RSVL-2 (Figure 1B), but not the methoxylated resveratrol analogue RSVL-3 (Figure 1C), was able to bind with COX-2 (Figure 2D). To further characterize the physical binding between RSVL and COX-2, we determined the binding affinity (K_d) of this complex using fluorescence spectroscopic techniques. Srivastava *et al.* [23] found that binding of resveratrol to type II phosphatidylinositol 4-kinase resulted in a several-fold increase of the fluorescence intensity of resveratrol at 389 nm, and thus could be used to determine the affinity of the resveratrol and type II phosphatidylinositol 4-kinase interaction [23]. Our results indicated that incubating COX-2 with increasing concentrations of resveratrol resulted in a dose-dependent increase in fluorescent intensity at 389 nm (Figure 3A). We further showed that the fluorescence signal increased after binding of 10 μ M resveratrol with 0.2 μ M COX-2 (Figure 3B) compared to resveratrol or COX-2 alone. These results provide evidence for the direct binding of resveratrol and COX-2. A K_d value of 58 μ M was determined by titrating COX-2 with increasing concentrations of resveratrol and then using fluorescence intensity at 389 nm to calculate the concentration of bound and free resveratrol (Figure 3C).

Resveratrol Inhibits COX-2 Enzyme Activity *In Vitro* and *Ex Vivo*

The activity of COX-2 was determined by measuring the synthesis of PGE₂. The incubation mixture contained COX-2 protein (0.5 μ g), various concentrations of RSVL, RSVL-2, RSVL-3, or CEL (positive control) dissolved in DMSO and reaction buffer (0.1 M Tris-HCl, pH 8.0) containing 5 mM EDTA and 2 mM phenol. The reaction was started by the addition of arachidonic acid, in a final volume of 100 μ l, and incubated for 30 min at 37 °C. The reaction was stopped by adding 1M HCl; and Ellman's Reagent was used to develop the PGE₂ at an absorbance of 405 nm according to the manufacturer's instructions. A standard curve with PGE₂ was used to quantify PGE₂ levels produced in the presence of test samples. Results (Figure 4A) showed that RSVL (IC_{50} = 50 μ M) or RSVL-2 (IC_{50} = 25 μ M), but not RSVL-3, inhibited PGE₂ synthesis *in vitro*. In addition, RSVL or RSVL-2, but not RSVL-3, dose-dependently suppressed PGE₂ synthesis (Figure 4B) *ex vivo* in COX-2^{+/+} cells with IC_{50} values of 60 and 50 μ M, respectively. As expected, CEL, a known inhibitor of COX activation,

strongly suppressed COX-2-mediated PGE₂ production (IC₅₀ = 10 μ M *in vitro* and IC₅₀ = 30 μ M *ex vivo*).

RSVL and RSVL-2 suppress colony formation of HT-29 colon cancer cells in soft agar

We next assessed the effect of RSVL, RSVL-2 or RSVL-3 on the growth curves of HT-29 cells over 5 days. Results demonstrated that RSVL or RSVL-2, but not RSVL-3, inhibited cell growth by day 4 compared to untreated control cells (Figure 5A). RSVL, RSVL-2, and RSVL-3 were compared for their ability to suppress anchorage-independent colony formation of HT-29 cells using the soft agar cell transformation assay. Data indicated that colony formation by HT-29 cells was inhibited by RSVL and even more strongly by RSVL-2 compared to untreated cells, whereas RSVL-3 had little effect (Figure 5B). We then compared the effect of RSVL, RSVL-2, RSVL-3 and CEL on the growth rate of COX-2^{+/+} or COX-2^{-/-} cells (Figure 6). Results after 6 days indicated that RSVL, RSVL-2, or CEL, but not RSVL-3, exhibited potent growth inhibitory effects on COX-2^{+/+} cells. None of the compounds affected the growth of COX-2^{-/-} cells, suggesting that COX-2 is absolutely required for the effects of RSVL, RSVL-2 or CEL. These results indicate that the anti-proliferative activity of resveratrol could be modulated by a slight modification in the chemical structure of the parent compound resulting in a more potent anticancer agent.

DISCUSSION

Some reports suggest that resveratrol can suppress neoplastic cell transformation [10,13]. From animal investigations, resveratrol has been shown to have a potent protective affect against the development of tumors [7]. For example, resveratrol was shown to inhibit the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture and also suppress tumorigenesis in a mouse skin cancer model [7]. These results suggest that resveratrol, a common constituent of the human diet, merits investigation as a potential cancer chemopreventive agent in humans [7]. Huang *et al.* [10] showed that resveratrol inhibited tumor promoter (TPA- or EGF)-induced JB6 Cl41 cellular transformation in a dose-dependent manner in the range 2.5–40 μ M and induced apoptosis through a p53-dependent pathway. Tang *et al.* [27] found that resveratrol induced nuclear accumulation of the COX-2 protein in human breast cancer cell lines and formed complexes with p53 and co-activator protein p300 thereby facilitating p53-dependent apoptosis. She *et al.* [13] investigated the effect of resveratrol and its structurally-related derivatives on EGF-induced JB6 Cl41 cellular transformation. Compared with the antitumor activity of resveratrol, the structural analogue, 3,5,3',4',5'-pentahydroxy-trans stilbene (RSVL-2) exhibited a more potent inhibitory effect on EGF-induced cell transformation [13]. The reported IC₅₀ values for inhibition of COX-2-mediated PGE₂ production by resveratrol vary. Jang *et al.* [7] reported that resveratrol did not inhibit the cyclooxygenase activity of COX-2 but instead suppressed the hydroperoxidase activity of COX-2 with an IC₅₀ of 85 μ M. On the other hand, Subbaramaiah *et al.* [18] showed that resveratrol inhibited COX-2 enzyme activity through a dose-dependent inhibition of PGE₂ production with an IC₅₀ of 30 μ M *in vitro* using a recombinant human COX-2 enzyme or with an IC₅₀ value of 32.2 μ M *ex vivo* in 184B5/HER cells. A possible explanation for these apparent contradictory results might be the difference in assays used to measure COX-2-mediated PGE₂ production and the use of different COX-2 proteins and resveratrol compounds. In addition, the possibility that resveratrol could also have targets other than COX-2 cannot be totally eliminated. Our experimental data showed that resveratrol inhibited COX-2-mediated PGE₂ production with an IC₅₀ of 50 μ M *in vitro* and an IC₅₀ of 60 μ M in COX2^{+/+} cells and COX-2 was absolutely required for the inhibitory effect. Murias *et al.* [19], used the same Chemiluminescent COX (ovine) Inhibitor Screening Assay kit (Cayman Chemicals) and showed a stronger inhibitory *in vitro* effect of resveratrol and pentahydroxystilbene on the synthesis of PGE₂ with IC₅₀ values of 0.996 μ M and 0.00138 μ M, respectively. One possible

explanation for these different results might be due to the use of different COX-2 proteins. Murias *et al.* used a COX-2 protein from the assay kit, whereas we used a recombinant human COX-2 enzyme purchased from Sigma. Our results indicated that resveratrol directly binds to the COX-2 protein both *in vitro* and *ex vivo*. In addition, we observed that the fluorescence signal of resveratrol dramatically increased upon binding with the COX-2 protein, a characteristic that was useful for determining the K_d. COX-2 expression increases early during colon carcinogenesis [28]. Vaticanol C, a novel resveratrol tetramer, has been shown to markedly suppress cell growth through the induction of apoptosis in three different human colon cancer cell lines [29]. The screening experiment showed that the sensitivity of colon cancer cell lines to vaticanol C was higher than that of other human tumor cell lines tested [27]. Piceatannol, a natural analog of resveratrol, inhibited progression through the S phase of the cell cycle in colorectal cancer cell lines independent of COX activity [30]. Resveratrol (8 mg/kg body weight, administered every day for 30 weeks), was reported to markedly reduce the size of tumors in 1,2-dimethylhydrazine-induced colon carcinogenesis in rats [31]. A phase-I clinical trial in colon cancer patients (University of California, Irvine) is intended to examine the effect of resveratrol treatment on colon cancer progression and colonic mucosa in these patients [32]. Experimental studies continue to identify important mechanisms and pathways by which COX-2 plays an important role in carcinogenesis [33]. Gastric, hepatic, esophageal, pancreatic, head and neck, lung, breast, bladder, cervical, endometrial, skin, and colorectal cancer all exhibit elevated COX-2 expression when compared with nonmalignant tissue [34]. For use in colorectal cancer, on the basis of the cumulative experimental and clinical evidence, selective COX-2 inhibitors may be considered as co-therapeutic agents [35]. In most preclinical studies, selective COX-2 inhibitors reduce the growth rate of established tumors rather than causing tumor regression [36].

We have shown that resveratrol and its analogue, RSVL-2, effectively suppressed the growth and colony formation ability in soft agar of HT-29 colon cancer cells, which express very high levels of COX-2. Importantly, each of these compounds inhibited cell growth on in COX-2^{+/+} cells, but were ineffective in COX-2^{-/-} cells suggesting that COX-2 might indeed be the primary target of these compounds. Overall, RSVL-2 was a more potent inhibitor than RSVL. All of these results indicate that the anti-proliferative activity of resveratrol could be modulated by a slight modification in the chemical structure of the parent compound resulting in a more potent but perhaps less toxic anticancer agent. The systematic analysis of analogues of resveratrol will provide a useful clue for the development of the new anticancer drugs.

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REFERENCES

1. Aziz MH, Kumar R, Ahmad N. Cancer chemoprevention by resveratrol: in vitro and in vivo studies and the underlying mechanisms (review). *Int J Oncol* 2003;23(1):17–28. [PubMed: 12792772]
2. Cal C, Garban H, Jazirehi A, Yeh C, Mizutani Y, Bonavida B. Resveratrol and cancer: chemoprevention, apoptosis, and chemo-immunosensitizing activities. *Curr Med Chem Anticancer Agents* 2003;3(2):77–93. [PubMed: 12678904]
3. Savouret JF, Quesne M. Resveratrol and cancer: a review. *Biomed Pharmacother* 2002;56(2):84–87. [PubMed: 12000139]
4. Oak MH, El Bedoui J, Schini-Kerth VB. Antiangiogenic properties of natural polyphenols from red wine and green tea. *J Nutr Biochem* 2005;16(1):1–8. [PubMed: 15629234]
5. Bode AM, Dong Z. Targeting signal transduction pathways by chemopreventive agents. *Mutat Res* 2004;555(1–2):33–51. [PubMed: 15476850]

6. Bianchini F, Vainio H. Wine and resveratrol: mechanisms of cancer prevention? *Eur J Cancer Prev* 2003;12(5):417–425. [PubMed: 14512807]
7. Jang M, Cai L, Udeani GO, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 1997;275(5297):218–220. [PubMed: 8985016]
8. Bhat KP, Pezzuto JM. Cancer chemopreventive activity of resveratrol. *Ann N Y Acad Sci* 2002;957:210–229. [PubMed: 12074974]
9. Gusman J, Malonne H, Atassi G. A reappraisal of the potential chemopreventive and chemotherapeutic properties of resveratrol. *Carcinogenesis* 2001;22(8):1111–1117. [PubMed: 11470738]
10. Huang C, Ma WY, Goranson A, Dong Z. Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. *Carcinogenesis* 1999;20(2):237–242. [PubMed: 10069459]
11. She QB, Bode AM, Ma WY, Chen NY, Dong Z. Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. *Cancer Res* 2001;61(4):1604–1610. [PubMed: 11245472]
12. She QB, Huang C, Zhang Y, Dong Z. Involvement of c-jun NH(2)-terminal kinases in resveratrol-induced activation of p53 and apoptosis. *Mol Carcinog* 2002;33(4):244–250. [PubMed: 11933078]
13. She QB, Ma WY, Wang M, Kaji A, Ho CT, Dong Z. Inhibition of cell transformation by resveratrol and its derivatives: differential effects and mechanisms involved. *Oncogene* 2003;22(14):2143–2150. [PubMed: 12687016]
14. Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *Faseb J* 2001;15(12):2057–2072. [PubMed: 11641233]
15. Gasparini G, Longo R, Sarmiento R, Morabito A. Inhibitors of cyclo-oxygenase 2: a new class of anticancer agents? *Lancet Oncol* 2003;4(10):605–615. [PubMed: 14554238]
16. Harris RE, Beebe-Donk J, Doss H, Burr Doss D. Aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs in cancer prevention: a critical review of non-selective COX-2 blockade (review). *Oncology reports* 2005;13(4):559–583. [PubMed: 15756426]
17. Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res* 1998;58(2):362–366. [PubMed: 9443418]
18. Subbaramaiah K, Chung WJ, Michaluart P, et al. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J Biol Chem* 1998;273(34):21875–21882. [PubMed: 9705326]
19. Murias M, Handler N, Erker T, et al. Resveratrol analogues as selective cyclooxygenase-2 inhibitors: synthesis and structure-activity relationship. *Bioorg Med Chem* 2004;12(21):5571–5578. [PubMed: 15465334]
20. Martin AR, Villegas I, La Casa C, de la Lastra CA. Resveratrol, a polyphenol found in grapes, suppresses oxidative damage and stimulates apoptosis during early colonic inflammation in rats. *Biochem Pharmacol* 2004;67(7):1399–1410. [PubMed: 15013856]
21. Kundu JK, Shin YK, Surh YJ. Resveratrol modulates phorbol ester-induced pro-inflammatory signal transduction pathways in mouse skin in vivo: NF-kappaB and AP-1 as prime targets. *Biochem Pharmacol* 2006;72(11):1506–1515. [PubMed: 16999939]
22. Wang M, Jin Y, Ho CT. Evaluation of resveratrol derivatives as potential antioxidants and identification of a reaction product of resveratrol and 2, 2-diphenyl-1-picrylhydrazyl radical. *J Agric Food Chem* 1999;47(10):3974–3977. [PubMed: 10552752]
23. Reese J, Paria BC, Brown N, Zhao X, Morrow JD, Dey SK. Coordinated regulation of fetal and maternal prostaglandins directs successful birth and postnatal adaptation in the mouse. *Proc Natl Acad Sci U S A* 2000;97(17):9759–9764. [PubMed: 10944235]
24. Srivastava R, Ratheesh A, Gude RK, Rao KV, Panda D, Subrahmanyam G. Resveratrol inhibits type II phosphatidylinositol 4-kinase: a key component in pathways of phosphoinositide turn over. *Biochem Pharmacol* 2005;70(7):1048–1055. [PubMed: 16102733]
25. Santra MK, Panda D. Detection of an intermediate during unfolding of bacterial cell division protein FtsZ: loss of functional properties precedes the global unfolding of FtsZ. *J Biol Chem* 2003;278(24):21336–21343. [PubMed: 12649276]

26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254. [PubMed: 942051]
27. Tang HY, Shih A, Cao HJ, Davis FB, Davis PJ, Lin HY. Resveratrol-induced cyclooxygenase-2 facilitates p53-dependent apoptosis in human breast cancer cells. *Mol Cancer Ther* 2006;5(8):2034–2042. [PubMed: 16928824]
28. Sano H, Kawahito Y, Wilder RL, et al. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res* 1995;55(17):3785–3789. [PubMed: 7641194]
29. Ito T, Akao Y, Tanaka T, Iinuma M, Nozawa Y. Vaticanol C, a novel resveratrol tetramer, inhibits cell growth through induction of apoptosis in colon cancer cell lines. *Biol Pharm Bull* 2002;25(1): 147–148. [PubMed: 11824549]
30. Wolter F, Clausnitzer A, Akoglu B, Stein J. Piceatannol, a natural analog of resveratrol, inhibits progression through the S phase of the cell cycle in colorectal cancer cell lines. *J Nutr* 2002;132(2): 298–302. [PubMed: 11823594]
31. Sengottuvelan M, Viswanathan P, Nalini N. Chemopreventive effect of trans-resveratrol--a phytoalexin against colonic aberrant crypt foci and cell proliferation in 1,2-dimethylhydrazine induced colon carcinogenesis. *Carcinogenesis* 2006;27(5):1038–1046. [PubMed: 16338953]
32. Athar M, Back JH, Tang X, et al. Resveratrol: a review of preclinical studies for human cancer prevention. *Toxicol Appl Pharmacol* 2007;224(3):274–283. [PubMed: 17306316]
33. Brown JR, DuBois RN. COX-2: a molecular target for colorectal cancer prevention. *J Clin Oncol* 2005;23(12):2840–2855. [PubMed: 15837998]
34. Koki AT, Masferrer JL. Celecoxib: a specific COX-2 inhibitor with anticancer properties. *Cancer Control* 2002;9(2 Suppl):28–35. [PubMed: 11965228]
35. Blanke CD. Celecoxib with chemotherapy in colorectal cancer. *Oncology (Williston Park)* 2002;16 (4 Suppl 3):17–21. [PubMed: 12014863]
36. Dannenberg AJ, Altorki NK, Boyle JO, et al. Cyclo-oxygenase 2: a pharmacological target for the prevention of cancer. *Lancet Oncol* 2001;2(9):544–551. [PubMed: 11905709]

Figure 1. Structure of resveratrol and its derivatives. Resveratrol (RSVL, A) and two of its analogues, RSVL-2 (B) and RSVL-3 (C), are polyphenolic compounds differing by the number of hydroxyl or methoxy groups, respectively.

Figure 2.

In vitro and *ex vivo* verification of resveratrol binding with COX-2. (A), Expression of COX-2 in HCT116 or HT-29 human colon cancer cell lines. (B), Resveratrol binding with COX-2 *in vitro* was confirmed by immunoblotting with an antibody against COX-2. Lane 1–input control: COX-2 recombinant protein; lane 2–negative control: Sepharose 4B was used to pull down COX-2 as described in Materials and Methods; lane 3–COX-2 was pulled down using resveratrol-Sepharose 4B affinity beads. (C), COX-2 binding with resveratrol *ex vivo* was confirmed by immunoblotting with an antibody against COX-2. Lane 1–input control: whole-cell lysate from HT-29 human colon cancer cells; lane 2–negative control: a lysate prepared from HT-29 human colon cancer cells was precipitated with Sepharose 4B beads as described

in Materials and Methods; lane 3—whole-cell lysate prepared from HT-29 cells was precipitated by resveratrol-Sepharose 4B affinity chromatography as described in Materials and Methods. (D), Comparison of sensograms representing the interaction of RSVL, RSVL-2 or RSVL-3 (each at 300 μ M) with 0.1 μ g His-COX-2 as visualized by SPR (surface plasmon resonance) produced by a BIAcore X as described in Materials and Methods. The dissociation phase was started at 180 seconds. One representative graph from results of two independent experiments is shown.

Figure 3.

Resveratrol directly binds with COX-2. Binding of resveratrol to COX-2 was determined by fluorescence spectroscopy. (A), Increasing concentrations of resveratrol (5–120 μ M) amplify the fluorescence signal indicating an enhanced dose-dependent binding of resveratrol with COX-2. (B), Resveratrol (10 μ M) was incubated with or without (negative control) 0.2 μ M COX-2 recombinant protein and the emission spectra were collected after excitation at 320 nm; (a.u.) = absorbance units at 389 nm. (C), The K_d (dissociation kinetic value) of resveratrol's interaction with COX-2 was determined. COX-2 (0.2 μ M) was incubated with increasing concentrations of resveratrol (0–110 μ M). Fluorescence intensity at 389 nm was used to observe the change in fluorescence associated with resveratrol and protein binding. The inset

shows the fluorescence unit change at 389 nm used to determine the concentration of bound and free resveratrol. The graph is representative of three independent experiments.

Figure 4. Inhibitory effect of RSVL, RSVL-2, RSVL-3 or CEL on COX-2-mediated PGE₂ production. The effects of different concentrations of RSVL, its analogues, or CEL on COX-2-mediated PGE₂ production *in vitro*, using a recombinant COX-2 protein (Sigma; A), and *ex vivo*, using COX-2^{+/+} cells (B), were determined by measuring prostaglandin E₂ production as described in the protocol of the COX Inhibitory Screening Kit (Cayman Chemical). Data are presented as means ± S.D. of three samples from two independent experiments. For A and B, the asterisk (*) indicates a significant decrease ($p < 0.001$) in COX-2-mediated PGE₂ production compared to untreated control.

A **Figure 5.**

Inhibitory effect of RSVL and its analogues on growth and colony formation of HT-29 colon cancer cells in soft agar. (A), HT-29 cells (1×10^4) were treated or not treated with RSVL, RSVL-2, or RSVL-3 (each at $10 \mu\text{M}$) and cell number was calculated each day over 5 days. Data are presented as means $\pm$ S.D. from 3 samples of 2 independent experiments. The asterisk (*) indicates a significant decrease ($p < 0.001$) in cell number of treated cells compared to respective untreated control cells. (B), Final average number of colonies formed in soft agar. HT-29 colon cancer cells (1×10^4) were subjected to a soft agar assay with or without resveratrol or its analogues (10 or $20 \mu\text{M}$). The numbers of colonies were counted after 10 days. Results are expressed as means $\pm$ S.D. of three independent experiments. The asterisk (*) indicates a significant decrease in colony number of treated cells compared to untreated control cells (* $p < 0.03$, $20 \mu\text{M}$ RSVL; ** $p < 0.006$, $20 \mu\text{M}$ RSVL-2).

Figure 6.

Effect of RSVL, its analogues, or CEL on COX-2^{+/+} and COX-2^{-/-} cell growth after 6 days. COX2^{+/+} and COX2^{-/-} cells (inset shows COX02 expression in respective cell lines) were treated with RSVL, analogues, or CEL (10 μ M) and cell number was determined each day over a 6-day period using a Coulter Z cell counter. Control represents untreated cells. Data are presented as means $\pm$ S.D. of three samples from two independent experiments. The asterisk (*) indicates a significant difference ($p < 0.001$) in number of treated COX-2^{+/+} or COX-2^{-/-} cells compared to respective untreated cells.