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Original Contribution

Finding more active antioxidants and cancer chemoprevention agents by elongating the conjugated links of resveratrol

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ABSTRACT

Resveratrol is the subject of intense research as a natural antioxidant and cancer chemopreventive agent. There has been a great deal of interest and excitement in understanding its action mechanism and developing analogs with antioxidant and cancer chemoprevention activities superior to that of the parent compound in the past decade. This work delineates that elongation of the conjugated links is an important strategy to improve the antioxidant activity of resveratrol analogs, including hydrogen atom- or electron-donating ability in homogeneous solutions and antihemolysis activity in heterogeneous media. More importantly, **C3,** a triene bearing 4,4′-dihydroxy groups, surfaced as an important lead compound displaying remarkably increased antioxidant, cytotoxic, and apoptosis-inducing activities compared with resveratrol.

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Numerous reports suggest that a diet rich in fruits and vegetables reduces cancer risk in humans, and therefore using dietary antioxidants has become a fascinating strategy for cancer chemoprevention [1–7]. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) (**A1** in Scheme 1), a naturally occurring phytoalexin derived from more than 72 plant species, including a wide variety of fruits and vegetables such as grapes, berries, and peanuts, is one such dietary antioxidant and cancer chemoprevention agent [8–18]. Since Jang et al. first demonstrated the ability of resveratrol to inhibit carcinogenesis at multiple stages and pointed out that it would be worthy of further investigation as a dietary cancer chemoprevention agent in humans [19], the interest in this molecule has increased exponentially. The major findings have shown that it can suppress proliferation and directly induce apoptosis of cancer cells from diverse tissue origins by modulating a variety of molecular targets in the development of cancer, as detailed in recent reviews [8–18]. Interestingly, some studies have shown that resveratrol exhibits its cytotoxicity selectively against malignant cells with minimal cytotoxicity against normal blood cells [20–22]. Currently, resveratrol is in early clinical trials as an anti-cancer drug for treatment of human colon cancer (http://www.clinicaltrials.gov).

However, the potential use of resveratrol in cancer chemoprevention or chemotherapy settings has been hindered by its short half-life and low bioavailability [23,24]. Moreover, compared with other chemotherapy agents, resveratrol is not cytotoxic enough, and usually, relatively high concentrations are required to induce apoptosis of cancer cells [25]. The limitations of resveratrol accompanied with its structural simplicity and low toxicity have prompted interest in designing novel resveratrol analogs with cancer chemoprevention or chemotherapy activity superior to that of the parent compound [26–39]. Structural modifications of resveratrol focus mainly on alterations in hydroxyl and methoxy moieties, as well as the *cis*/*trans* configuration of stilbene, and some of these analogs have turned out to be more cytotoxic against various human cancer cells than resveratrol [26–39]. On the other hand, because free radical-mediated peroxidation of membrane lipids and oxidative damage of DNA have been implicated as a causative mechanism in cancer [40,41], the antioxidant activity and mechanism of resveratrol and its analogs have attracted much attention in the past decade [42–54]. It has been demonstrated that 4′-OH is more active than 3- and 5-OH in the antioxidant reaction of resveratrol by stationary γ-radiolysis and pulse radiolysis experiments [49,51], oxidation product analysis [52], experimental X-ray structure [47], and theoretical calculations [42,53]. Additionally, the double bond and its *trans*-isomery between the phenyl rings are also very important structural determinants for the antioxidant potency of resveratrol [50,53]. Specifically, the bond dissociation energy (BDE) values for 4′-OH of *trans*-resveratrol, *cis*-resveratrol, and α,β-dihydro-3,5,4′-trihydroxy-*trans*-stilbene in the gas phase have been calculated to be 78.683, 80.026, and 82.401 kcal/mol, respectively [53]. Our previous study showed that the introduction of electron-donating (ED) groups into the stilbene scaffold of resveratrol (ene compounds of series A in Scheme 1) could significantly increase its galvinoxyl (GO•) and 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity [54], antioxidant activity [55], pro-oxidant activity on DNA

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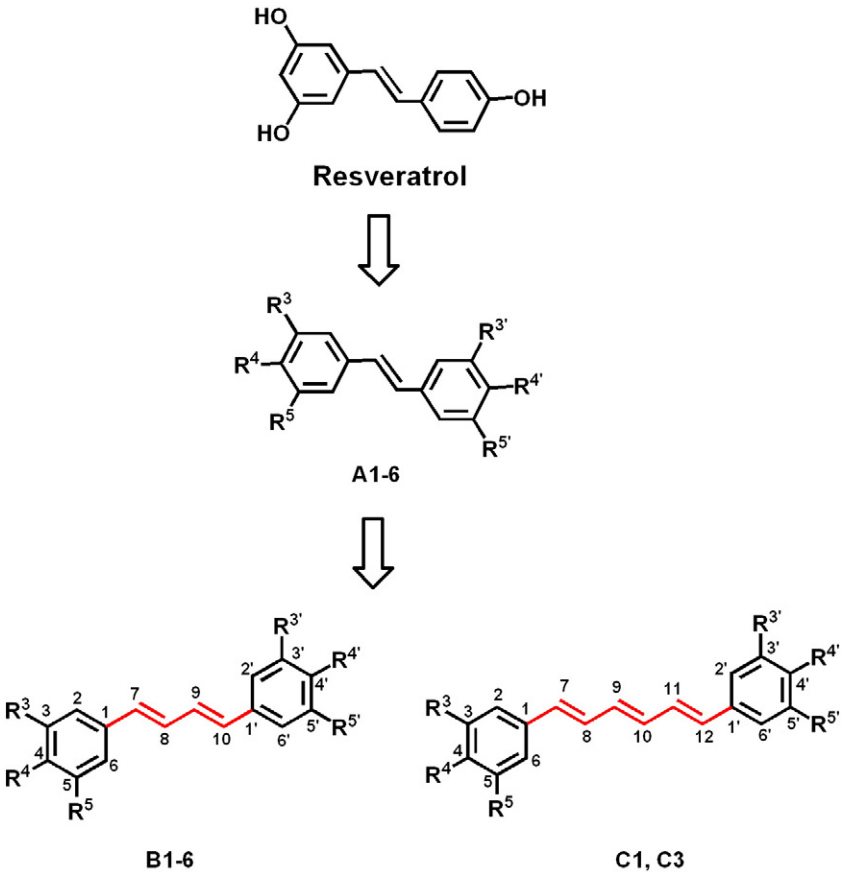
damage in the presence of Cu(II) ions [56], and cytotoxicity on human promyelocytic leukemia cells [57]. More recently, we have constructed resveratrol derivatives by incorporating a chroman moiety of vitamin E, resulting in the remarkable enhancement of tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl radical-scavenging activity [58].

From a free radical chemistry point of view, the high free radical-scavenging activity (antioxidant activity) of phenolic compounds relies on the resonance stabilization of the resulting phenoxyl radicals. Therefore, the double bond in the stilbene scaffold of resveratrol plays a pivotal role in stabilizing resonantly the phenoxyl radical to decrease the BDE of the phenolic O–H bond. We believe that one promising way to achieve the enhanced antioxidant activity would be elongation of the conjugated links of resveratrol, that is, the insertion of additional double bonds between two aromatic rings (diene and triene compounds of series B and C in Scheme 1). This chemical modification should not only improve the hydrogen atom- or electron-donating ability of resveratrol (see the Discussion) but also provide adequate lipophilicity, which is needed to penetrate the cell membrane. Thus, it might be valuable in the development of resveratrol-directed antioxidants and cancer chemoprevention agents. Considering the above remarks and as an extension of our previous studies [54–58], we synthesized resveratrol analogs by elongation of the conjugated links (diene and triene compounds of series B and C in Scheme 1) based on the concept of free radical chemistry, in which not only was 4- or 4′-OH on the phenyl rings and *trans*-isomery of the double bond between the phenyl rings retained, but also the ED groups (methoxy and hydroxyl) were introduced into the *ortho*- or *para*-position of 4-OH or 4′-OH. Some of them, such as 3,5,4′-trihydroxybiphenylbutadiene (**B1**) and 4,4′-dihydroxybiphenylhexatriene (**C3**), have been synthesized previously and can be characterized as an estrogen agonist [59] and a neuroprotective agent [60], as well as a selective probe potentially useful for detecting amyloid aggregates in the brain [61], respectively. Additionally, the backbone structure of diphenylhexatriene has been used as the fluorescent probe for monitoring low-density lipoprotein peroxidation [62]. Amazingly, however, virtually no effort has been devoted to evaluating systematically the antioxidant, cytotoxic, and apoptosis-inducing activities of the hydroxystilbenoid compounds with elongated conjugated links. We report herein the amplification of the above activities by a simple strategy, aimed at providing useful information for resveratrol-based antioxidant drug design.

Materials and methods

Materials

Galvinoxyl radical and 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox) were obtained from Acros Organics. Etoposide (VP-16), 5-fluorouracil (5-Fu), 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH), propidium iodide (PI), ribonuclease A (RNase A), 3-



Scheme 1. Molecular structures of resveratrol and its hydroxystilbenoid analogs.

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and sodium dodecyl sulfate (SDS) were purchased from Sigma–Aldrich and used as received. RPMI medium 1640 and 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) were from GIBCO and Alfa Aesar, respectively. Other chemicals used were of analytical grade.

Synthesis of compounds

The ene compounds in Scheme 1 were synthesized by the Wittig–Horner reaction as described in our previous papers [54,56]. The diene and triene compounds in Scheme 1 were synthesized by the Wittig–Horner reaction between appropriate aldehydes and corresponding phosphonates (Scheme 2; for details and characterization of all the hydroxystilbenoid compounds **B1–6, C1,** and **C3,** see the supplementary material). Structural determinations of the compounds were based on ^{1}H NMR, ^{13}C NMR spectra, HRMS (ESI), and EI–MS analysis. The purity of the compounds was checked by HPLC (see the supplementary material).

*trans,trans-3,5,4′-Trihydroxybistyryl (**B1**)*

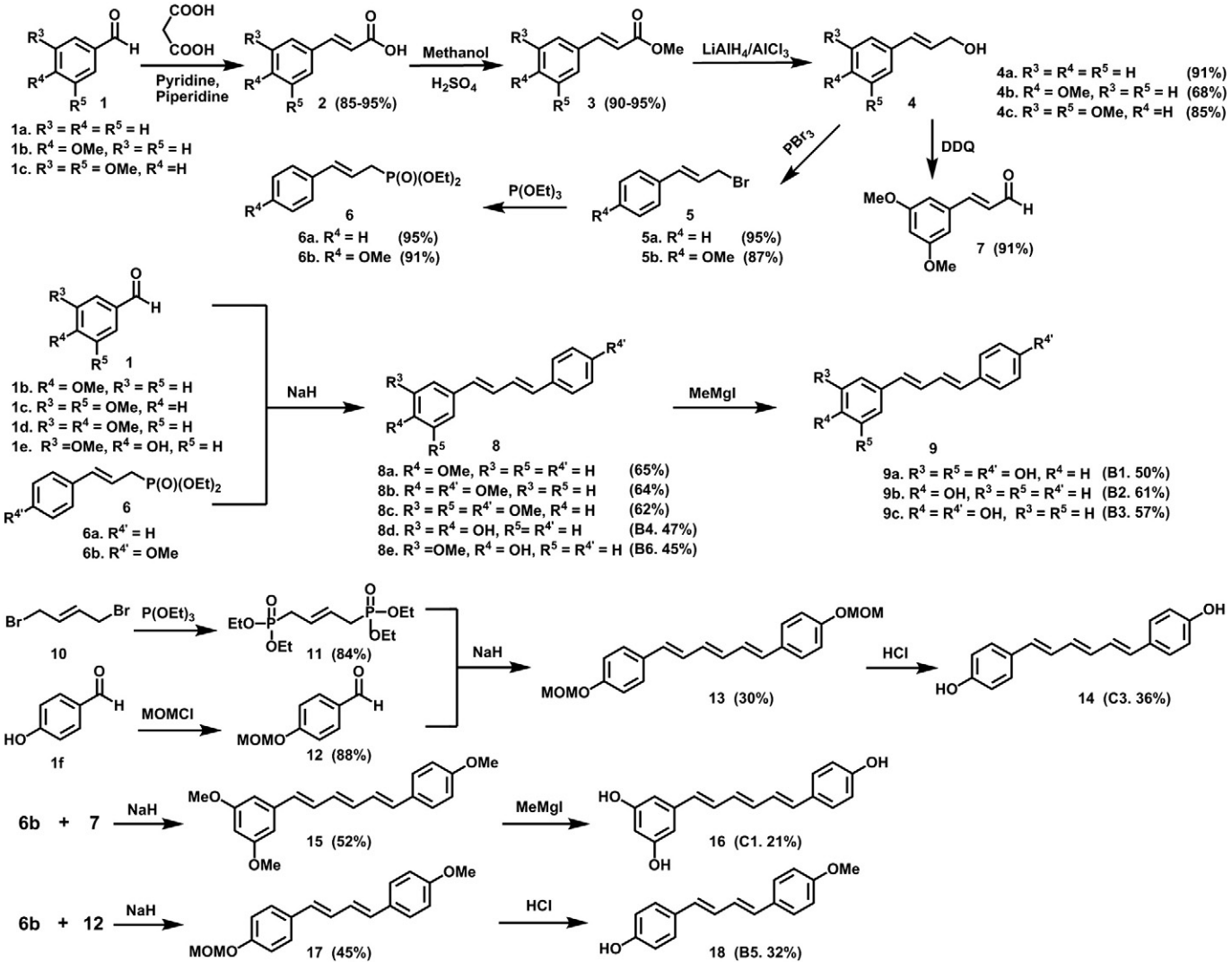
A light brown solid; mp 193–196 °C (lit. [59] mp 198–202 °C); ^{1}H NMR (400 MHz, acetone-d_6) δ 6.27 (t, J = 2.0 Hz, 1 H, H-4), 6.48–6.52 (m, 3 H, H-2, H-6, and H-10), 6.63 (d, J = 14.8 Hz, 1 H, H-7), 6.82 (d, J = 8.8 Hz, 2 H, H-3′ and H-5′), 6.85–6.94 (m, 2 H, H-8 and H-9), 7.36 (d, J = 8.8 Hz, 2 H, H-2′ and H-6′), 8.18 (s, 2 H, OH), 8.46 (s, 1 H, OH); ^{13}C NMR (100 MHz, acetone-d_6) δ 102.9 (1 C), 105.7 (2 C), 116.5 (2 C), 127.4 (1 C), 128.7 (2 C), 130.1 (1 C), 130.5 (1 C), 132.4 (1 C), 133.6 (1 C), 140.7 (1 C), 158.1 (1 C), 159.6 (2 C); HRMS (ESI) m/z calcd for $C_{16}H_{14}O_3$ (M-H): 253.0870. Found: 253.0865. Error 2.0 ppm.

*trans,trans-4-Hydroxybistyryl (**B2**)*

A pink solid; mp 192–194 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 6.60 (d, J = 15.6 Hz, 1 H, H-7), 6.80 (d, J = 15.6 Hz, 1 H, H-10), 6.83 (d, J = 8.4 Hz, 2 H, H-3 and H-5), 6.92 (dd, J = 15.6, 10.4 Hz, 1 H, H-8), 7.05 (dd, J = 15.6, 10.4 Hz, 1 H, H-9), 7.21 (t, J = 7.2 Hz, 1 H, H-4′), 7.33 (t, J = 7.6 Hz, 2 H, H-3′ and H-5′), 7.37 (d, J = 8.4 Hz, 2 H, H-2 and H-6), 7.48 (d, J = 7.6 Hz, 2 H, H-2′ and H-6′), 8.48 (s, 1 H, OH); ^{13}C NMR (100 MHz, acetone-d_6) δ 116.5 (2 C), 127.1 (2 C), 127.5 (1 C), 128.1 (1 C), 128.7 (2 C), 129.5 (2 C), 130.1 (1 C), 130.7 (1 C), 132.0 (1 C), 133.9 (1 C), 138.7 (1 C), 158.3 (1 C); MS (EI) m/z 222 [M$^+$].

*trans,trans-4,4′-Dihydroxybistyryl (**B3**)*

A white solid; mp 282–284 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 6.54–6.62 (m, 2 H, H-7 and H-10), 6.82 (m, 4 H, H-3, H-5, H-3′ and H-5′), 6.84–6.87 (m, 2 H, H-8 and H-9), 7.34 (d, J = 8.4 Hz, 4 H, H-2, H-6, H-2′ and H-6′); ^{13}C NMR (100 MHz, acetone-d_6) δ 116.5 (4 C), 127.9 (2 C), 128.5 (4 C), 130.4 (2 C), 132.2 (2 C) , 158.0 (2 C); MS (EI) m/z 238 [M$^+$].



Scheme 2. Synthesis of resveratrol analogs with elongation of the conjugated links.

*trans,trans-3,4-Dihydroxybistyryl (**B4**)*

A spongy white solid; mp 184–187 °C; ^{1}H NMR (300 MHz, acetone-d_6) δ 6.62 (d, J = 15.6 Hz, 1 H, H-7), 6.65 (d, J = 15.6 Hz, 1 H, H-10), 6.79–6.88 (m, 3 H, H-5, H-6 and H-8), 7.00–7.07 (m, 2 H, H-2 and H-9), 7.21 (t, J = 7.6 Hz, 1 H, H-4′), 7.32 (t, J = 7.6 Hz, 2 H, H-3′ and H-5′), 7.47 (d, J = 7.6 Hz, 2 H, H-2′ and H-6′), 7.97 (s, 2 H, OH); ^{13}C NMR (100 MHz, acetone-d_6) δ 113.8 (1 C), 116.4 (1 C), 120.0 (1 C), 127.1 (2 C), 127.6 (1 C), 128.1 (1 C), 129.5 (2 C), 130.7 (1 C), 130.9 (1 C), 132.0 (1 C), 134.2 (1 C), 138.8 (1 C), 146.2 (1 C), 146.3 (1 C); MS (EI) m/z 238 [M^+].

*trans,trans-4-Hydroxy-4′-methoxybistyryl (**B5**)*

A violet solid; mp 222–224 °C; ^{1}H NMR (300 MHz, acetone-d_6) δ 3.80 (s, 3 H, OCH_3), 6.60 (d, J = 14.7 Hz, 2 H, H-7 and H-10), 6.81–6.90 (m, 6 H, H-8, H-9, H-3, H-5, H-3′ and H-5′), 7.35 (d, J = 8.7 Hz, 2 H, H-2 and H-6), 7.42 (d, J = 8.7 Hz, 2 H, H-2′ and H-6′), 8.43 (s, 1 H, OH); ^{13}C NMR (75 MHz, acetone-d_6) δ 27.8 (1 C), 87.2 (2 C), 88.7 (2 C), 100.0 (1 C), 100.5 (1 C), 100.7 (2 C), 100.8 (2 C), 102.5 (1 C), 103.6 (1 C), 104.0 (1 C), 104.8 (1 C), 130.2 (1 C), 132.4 (1 C); MS (EI) m/z 252 [M^+].

*trans,trans-4-Hydroxy-3-methoxybistyryl (**B6**)*

A spongy white solid; mp 147–148 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.93 (s, 3 H, OCH_3), 5.69 (s, 1 H, OH), 6.61 (d, J = 14.7 Hz, 1 H, H-7), 6.64 (d, J = 14.7 Hz, 1 H, H-10), 6.81 (d, J = 14.7 Hz, 1 H, H-8), 6.82–7.00 (m, 4 H, H-2, H-5, H-6, and H-9), 7.23 (t, J = 7.8 Hz, 1 H, H-4′), 7.34 (t, J = 7.8 Hz, 2 H, H-3′ and H-5′), 7.44 (d, J = 7.8 Hz, 2 H, H-2′ and H-6′); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.1 (1 C), 108.3 (1 C), 114.8 (1 C), 120.7 (1 C), 126.5 (2 C), 127.4 (1 C), 127.6 (1 C), 128.9 (2 C), 129.6 (1 C), 130.3 (1 C), 132.0 (1 C), 133.1 (1 C), 137.8 (1 C), 145.8 (1 C), 146.9 (1 C); MS (EI) m/z 252 [M^+].

*trans,trans,trans-1-(3,5-Dihydroxyphenyl)-6-(4-hydroxyphenyl)-1,3,5-hexatriene (**C1**)*

A brown solid; mp 207–210 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 6.26 (s, 1 H, H-4), 6.45–6.60 (m, 6 H, H-2, H-6, H-7, H-9, H-10, and H-12), 6.79–6.89 (m, 4 H, H-8, H-11, H-3′, and H-5′), 7.34 (d, J = 8.4 Hz, 2 H, H-2′ and H-6′), 8.18 (s, 2 H, OH), 8.47 (s, 1 H, OH); ^{13}C NMR (100 MHz, acetone-d_6) 103.1 (1 C), 105.8 (2 C), 116.5 (2 C), 127.4 (1 C), 128.7 (3 C), 130.2 (1 C), 133.0 (1 C), 133.1 (1 C), 133.6 (1 C), 135.1 (1 C), 140.7 (1 C), 158.3 (1 C), 159.7 (2 C). HRMS (ESI) m/z calcd for $C_{18}H_{16}O_3$ (M-H): 279.1027. Found: 279.1023. Error 1.4 ppm.

*trans,trans,trans-1,6-Dis(4-hydroxyphenyl)-1,3,5-hexatriene (**C3**)*

A brown solid; mp 269–272 °C (lit. [63] mp 273–274 °C); ^{1}H NMR (400 MHz, acetone-d_6) δ 6.48 (dd, J = 7.2, 2.8 Hz, 2 H, H-9 and H-10), 6.54 (d, J = 15.2 Hz, 2 H, H-7 and H-12), 6.78–6.85 (m, 6 H, H-3, H-5, H-3′, H-5′, H-8, and H-11), 7.33 (d, J = 8.8 Hz, 4 H, H-2, H-6, H-2′, and H-6′), 8.45 (s, 2 H, OH); ^{13}C NMR (100 MHz, acetone-d_6) δ 116.5 (4 C), 127.6 (2 C), 128.6 (4 C), 130.3 (2 C), 132.8 (2 C), 133.6 (2 C), 158.1 (2 C); MS (EI) m/z 264 [M^+].

Assay for GO$^\bullet$-scavenging activity

The GO$^\bullet$-scavenging reaction kinetics in ethyl acetate was determined following the procedure described previously [54] (see also the supplementary material). All the hydroxystilbenoid compounds were stable in ethyl acetate. The concentration of GO$^\bullet$ in ethyl acetate was measured from its molar extinction coefficient value, ε, of 16,100 $M^{-1}cm^{-1}$ (λ_{max} 428 nm).

Assay for ferric reducing/antioxidant power (FRAP)

FRAP assay was used to evaluate the reducing capacity of resveratrol and its analogs according to the procedure of Benzie and Strain [64]. Briefly, 1.5 ml of FRAP reagent was incubated for 10 min at 37 °C. Then, 150 μl of water and 50 μl of various concentrations of resveratrol or its analogs (methanol solution) were added and the absorbance was measured at 593 nm after 4 min. The FRAP reagent was prepared fresh by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM ferric chloride in distilled water, in the ratio 10:1:1, respectively. The standard curve was built with methanol solution of known ferrous concentrations. The number of donated electrons was calculated from the slopes of the lineal adjustments between the concentrations of resveratrol or its analogs and the FRAP activity. Readings at the absorption maximum (593 nm) were taken using a Varian Cary 300 spectrophotometer equipped with a thermostated auto-cell-holder (accessory controller). Temperature was kept at 37 °C.

Assay for antihemolysis activity

Human red blood cells (RBCs; blood type B) were provided by the Red Cross Center for Blood (Gansu, China), which was authorized legally to be responsible for collecting blood from healthy subjects and provides blood products for clinical and scientific usage in our city. The extent of hemolysis was determined spectrophotometrically by measuring the absorbance of hemolysate at 540 nm as described previously [65] (see also the supplementary material). Resveratrol and its analogs were dissolved in dimethyl sulfoxide (DMSO) before experiments, and the volume of DMSO solution added to RBCs was less than 0.1% (v/v) of the reaction mixture.

Cell culture

Human promyelocytic leukemia (HL-60) and human hepatocellular (HepG2) cell lines were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and kept at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 kU/L), streptomycin (100 kU/L), and 2 mM glutamine. Exponentially growing cells were used throughout these experiments. Resveratrol and its analogs were dissolved in DMSO before the experiments and the volume of DMSO solution added to the cell suspension was less than 0.1% (v/v) of the culture medium.

Assay for cytotoxicity

The cell cytotoxicity was assessed by the MTT colorimetric assay, which is based on the reduction of MTT by the mitochondrial succinate dehydrogenase of intact cells to a purple formazan product [66]. HL-60 cells (5×10^4/ml) were added to each well of 96-well flat microtiter plates and incubated with various concentrations of resveratrol or its analogs for 48 h. HepG2 cells (2.0×10^4/ml) were added to each well for adherence for 24 h, then the medium was replaced by fresh medium and the cells were incubated with various amounts of resveratrol or its analogs for an additional 72 h. Four replicate wells were used for each point in the experiments. After 48 and 72 h for HL-60 and HepG2 cells, respectively, MTT solution (5 mg/ml in PBS) stored at 4 °C in the dark was added to each well and plates were incubated for 4 h at 37 °C. For HL-60 cells, 100 μl extraction buffer (10% SDS–5% isobutanol–0.1 M HCl) was added. The absorbance at 570 nm was measured after an overnight incubation at 37 °C. For HepG2 cells, after incubation for 4 h at 37 °C, the culture medium was removed and then 100 μl DMSO was added. The absorbance was read at 570 nm using a Bio-Rad M680 enzyme-linked immunosorbent assay microplate

reader. The percentage of cell viability was calculated relative to control wells designated as 100% viable cells.

Cell cycle analysis using flow cytometry

HepG2 cells were seeded in six-well plates and incubated for 24 h. After treatment with resveratrol or its selective analogs (**A3, B3,** and **C3**) for 48 h, the cells were harvested, washed with PBS (containing 1% fetal bovine serum), and fixed in 70% ethanol at 4 °C overnight. After centrifugation, the cells were resuspended in PBS and incubated in PI staining buffer (200 μg/ml DNase-free RNase A, 0.1% Triton X-100, 50 μg/ml PI in PBS) for 30 min at room temperature in the dark and analyzed using a FACSCanto flow cytometer (Becton–Dickinson, San Jose, CA, USA) as described previously [67]. The FACSDiva software program was used for the acquisition of the cells and cell cycle distributions were analyzed using Modfit LT 3.0 software. Twenty thousand events were collected per sample. Mean values from three independent experiments are presented.

Analysis of chromatin condensation

Condensation of chromatin is usually the late event in apoptosis and is detected by nuclear staining with Hoechst 33258 as described previously [68]. HepG2 cells were cultured on coverslips, which were kept in six-well plates for 24 h before treatment. To observe cells undergoing apoptosis after 48 h treatment with resveratrol or its selective analogs (**A3, B3,** and **C3**), Hoechst 33258 staining was performed according to the kit's instructions (Beyotime Institute Biotechnology, China). The cells were observed using a fluorescence microscopy with a × 40 objective lens (Leica DMI4000 B; Germany).

Annexin V–FITC and PI assays

The apoptosis in HepG2 cells was further detected with an annexin V–FITC/PI apoptosis detection kit (BD Biosciences) as described previously [69]. Seventy-two hours after treatment with resveratrol (50 μM) or **C3** (10 μM), cells were harvested, washed with ice-cold PBS, and then labeled with annexin V–FITC/PI according to the manufacturer's instructions. The cells were subsequently resuspended in 400 μl of binding buffer, and flow cytometric analysis (FACSCanto; Becton–Dickinson) was then performed. A total of 10,000 cells were acquired per sample and data were analyzed using FACSDiva software (Becton–Dickinson).

Results

Synthesis of resveratrol analogs with elongation of the conjugated links

Construction of the *trans*-stilbenoid skeletons with elongation of the conjugated links was accomplished by the Wittig–Horner reaction between appropriate aldehydes and corresponding phosphonates in the presence of NaH in tetrahydrofuran (Scheme 2; for synthetic details and characterization of all the hydroxystilbenoid compounds **B1–6, C1,** and **C3** see the supplementary material). **B1–3** and **C1** were finally obtained by demethylation with a large excess of the Grignard reagent MeMgI. In the case of **B4** and **B6,** bearing *ortho*-dihydroxy and *ortho*-methoxyhydroxy groups, protecting groups were not needed for the final Wittig–Horner reaction, and the products were obtained in 47 and 45% yields, respectively. Moreover, methoxymethyl chloride was used as a protective group for the synthesis of **B5** and **C3** because of the selective deprotection of phenolic OH and readily accessible routine, respectively.

GO•-scavenging activity of the hydroxystilbenoid compounds

Galvinoxyl radical is a stable phenoxyl radical and commonly applied in the estimation of reactivity of an antioxidant (ArOH) toward radicals [Eq. (1)] [70]:

$$\mathrm{ArOH} + \mathrm{GO}^{\bullet} \rightarrow \mathrm{ArO}^{\bullet} + \mathrm{GOH}. \quad (1)$$

We have determined previously the second-order rate constants for the reaction of GO• with resveratrol and its hydroxystilbene analogs (ene compounds in Scheme 1) in ethyl acetate by monitoring spectrophotometrically at 428 nm the loss of GO• over time [54]. For easy comparison the same method was also used to evaluate GO•-scavenging activity of the hydroxystilbenoid compounds with elongation of the conjugated links (diene and triene compounds in Scheme 1). The determined second-order rate constants (k_2) of the hydroxystilbenoid compounds are listed in Table 1. A comparison of k_2 values of the compounds with three different backbones clearly indicates that their GO•-scavenging activity follows the sequence of triene > diene > ene, highlighting the importance of elongation of the conjugated links. For example, the k_2 values of **C1** (triene) and **B1** (diene) bearing the same 3,5,4′-trihydroxy groups are approximately 9 and 6 times larger, respectively, than that of the corresponding ene, resveratrol (**A1**). Moreover, introduction of ED groups, such as methoxy and hydroxyl, in the *ortho*- or *para*-position of 4-OH or 4′-OH significantly increases the GO•-scavenging activity as suggested by the rank order **4**>**6**≈**3**>**5**>**2** in series A and B. Noticeably, **B4,** with two double bonds and one 3,4-dihydroxy moiety, is the most active among these compounds examined, giving a k_2 value that is 200 times larger than that of resveratrol.

Reducing capacity of the hydroxystilbenoid compounds

The reducing capacity of the compounds in three series was also estimated through the FRAP assay according to the procedure of Benzie and Strain [64]. The method measures the reduction of Fe^{3+} (ferric iron) to Fe^{2+} (ferrous iron) in the presence of antioxidants or reductants (ArOH) [Eq. (2)]:

$$\mathrm{ArOH} + \mathrm{Fe}^{3+} \rightarrow \mathrm{ArOH}^{\bullet+} + \mathrm{Fe}^{2+}. \quad (2)$$

The results are expressed as the number of donated electrons per molecule and summarized in Table 1. In accordance with what was found in the GO•-scavenging reaction, reducing capacity increases with increasing the electron-rich environment in the molecules and further improves by elongation of the conjugated links. The best result was again obtained with **B4,** whose reducing capacity is three times more active than that of resveratrol.

Antihemolysis activity of the hydroxystilbenoid compounds

One of the well-recognized targets of free radical-mediated injury is lipid peroxidation. Free radical-mediated peroxidation of polyunsaturated fatty acids (PUFAs) leads to oxidative damage of cell membranes, which is termed oxidative stress and might play a causative role in cancer [40,41,71]. Morrow et al. have previously provided clear evidence of free radical-induced oxidative injury in that a series prostaglandin F_2-like compounds derived from PUFAs is produced in vivo in humans by a noncyclooxygenase, free radical-catalyzed mechanism [71].

Thermal decomposition of AAPH in the aqueous dispersion of human RBCs produces an initiating radical, which can attack the PUFAs in RBC membranes to induce lipid peroxidation. This ultimately results in hemolysis, as detected easily by spectroscopy at 540 nm [65]. Therefore, the AAPH-induced RBC hemolysis model was used to probe antioxidant properties of the compounds in heterogeneous media. Fig. 1A presents a set of hemolytic curves in the absence and presence of various concentrations of AAPH. The RBCs were stable and

Table 1
Antioxidant and cytotoxic activities of resveratrol and its hydroxystilbenoid analogs (ArOHs).

ArOH	k_2[a] (M^{-1} s^{-1})	Reducing capacity[b]	t_{eff}[b] (min)	n	C log P[c]	IC_{50}[d] (μM)	
						HL-60	HepG2
A1	15.7[f]	1.53 ± 0.05	35	1.9	2.833	36.3 ± 1.8[e]	182 ± 3
B1	90.8 ± 3.3	1.67 ± 0.05	39	2.2	3.037	29.3 ± 1.1	167 ± 12
C1	142 ± 1	1.70 ± 0.04	47	2.6	3.641	24.7 ± 2.6	>200
A2	9.7[e]	1.29 ± 0.02	37	2.1	4.167	32.9 ± 1.5	>200
B2	68.0 ± 4.2	1.78 ± 0.03	54	3.0	4.371	37.5 ± 3.6	70.9 ± 2.4
A3	109[e]	2.15 ± 0.11	50	2.8	3.500	6.5 ± 0.1[f]	18.5 ± 0.5
B3	274 ± 5	2.56 ± 0.02	81	4.5	3.704	3.9 ± 0.7	23.5 ± 1.5
C3	411 ± 13	2.82 ± 0.11	101	5.6	4.308	1.7 ± 0.6	7.7 ± 0.3
A4	1.47×10^{3}[e]	4.07 ± 0.30	78	4.3	3.570	16.8 ± 0.8	103 ± 14
B4	$(3.36 \pm 0.12) \times 10^{3}$	4.65 ± 0.15	92	5.1	3.774	5.4 ± 0.8	35.9 ± 2.0
A5	33.0[e]	1.79 ± 0.12	—	—	4.086	23.8 ± 0.7	49.3 ± 3.3
B5	171 ± 7	2.25 ± 0.17	—	—	4.290	9.4 ± 1.1	15.2 ± 1.4
A6	101[e]	1.51 ± 0.11	—	—	4.016	>80	>200
B6	282 ± 4	2.03 ± 0.11	—	—	4.220	65.6 ± 2.2	>200
VP-16	—	—	—	—	—	3.3 ± 0.3	—
5-Fu	—	—	—	—	—	—	5.3 ± 0.1
Trolox	—	2.29 ± 0.10	36	2.0	3.017	—	—

[a] The rate constants of **B1**, **C1**, **B2**, **B3**, **C3**, **B5**, and **B6** were determined by pseudo-first-order kinetics, whereas the rate constant of **B4** was measured by second-order kinetics with the ratio of [**B4**]/[GO•] being 1/1. Data are expressed as the mean ± SD for three determinations.
[b] Data are the average of three determinations, which were reproducible with deviation less than ± 10%.
[c] Calculated using Bio-Loom software [77,78].
[d] Data are expressed as the mean ± SD for three determinations. HL-60 and HepG2 cells were treated with ArOHs for 48 and 72 h, respectively.
[e] Cited from Ref. [54].
[f] Cited from Ref. [57].

no hemolysis took place within 4.5 h in the absence of AAPH (line a). When 25 mM AAPH was used, the rate of hemolysis was very slow (line b). However, addition of 50, 75, or 100 mM AAPH induced fast hemolysis after an inhibition time (t_{inh}) (100, 80, or 60 min, respectively; lines c–e). This inhibition time stems from the presence of the endogenous antioxidant such as vitamin E and/or ubiquinol-10 in the RBC membrane [72]. It can be seen from Fig. 1A and its inset that the rate of hemolysis and the inhibition time are inversely correlated with the concentration of AAPH. A concentration of 50 mM AAPH was selected to conduct the following experiment, as it was found capable of producing an adequate inhibition time (100 min) followed by a fast hemolysis. Incubation of resveratrol with RBCs significantly increased the intrinsic inhibition time of the RBCs (Fig. 1B). The inhibition time was 117, 135, 150, and 170 min when the concentration of resveratrol was 10, 20, 30, and 40 μM, respectively. This corresponds to the additional, or effective, inhibition time, t_{eff}, being 17, 35, 50, and 70 min, respectively. The effective inhibition time produced by resveratrol depended on its concentration, as illustrated in the inset of Fig. 1B. To easily compare the antioxidant capacity of resveratrol and its analogs to inhibit RBC hemolysis, a concentration of 20 μM was chosen for the compounds in the subsequent experiment (Fig. 1C). The introduction of double bonds showed a positive impact on t_{eff} as shown, for example, for resveratrol (**A1**), **B1,** and **C1,** with the same 3,5,4′-trihydroxy groups (Fig. 1C and Table 1). The impact was more evident in the compounds with the same 4,4′-dihydroxy groups but bearing different numbers of double bonds (**A3, B3,** and **C3;** Fig. 1C and Table 1). Furthermore, the total number of LOO• (lipid peroxyl radicals) trapped per molecule of resveratrol and its analogs, that is, the stoichiometric factor ($n_{LOO\bullet}$), was also calculated by using the equation $t_{eff} = n_{LOO\bullet}$ [antioxidant]/R_i, where R_i is the apparent rate of chain initiation, which can be determined with the reference antioxidant, Trolox, whose $n_{LOO\bullet}$ is taken as 2 [73]. The t_{eff} and $n_{LOO\bullet}$ values for all of these compounds are listed in Table 1. It can be concluded from the t_{eff} and $n_{LOO\bullet}$ indexes that both introduction of ED groups and elongation of the conjugated links are important for enhancing antihemolysis activity. The results obtained from this experiment are similar to those obtained from GO•-scavenging and ferric-reducing experiments mentioned above. However, in the anti-hemolysis experiment, compound **C3** (a triene bearing 4,4′-dihydroxy groups) is the most active among the compounds investigated, with the highest t_{eff} and $n_{LOO\bullet}$ values being 101 min and 5.6, respectively.

Cytotoxicity of the hydroxystilbenoid compounds

The cytotoxicity of these hydroxystilbenoid compounds in three series was further investigated together with that of resveratrol in human promyelocytic leukemia HL-60 and human hepatoma HepG2 cells by MTT assay [66]. The results are expressed as the IC_{50}, the concentration for the compound to cause 50% inhibition of cell viability, and are summarized in Table 1. A striking feature of our data was that the compounds bearing 4,4′-dihydroxy groups (**A3, B3,** and **C3**) exhibited remarkably higher cytotoxicity on HL-60 and HepG2 cells than resveratrol. Compound **C3** is the most cytotoxic among the compounds examined, with IC_{50} values of 1.7 and 7.7 μM in HL-60 and HepG2 cells, respectively. The most promising compound **C3** is at least 20-fold more efficacious than resveratrol in HL-60 and HepG2 cells and is superior to etoposide (VP-16) in HL-60 cells as well as being comparable to 5-Fu in HepG2 cells, the two chemotherapeutic agents commonly used.

Effects of resveratrol and its active analogs on cell cycle of HepG2 cells

To further explore the mechanisms by which resveratrol and its active analogs (**A3, B3,** and **C3**) exert their cytotoxic potencies, their effects on the cell cycle distribution of HepG2 cells were also evaluated by flow cytometry after cells were stained with propidium iodide [67]. As shown in Fig. 2, treatment with 50 μM resveratrol resulted in a remarkable accumulation of cells in S phase at 48 h. In contrast, compounds **A3, B3,** and **C3** caused a clear block of cells in G0/G1 phase, suggesting that they impaired cell cycle progression with a mechanism different from that of resveratrol.

Apoptosis-inducing effects of resveratrol and its active analogs in HepG2 cells

Finally, the apoptosis-inducing effects of the compounds in HepG2 cells were characterized by changes in nuclear morphology (Fig. 3A)

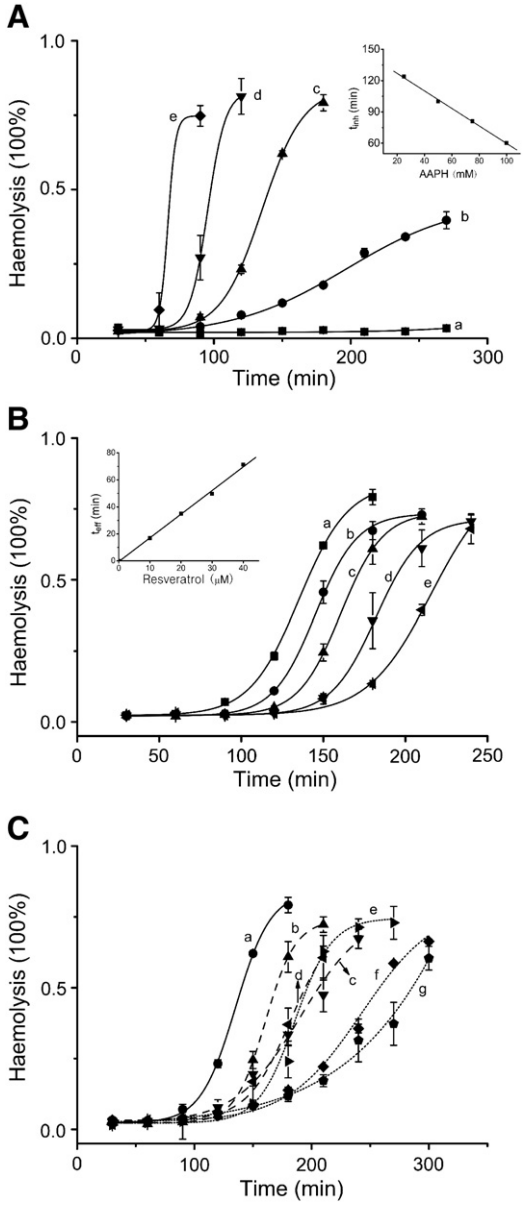


Fig. 1. (A) AAPH-induced hemolysis of 5% human RBCs in 0.15 M PBS (pH 7.4) under air atmosphere at 37 °C. The initial concentrations of AAPH were: (curve a) 0, (b) 25 mM, (c) 50 mM, (d) 75 mM, and (e) 100 mM. The inset shows the relationship between the intrinsic inhibition time, t_{inh}, and the initial concentration of AAPH. (B) Inhibitory effect of resveratrol at various concentrations against 50 mM AAPH-induced RBC hemolysis. The initial concentrations of resveratrol were: (curve a) 0, (b) 10 μM, (c) 20 μM, (d) 30 μM, and (e) 40 μM. The inset shows the relationship between the additional, or effective, inhibition time, t_{eff}, and the initial concentration of resveratrol. (C) Inhibitory effect of resveratrol and its hydroxystilbenoid analogs (ArOHs) against 50 mM AAPH-induced RBC hemolysis. The initial concentration of the ArOHs was 20 μM. (Curve a) native RBCs, (b) inhibited with resveratrol (**A1**), (c) inhibited with **B1**, (d) inhibited with **C1**, (e) inhibited with **A3**, (f) inhibited with **B3**, and (g) inhibited with **C3**. Data are expressed as the mean of three RBC samples.

[68] and flow cytometric analysis (Fig. 3B) [69]. Staining with Hoechst 33258 showed fragmentation and condensation of chromatin in HepG2 cells treated with 100 μM resveratrol for 48 h, compared with the untreated control (Fig. 3A), demonstrating a proapoptotic activity of resveratrol. This apoptotic tendency was more apparent in **A3, B3,** and **C3** at relatively low concentration (50 μM; Fig. 3A). Flow cytometric analysis by staining cells with annexin V–FITC and propidium iodide revealed that treatment with 50 μM resveratrol for 72 h induced 16.5 and 10.8% early and late apoptotic cell death, respectively (Fig. 3B). Notably, 10 μM **C3** showed very high apoptosis-inducing activity, with 31.5 and 22.6% early and late apoptotic cells, respectively (Fig. 3B).

Discussion

Resveratrol, a natural polyphenol with stilbenoid core, features prominently in dietary antioxidants and cancer chemoprevention agents. Over the course of the past decade, a tremendous amount of work has been directed toward improving its antioxidant and cancer chemoprevention activities [26–39,43,45,46,48,50,54–58]. This work describes efforts to find more active antioxidants and cancer chemoprevention agents by elongating the conjugated links of resveratrol.

The formal abstraction of hydrogen atoms of a phenolic antioxidant (ArOH) by a free radical ($X^{\bullet}$) can occur by at least two different chemical pathways: direct hydrogen atom transfer (HAT) [Eq. (3)] and single-electron transfer (SET) [Eq. (4)] [74]:

$$ArOH + X^{\bullet} \rightarrow ArO^{\bullet} + XH, \quad (3)$$

$$ArOH + X^{\bullet} \rightarrow ArOH^{\bullet+} + X^{-} \rightarrow ArO^{\bullet} + XH. \quad (4)$$

In the SET mechanism, a phenolic antioxidant first loses an electron to form the radical cation ($ArO^{\bullet+}$) followed by rapid and reversible deprotonation in solution, resulting in the same net result ($ArO^{\bullet}$ and XH) as in the HAT mechanism. However, the essence of the HAT mechanism is different from that of SET. The former is relevant to the hydrogen atom-donating ability of phenolic antioxidants and the stability of the resulting phenoxyl radicals ($ArO^{\bullet}$), which are characterized by the BDE of the phenolic O–H bond, whereas the latter corresponds to the electron-donating ability of phenolic antioxidants and stability of the radical cations, which deal with the ionization potential (IP). Both the BDE and the IP are of particular importance for antioxidant molecules, and the molecules with lower values of BDE and IP are endowed with higher antioxidant activity. It should be pointed out that very low IP will result in air instability of electron-rich antioxidants, which limits their antioxidant efficacy, because they might react with molecular oxygen to generate superoxide. However, the compounds used in this study were stable in either solid or solution form under air during the experiments.

We have demonstrated previously that in ethyl acetate, with a low ability to ionize phenolics, the $GO^{\bullet}$-scavenging reaction of resveratrol and its hydroxystilbene analogs (ene compounds in Fig. 1) occurs primarily by the HAT mechanism [Eqs. (1) and (3)] [54]. Consequently, we first evaluated the hydrogen atom-donating ability of the hydroxystilbenoid compounds by a quantitative decay kinetic determination of $GO^{\bullet}$ in ethyl acetate. Based on the determined second-order rate constants (k_2) (Table 1) the hydrogen atom-donating ability of the hydroxystilbenoid compounds can be arranged in the order **4** (3,4-dihydroxy groups) >**6** (4-hydroxy-3-methoxy groups) ≈**3** (4,4′-dihydroxy groups) >**5** (4-hydroxy-4′-methoxy groups) >**1** (3,5,4′-trihydroxy groups) >**2** (4-hydroxy group) within each series. This result shows that the introduction of ED groups (methoxy and hydroxyl) in the *ortho*- or *para*-position of 4-OH or 4′-OH decreases significantly the BDE of 4-OH or 4′-OH. Wright and coworkers have pointed out by theoretical calculations that the introduction of an extra hydroxy group into the *ortho*- and *para*-position of phenol decreases the BDE of O–H by 9.2 and 5.9 kcal/mol, respectively [74]. Compounds **A4** and **B4,** bearing 3,4-dihydroxy groups, displayed the highest hydrogen atom-donating ability in series A and B, respectively. This could be understood because the oxidative intermediate, *o*-hydroxyphenoxyl radical, is more stable because of the intramolecular hydrogen bonding interaction, as evidenced from theoretical calculations that suggest that the hydrogen bond in the *o*-hydroxyphenoxyl radical is approximately

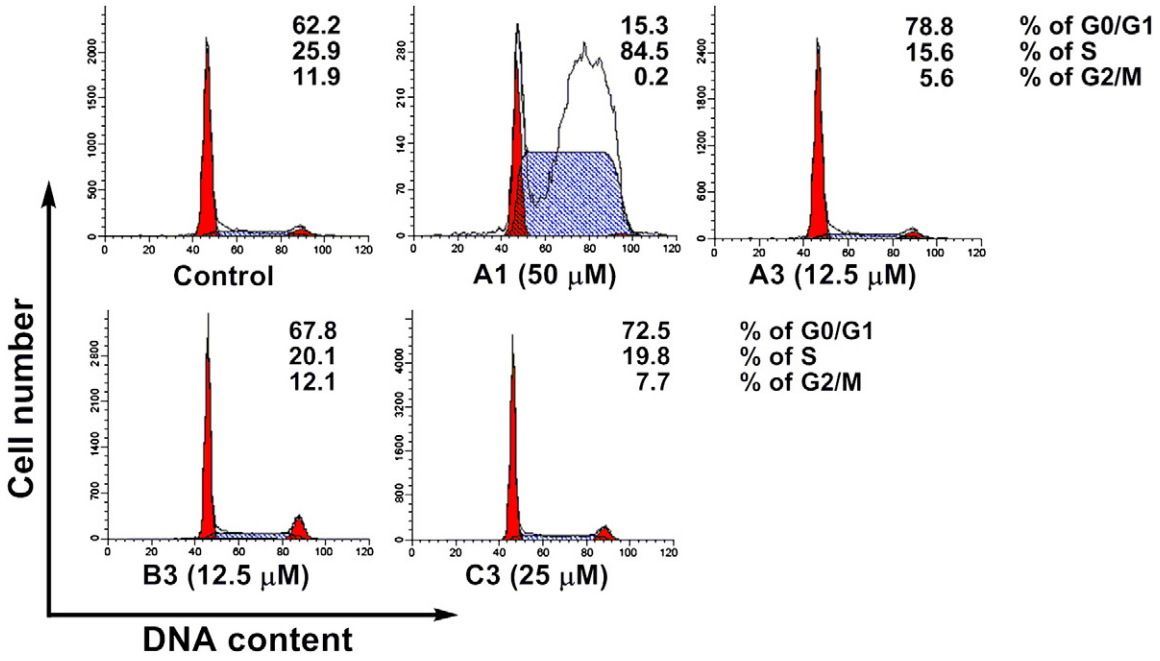


Fig. 2. Effects of resveratrol (**A1**) and its active analogs (**A3**, **B3**, and **C3**) at the indicated concentrations on the cell cycle of HepG2 cells. The cultured cells were treated with these compounds for 48 h, then harvested, and analyzed by flow cytometry as described in the materials and methods. Each experiment was performed in triplicate.

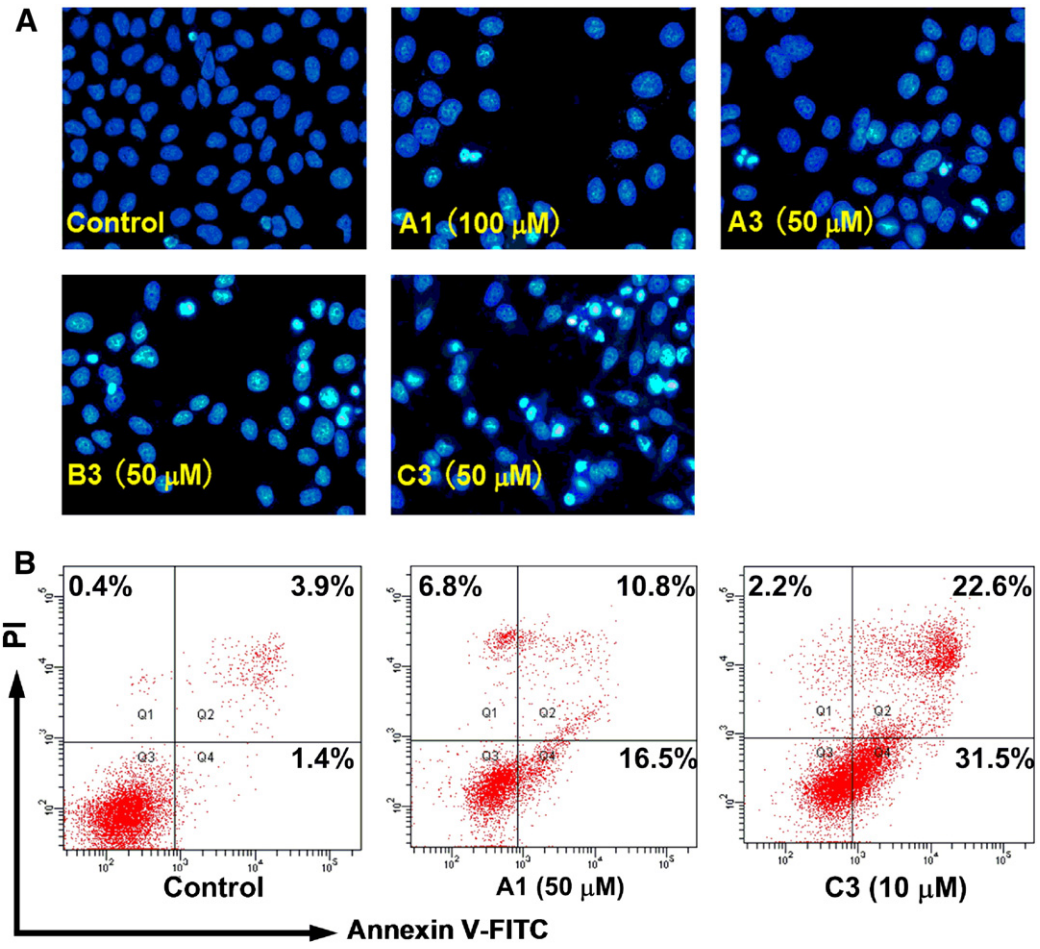


Fig. 3. Induction of apoptosis by resveratrol (**A1**) and its active analogs (**A3**, **B3**, and **C3**) at the indicated concentrations in HepG2 cells. (A) Fluorescence microscopic analysis of cells stained with Hoechst 33258 after 48 h of treatment with these compounds. (B) Flow cytometric analysis of viable, apoptotic, and necrotic cells after 72 h of treatment with resveratrol (**A1**) and **C3**. Cells in the lower left quadrants are alive, in the lower right quadrants are in early apoptosis, in the upper right quadrants are in late apoptosis, and in the upper left quadrants are necrotic. Percentage of total signal within the quadrant is indicated. Each experiment was performed in triplicate.

4 kcal/mol stronger than that in the parent catechol [74]. Additionally, Mikulski et al. have also reported the BDE values in the gas phase for 4′-OH of *trans*-resveratrol (**A1**) and 4-OH of 4,4′-dihydroxy-*trans*-stilbene (**A3**) and 3,4-dihydroxy-*trans*-stilbene (**A4**) to be 78.683, 77.663, and 69.734 kcal/mol, respectively [53], in line with our experimental results. It can also be seen from the k_2 values of Table 1 that the hydrogen atom-donating ability order of the compounds bearing the same substituents but having different backbones is triene > diene > ene, that is, the longer the conjugated chain is, the stronger the hydrogen atom-donating ability is. Taken together, these observations support the notion that in addition to the introduction of ED groups, elongation of the conjugated links is also of great importance for improving the hydrogen atom-donating ability of resveratrol analogs because of the resonance stabilization of the resulting phenoxyl radical. This leads to **B4,** with two double bonds and one 3,4-dihydroxy moiety, being the strongest hydrogen atom donor among all compounds tested.

We next employed the reduction of ferric iron (FRAP assay) [64] to assess the electron-donating ability of resveratrol and its analogs [Eqs. (2) and (4)]. In the experiment, compound **B4** proved to be the strongest electron donor among all compounds examined, and the number of donated electrons per molecule was 4.65. Comparing the reducing capacity of resveratrol analogs, it is clear that electron-donating ability increases with introduction of ED groups and further improves by elongation of the conjugated links, suggesting that both structural factors help to enhance the stability of radical cations and thus lower the IP.

It has been recognized that the antioxidant activity in homogeneous solutions may not be the same as that in heterogeneous media such as micelles, liposomes, and biomembranes, let alone in vivo [73,75,76]. One of the reasons for this inconsistency is apparently that the microenvironment that the antioxidant experiences in heterogeneous media is significantly different from that in homogeneous solutions. Because the system used in the GO$^{\bullet}$-scavenging (ethyl acetate) or FRAP (water) experiments is a homogeneous solution, we further employed an AAPH-induced RBC hemolysis model to assess the antioxidant activity of resveratrol and its analogs in heterogeneous media (Fig. 1). Comparing the antihemolysis activity (t_{eff} and $n_{LOO\bullet}$) of resveratrol and its analogs, we found a trend similar to that obtained from GO$^{\bullet}$-scavenging and ferric-reducing experiments and can conclude that the two structural factors contribute to enhancing antihemolysis activity, that is, introduction of ED groups and elongation of the conjugated links. Interestingly, although compound **C3** (a triene bearing 4,4′-dihydroxy groups) has relatively weak hydrogen atom- and electron-donating abilities compared with **B4,** it is the most active among the compounds examined in the antihemolysis experiment, with the highest t_{eff} and $n_{LOO\bullet}$ values being 101 min and 5.6, respectively. This implies that the lipophilicity of the compound could be another predominant factor influencing the efficiency of the antioxidant in heterogeneous media. The interaction of a compound with biomembranes or the uptake of a compound into the membranes strongly depends on its lipophilicity, which can be represented by the octanol–water partition coefficient, *P*. Therefore, the calculated log *P* (C log *P*) values of resveratrol and its analogs by Bio-Loom software [77,78] are listed in Table 1. As expected, the lipophilicity (C log *P* value) of the compounds with the same substituents increases with elongation of the conjugated links (Table 1), and compound **C3** (C log $P = 4.308$) indeed has relatively high lipophilicity compared with **B4** (C log $P = 3.774$), hence facilitating its penetration into the membrane and subsequent reaction with LOO$^{\bullet}$ within the membrane. In addition to the hydrogen atom- and electron-donating abilities, many factors may also play an important role in determining what makes an effective antioxidant in vivo, including the lipophilicity, stabilization, localization, transport to specific tissue, and so on. In view of RBCs being a biologically relevant system, **C3** would be more suitable as a lead compound in resveratrol-directed antioxidant design than **B4.**

Finally, we evaluated the cytotoxic, cell cycle arrest, and apoptosis-inducing effects of resveratrol and its analogs on cancer cells. MTT assay [66] demonstrated that the compounds bearing 4,4′-dihydroxy groups (**A3, B3,** and **C3**) exhibited remarkably higher cytotoxicity on HL-60 and HepG2 cells than resveratrol. We have noted previously that 4,4′-dihydroxy-*trans*-stilbene (**A3**) displays more potent cytotoxicity against HL-60 than resveratrol [57]. Additionally, the compound has also been reported to selectively induce the down-regulation of estrogen receptor α on MCF-7 breast cancer cells [32] and to inhibit proliferation of LF1 human lung fibroblasts with higher efficiency but a different mechanism compared to resveratrol [29]. However, in the present study, the introduction of a double bond to form the corresponding triene, **C3,** leads to further improvement in the activity against HL-60 and HepG2 cells. As a matter of fact, **C3** displayed the highest cytotoxic activity among the compounds tested, with IC_{50} values of 1.7 and 7.7 μM in HL-60 and HepG2 cells, respectively. On the basis of the cytotoxicity study, resveratrol and its active analogs (**A3, B3,** and **C3**) were selected for further investigation to determine whether the cytotoxicity against HepG2 cells is mediated by cell cycle arrest and induction of apoptosis.

Cell cycle distribution analysis revealed that resveratrol induced a remarkable accumulation of HepG2 cells in S phase. Similar accumulation of cells in S phase has been reported previously for various carcinoma cells exposed to resveratrol [79–84]. However, treatment of the compounds (**A3, B3,** and **C3**) resulted in a clear block of cells in G0/G1 phase, revealing that the superior cytotoxicity of the compounds over resveratrol was associated with a mechanism different from that of resveratrol in cell cycle progression. Stivala and co-workers have found recently that compound **A3** induces an accumulation of human lung fibroblasts predominantly in G1 phase, whereas resveratrol perturbs the G1/S phase transition [29].

It is well recognized that apoptosis is a very important mechanism involved in the anti-cancer effect induced by chemopreventive and chemotherapeutic agents and can be characterized by changes in nuclear morphology [68] and annexin-V/PI binding assay [69]. It is seen from Fig. 3 obtained from the two assays that **A3, B3,** and **C3** show higher apoptosis-inducing activity against HepG2 cells than resveratrol, and **C3** again ranks top among the compounds investigated.

To sum up, hydroxystilbenoid compounds with elongation of the conjugated links were constructed based on resveratrol, a well-known natural antioxidant and cancer chemopreventive agent, and their antioxidant, cytotoxic, and apoptosis-inducing activities were evaluated systematically. The data strongly suggest that elongation of the conjugated links is an important strategy to improve the antioxidant activity of resveratrol analogs, including hydrogen atom- or electron-donating ability in homogeneous solutions and antihemolysis activity in heterogeneous media. Most impressively, a triene bearing 4,4′-dihydroxy groups, **C3,** surfaced as an important lead compound, displaying remarkably increased cytotoxic and apoptosis-inducing activities against HL-60 and HepG2 cells by comparison with resveratrol. Therefore, this compound deserves further investigation to ascertain its potential benefits for cancer chemoprevention including the related apoptotic mechanism and metabolic stability.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2011.02.028.

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