Antioxidant-Based Lead Discovery for Cancer Chemoprevention: The Case of Resveratrol

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Resveratrol is a well-known natural antioxidant and cancer chemopreventive agent that has attracted much interest in the past decade. Resveratrol-directed compounds were synthesized, and their antioxidant effects against reactive oxygen species (ROS)-induced DNA damage, their prooxidant effects on DNA damage in the presence cupric ions, and their cytotoxic and apoptosis-inducing effects on human promyelocytic leukemia (HL-60) cells were investigated in vitro. It was found that the compounds bearing *o*-diphenoxyl groups exhibited remarkably higher activities in inhibiting ROS-induced DNA damage, accelerating DNA damage in the presence cupric ions, and inducing apoptosis of HL-60 cells compared with the ones bearing no such groups. The detail mechanism of the structure−activity relationship was also studied by the oxidative product analysis of resveratrol and its analogues with galvinoxyl radical or cupric ions and UV−visible spectra change in the presence cupric ions. This study reveals a good and interesting correlation between antioxidant and prooxidant activity, as well as cytotoxicity and apoptosis-inducing activity against HL-60 cells, and provides an idea for designing antioxidant-based cancer chemoprevention agents.

Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a phytoalexin found in a wide variety of dietary sources including grapes, plums, and peanuts.[1] It is also present in red wine at high levels (0.1−14.3 mg/L)[1b] and has been suggested to be linked to the low incidence of heart diseases in some regions of France, the so-called "French paradox"; i.e., despite high fat intake, mortality from coronary heart disease is lower because of the regular drinking of wine.[2] Recently, Sinclair's group and others reported that resveratrol could activate sirtuin deacetylases and extend the life span of yeast and flies,[3] implicating its potential as an antiaging agent in treating age-related human diseases. More recently, Baur et al. showed that this compound could improve the health of middle-aged mice on a high-calorie diet and significantly increase their survival.[4] One of the most striking biological activities of resveratrol intensely investigated during the past years has been its cancer chemopreventive or anticancer properties.[5] These properties were first appreciated when Pezzuto and colleagues demonstrated the ability of resveratrol to inhibit carcinogenesis at multiple stages.[5a] Since then, resveratrol has been becoming a synonym of naturally occurring molecules possessing chemopreventive activities and research on the compound has been gathering momentum. This has resulted in a flurry of papers reporting that resveratrol can activate various signal pathways to inhibit tumor cells growth and directly induce an apoptotic event, such as activation of caspases, p53, and Bax and inhibition of Bcl2 and NF-κB (reviewed in refs 5b−e).

The cancer chemoprevention effect elicited by resveratrol can be traced back to its antioxidant activity,[1a,6] since free-radical-mediated oxidative damage of DNA might play a causative role in cancer.[7] Therefore, the antioxidant activities of resveratrol have attracted considerable attention.[8] However, growing experimental evidence suggests that antioxidants present in food as chemopreventive agents are independent of their ability to scavenge reactive oxygen species (ROS[a]).[9] Every antioxidant is in fact a redox agent and thus might become a prooxidant to accelerate DNA damage under special conditions. Actually, resveratrol, as a prooxidant, could induce DNA damage in the presence of cupric ions by the production of ROS,[10] and its prooxidant action is believed to play an important role in its cancer chemopreventive and apoptosis-inducing properties, as ROS can mediate apoptotic DNA fragmentation.[6,10d,11]

The structure simplicity and low toxicity of resveratrol offer the promise for designing new chemopreventive agents from an antioxidant/prooxidant point of view. In our ongoing research project on bioantioxidants, we previously found that simple structural modification of resveratrol could significantly enhance its antioxidative activity against free radical induced lipid peroxidation in micelles, rat liver microsomes, and low-density lipoprotein.[12] Therefore, it is desirable to see if the same structure−activity relationship (SAR) is also valid in inhibiting ROS-induced DNA damage, accelerating DNA damage in the presence of cupric ions, and inducing apoptosis of human leukemia (HL-60) cells, with emphasis placed on the detail mechanistic study of the SAR. Resveratrol-directed compounds studied were 3,4-dihydroxy-*trans*-stilbene (3,4-DHS), 3,4,4′-trihydroxy-*trans*-stilbene (3,4,4′-THS), 3,4,5-trihydroxy-*trans*-stilbene (3,4,5-THS), 2,4-dihydroxy-*trans*-stilbene (2,4-DHS),

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[a] Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane hydrochloride); 3,4-DHS, 3,4-dihydroxy-*trans*-stilbene; 3,4,4′-THS, 3,4,4′-trihydroxy-*trans*-stilbene; 3,4,5-THS, 3,4,5-trihydroxy-*trans*-stilbene; 2,4-DHS, 2,4-dihydroxy-*trans*-stilbene; 3,5-DHS, 3,5-dihydroxy-*trans*-stilbene; 3,5,4′-TMS, 3,5,4′-trimethoxy-*trans*-stilbene; ROHs, resveratrol and its analogues; EDTA, ethylenediaminetetraacetic acid; EB, ethidium bromide; HL-60 cells, human leukemia cells; HPLC, high performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; PBS, phosphate-buffered saline; PI, propidium iodide; SDS, sodium dodecyl sulfate; SAR, structure−activity relationship.

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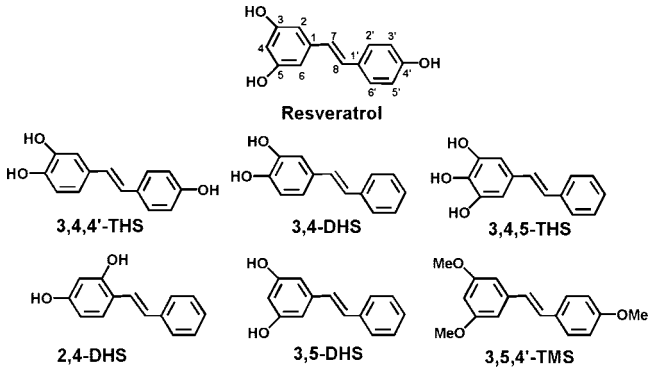
Chart 1



3,5-dihydroxy-*trans*-stilbene (3,5-DHS) and 3,5,4′-trimethoxy-*trans*-stilbene (3,5,4′-TMS) (Chart 1).

Results

Antioxidant Effects of Resveratrol and Its Analogues (ROHs). Inhibition of AAPH-Initiated DNA Strand Breakage by ROHs. The single strand breakage in supercoiled plasmid DNA leads to the formation of open circular form, whereas the formation of the full length linear form of the plasmid is indicative of a double-strand breakage.[13] Since 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) is water-soluble and the rate of free radical generation from AAPH can be easily controlled and measured, it has been extensively used as a free radical initiator for biological studies. Thermal decomposition of AAPH at physiological temperature generates alkyl radicals that can react with oxygen and give alkylperoxyl radicals. Alkylperoxyl radicals then attack plasmid DNA and lead to DNA strand breakage. AAPH-induced plasmid pBR322 DNA strand breakage and its inhibition by ROHs were assessed by agarose gel electrophoresis analysis (parts A−C of Figure 1). As shown in Figure 1A, the supercoiled DNA was gradually converted into the circular and linear forms with an increase of concentration of AAPH. The inhibition effect produced by resveratrol depended on the concentration of resveratrol, as illustrated in Figure 1B. With the increase of the concentration of resveratrol, its inhibition effect became clearer and the content of supercoiled DNA increased. The inhibition effects of resveratrol analogues also depended on the specific compound used, as exemplified in Figure 1C. Resveratrol analogues (10 μM) exhibited significant inhibition effects on the DNA breakage excepting 3,5,4′-TMS. On the basis of the percentage of intact supercoiled DNA, the activity for the inhibition of DNA strand breakage followed the sequence of 3,4-DHS ∼ 3,4,4′-THS > resveratrol > 3,4,5-THS > 2,4-DHS > 3,5-DHS > 3,5,4′-TMS (Figure 1D).

Protective Effects of ROHs against H_2O_2-Induced Thymocytes Death. Thymocytes from BALB/c mice were exposed to H_2O_2 for 24 h, and its viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.[14] Upon addition of H_2O_2 to the cells, its viability was decreased in a dose-dependent manner (Figure 2A). Figure 2B showed the effects of increasing concentration of ROHs in preventing H_2O_2-induced thymocytes death. It is seen from the figure that the compounds bearing *o*-diphenoxyl groups (3,4-DHS, 3,4,5-THS, and 3,4,4′-THS) exhibit remarkably higher activities in preventing the cells death than resveratrol. However, other analogues were ineffective in this experimental condition.

Moreover, nuclear DNA was isolated from thymocytes treated with 50 μM H_2O_2 for 6 h in the presence or absence of ROHs and subjected to agarose gel electrophoresis (Figure 2C). The addition of H_2O_2 resulted in the cleavage of DNA into discontinuous mono- and oligonucleosomal size fragments that formed a typical DNA ladder, a biochemical hallmark of apoptosis (lane 2 in Figure 2C). It is clearly seen that 100 μM 3,4-DHS, 3,4,4′-THS, and 3,4,5-THS could significantly reduce the formation of DNA ladder (lanes 3, 4, and 9 in Figure 2C). Resveratrol and 3,5,4′-TMS showed a similar but somewhat weaker effect (lanes 5 and 8 in Figure 2C). The SAR obtained from this experiment is similar to that obtained from the strand breakage of DNA by gel electrophoresis mentioned above.

Protective Effects of ROHs against H_2O_2-Induced DNA Damage in Human Peripheral Blood Lymphocytes. Furthermore, we also investigated the inhibitory effects of ROHs on H_2O_2-induced DNA damage in human peripheral blood lymphocytes using the single-cell alkaline electrophoresis (comet) assay.[15] It was found that human lymphocytes showed increased DNA damage after treated with H_2O_2, and the percentage of DNA damage was about 95% in the presence of 50 μM H_2O_2 (parts A and B of Figure 3). DNA damage was inhibited by 3,4-DHS in a concentration-dependent manner (parts C and D of Figure 3). Figure 3E showed the protective effects of 12.5 μM ROHs against H_2O_2-induced DNA damage. All of these analogues prevented the DNA damage of human lymphocytes treated with H_2O_2 in the order of 3,4-DHS ∼ 3,4,4′-THS > 3,4,5-THS > resveratrol > 2,4-DHS > 3,5-DHS > 3,5,4′-TMS (Figure 3E).

Oxidative Products of Resveratrol and 3,4-DHS in the Presence of Galvinoxyl Radical. Galvinoxyl radical is a relatively stable oxygen radical and has been widely used for evaluating antioxidant activities. To investigate the antioxidation mechanism of ROHs, we isolated and identified the reaction products of resveratrol and 3,4-DHS oxidized by galvinoxyl radical in ethanol at room temperature. The major products (Figure 4) were dihydrofuran dimer in the case of resveratrol and a dioxane-like dimer in the case of 3,4-DHS by characterizing with HRMS (ESI) and 1D and 2D NMR (see Supporting Information). More than eight values for coupling constant (^3J) between H-7 and H-8 in the dimers suggested a predominant pseudo-trans-axial arrangement for these two aliphatic protons. Furthermore, enantiomeric composition of the dimers was evaluated by high performance liquid chromatography (HPLC) analysis on a chiral column and it was found that the dimer was a racemic mixture. It is suggested from the oxidative products that the hydroxyl group at the 4-position is much easier to subject to oxidation than other hydroxyl groups, and the dioxane-like dimer is formed via an *o*-quinone intermediate (vide infra). The oxidative product analysis also suggests the importance of *o*-dihydroxyl groups and 4-hydroxyl groups.

Prooxidant Activities Analysis of ROHs. Calf Thymus DNA Damage Induced by ROHs in the Presence of Cu(II). The calf thymus DNA damage by ROHs in combination with Cu(II) was assessed by fluorometric analysis with the ethidium bromide (EB) binding assay[16] (Figure 5). It was found that neither resveratrol analogues nor Cu(II) alone caused detectable DNA damage (data not shown). However, in the presence of 250 μM Cu(II) the calf thymus DNA was remarkably damaged by 3,4-DHS, 3,4,4′-THS, and 3,4,5-THS in a concentration-dependent manner, and to a lesser extent, by resveratrol and 2,4-DHS (Figure 5). 3,5-DHS and 3,5,4′-TMS were inactive (data not shown). It is worth noting that the compounds bearing *o*-dihydroxyl groups (3,4-DHS, 3,4,4′-THS, and 3,4,5-THS) are

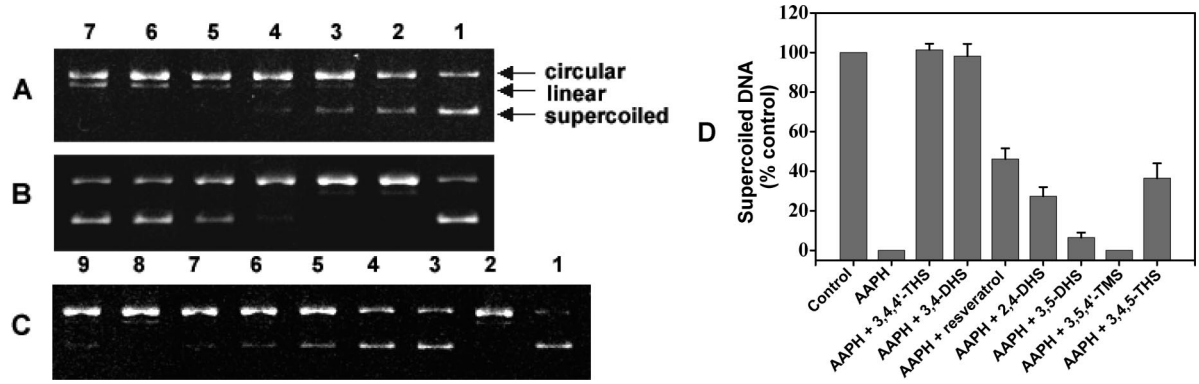


Figure 1. Agarose gel electrophoresis pattern of pBR322 DNA strand breakage induced by AAPH and inhibited by ROHs. Supercoiled plasmid DNA (100 ng) was incubated with AAPH and/or ROHs in phosphate-buffered saline (PBS, pH 7.4) at 37 °C for 60 min. (A) DNA strand breakage induced by the indicated concentration of AAPH: (lane 1) control; (lanes 2−7) 1.25, 2.5, 5, 10, 20, and 40 mM AAPH, respectively. (B) Inhibitory effect of resveratrol on AAPH (10 mM) induced DNA strand breakage: (lane 1) control; (lanes 2−7) 0, 2.5, 5, 10, 20, and 40 μM resveratrol, respectively. (C) Inhibitory effects of ROHs (10 μM) on AAPH (10 mM) induced DNA strand breakage: (lane 1) control; (lane 2) 10 mM AAPH alone; (lanes 3−9) 3,4,4′-THS, 3,4-DHS, resveratrol, 2,4-DHS, 3,5-DHS, 3,5,4′-TMS, and 3,4,5-THS, respectively. (D) Quantitative analysis of protective effects of ROHs (10 μM) against AAPH (10 mM)-induced DNA strand breakage. DNA damage is represented by the percentage of supercoiled DNA to native DNA. Experimental details are described in the section Material and Methods. Values represent the mean ± SD (n = 3).

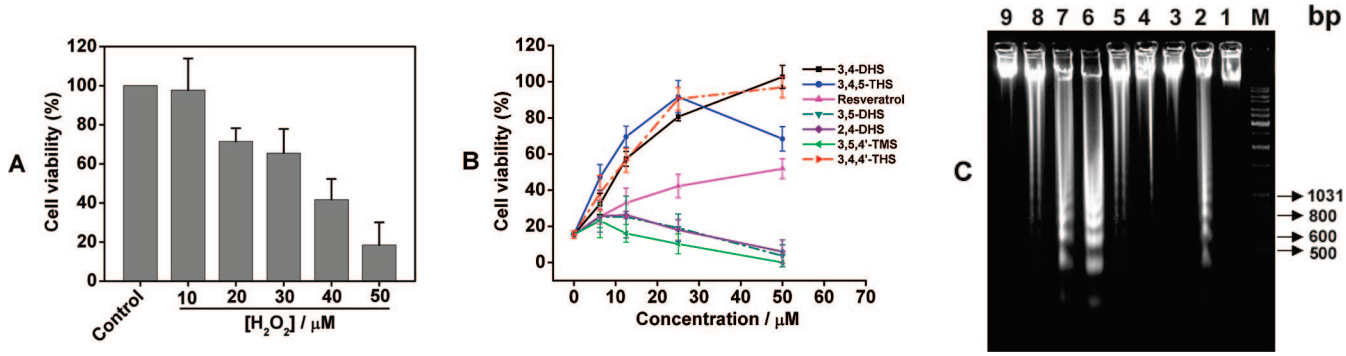


Figure 2. (A) Effect of H_2O_2 on cell viability. Viable thymocytes were determined using MTT reduction assay as described in the section Materials and Methods and expressed as a percentage of the untreated control cell samples. Samples were incubated for 24 h in the presence or absence of H_2O_2 (10−50 μM). Values are the mean ± SD (n = 4). (B) Inhibitory effects of ROHs on H_2O_2-induced thymocyte death. Thymocytes were preincubated with resveratrol or its analogues for 30 min, and 50 μM H_2O_2 was then added to the medium. After incubation for 24 h, cell viability was determined using MTT reduction assay. Values are the mean ± SD (n = 4). (C) Inhibitory effects of ROHs on DNA fragmentation induced by H_2O_2 in thymocytes. Thymocytes were preincubated with resveratrol or its analogues (100 μM) for 30 min, and 50 μM H_2O_2 was then added to the medium. After incubation for 6 h, nuclear DNA was extracted from thymocytes and analyzed by agarose gel electrophoresis: (lane M) DNA marker; (lane 1) control; (lane 2) 50 μM H_2O_2 alone; (lanes 3−9) 3,4,4′-THS, 3,4-THS, resveratrol, 2,4-DHS, 3,5-DHS, 3,5,4′-TMS, 3,4,5-THS, respectively, in the presence of 50 μM H_2O_2.

most active, followed by compounds bearing 4-hydroxyl groups (resveratrol and 2,4-DHS), while those bearing no such groups (3,5-DHS and 3,5,4′-TMS) are inactive.

UV−Visible Spectral Changes of ROHs in the Presence of Cu(II). In order to clarify the mechanism of the DNA damage, the UV−visible absorption changes of ROHs in the presence of Cu(II) were examined. The addition of Cu(II) to 3,4-DHS resulted in a rapid disappearance of the maximal absorption at 317 nm accompanied by its bathochromic shift to 342 nm (Figure 6A). This peak (342 nm) is characteristic of the formation of a chelate complex of 3,4-DHS with Cu(II). Its intensity decreased with an increase of time, and a new peak appeared at 459 nm because of the formation of the *o*-semiquinone anions or *o*-quinone (vide infra). The isosbestic point at 385 nm suggested a direct transition from one form (3,4-DHS−Cu(II) chelate) to another one (*o*-semiquinone anion). Similar slight bathochromic shift from 329 to 334 nm and appearance of a new peak at 439 nm, as well as one isobestic point, were observed in the case of 3,4,4′-THS (Figure 6B), but its decay was faster than that of 3,4-DHS. The decay of 3,4,5-THS was the fastest one among these compounds. It was too fast to make the new peak corresponding to the formation of the *o*-semiquinone anion or *o*-quinone observable (Figure 6C). In the case of resveratrol, its decay was significantly slower than that of 3,4-DHS and a similar bathochromic shift and a new peak of *o*-semiquinone anion or *o*-quinone were not observed (inset in Figure 6A). There were no clear spectral changes for 3,5-DHS and 3,5,4′-TMS in the presence of Cu(II). The decay rate of ROHs and/or ROH-Cu(II) chelate complexes follows the sequence of 3,4,5-THS > 3,4,4′-THS > 3,4-DHS > resveratrol > 3,5-DHS and 3,5,4′-TMS. This sequence correlates well with the activity sequence of the DNA damage mentioned above.

The formation of 3,4-DHS−Cu(II) chelate was confirmed by the reaction with ethylenediaminetetraacetic acid (EDTA), a well-known chelating agent for metal ions (Figure 7). EDTA was added after Cu(II) reacted with 3,4-DHS for 10 min. Upon addition of EDTA, the red-shifted bands (342 nm) returned to its initial position with a decrease in absorbance, but the new produced band (459 nm) did not show any change. If EDTA was added together with Cu(II), the spectrum of 3,4-DHS (line 1) did not show any change. These results indicate unambigu-

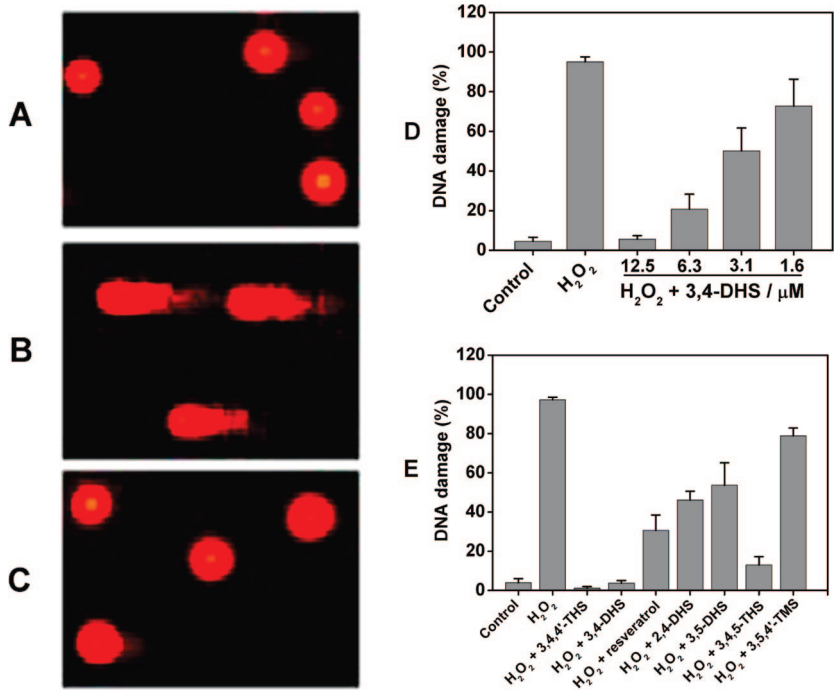


Figure 3. Representative single-cell alkaline electrophoresis patterns of human peripheral blood lymphocytes. Human lymphocytes in PBS were pretreated with 12.5 μM ROHs analogues, and 50 μM H_2O_2 was then added to the medium. After incubation for 10 min, DNA damage of individual lymphocytes was estimated by comet assay as described in the section Materials and Methods. (A) Untreated control. (B) H_2O_2 alone. (C) H_2O_2 + 3,4-DHS. (D) Protection of the indicated concentrations of 3,4-DHS from H_2O_2-induced DNA damage in human peripheral blood lymphocytes. Values are the mean ± SD ($n = 3$). (E) Protection of ROHs from H_2O_2-induced DNA damage in human peripheral blood lymphocytes. Values are the mean ± SD ($n = 3$).

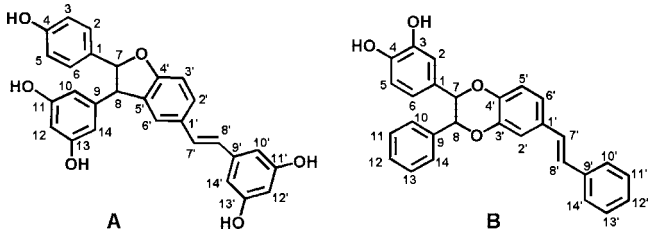


Figure 4. Oxidation products of resveratrol (A) and 3,4-DHS (B) in the presence of galvinoxyl radical.

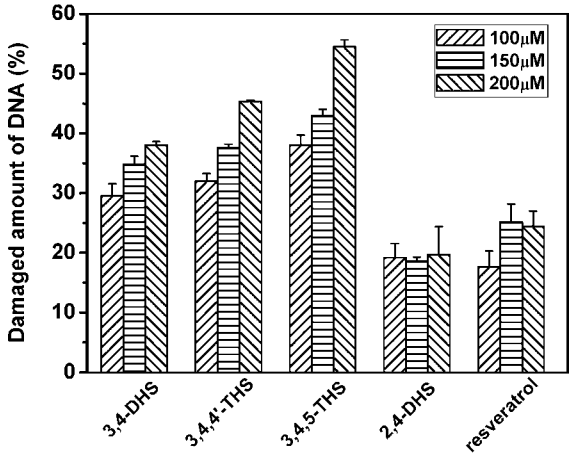


Figure 5. Extent of calf thymus DNA damages induced by ROHs in the presence of Cu(II). DNA (80 μg) was incubated at 37 °C for 60 min with different concentrations of ROHs in the presence of 250 μM Cu(II) in PBS buffer (pH 7.4) under air.

ously that 3,4-DHS can chelate with Cu(II) as a bidentate ligand, hence facilitating intramolecular electron transfer to form the stable *o*-semiquinone anions (vide infra). The participation of oxygen in the reaction of 3,4-DHS with Cu(II) was studied by mixing 3,4-DHS with Cu(II) under an inert atmosphere. It can be seen from line 4 in the inset of Figure 6 that under an inert atmosphere, although the 3,4-DHS−Cu(II) chelate still formed, the band at 459 nm did not appear. That is, Cu(II) could not induce the reaction of 3,4-DHS in the absence of O_2.

Oxidative Products of Resveratrol and 3,4-DHS in the Presence of Cu(II). To investigate the prooxidation mechanism of ROHs, we also isolated and identified the oxidative products of resveratrol and 3,4-DHS in the presence of Cu(II) in acetonitrile at room temperature. The dimer products in the presence of Cu(II) were the same as that in the presence of galvinoxyl radical. Other ROHs bearing no 4-hydroxyl group (3,5-DHS and 3,5,4′-TMS) gave no corresponding dimers. The result also indicates unambiguously that *o*-quinone may be the reaction intermediate in the case of 3,4-DHS.

Apoptosis-Inducing Activities of ROHs for HL-60 Cells. The antiproliferative effects of ROHs on HL-60 cells were assessed by the colorimetric MTT assay,[14] and results are demonstrated in Figure 8A. It is seen from the figure that ROHs exhibit dose-dependent inhibitory effects on HL-60 cell proliferation. The cell viability was reduced to 70%, 54%, and 38% after treatment with 10, 15, and 20 μM, respectively, in the case of 3,4,5-THS for 48 h. Other analogues showed similar effects, but to a lesser extent, on suppression of the cell viability as exemplified in Figure 8A. The IC_{50} values for all of these compounds are shown in Figure 8B. It indicates that 3,4-DHS, 3,4,4′-THS, and 3,4,5-THS exhibited higher cytotoxicity than resveratrol and other analogues.

It is now well recognized that apoptosis is implicated for multistage carcinogenesis and removal of damaged precancerous cells via apoptosis provides an important strategy for the treatment of cancer.[17] Apoptosis can be characterized by the cleavage of DNA into discontinuous mono- and oligo-nucleo-

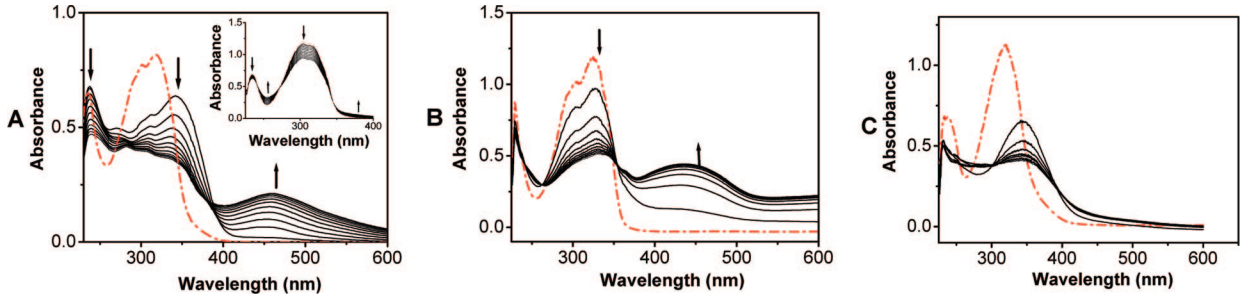


Figure 6. Absorption spectral changes of ROHs (50 μM) in the absence (dash line) and in the presence of 25 μM Cu(II) in PBS buffer (pH 7.4) under air: (A) 3,4-DHS (inset, resveratrol) (interval, 3 min); (B) 3,4,4′-THS (interval, 1 min); (C) 3,4,5-THS (interval, 1 min). Arrows show the time-related absorbance changes.

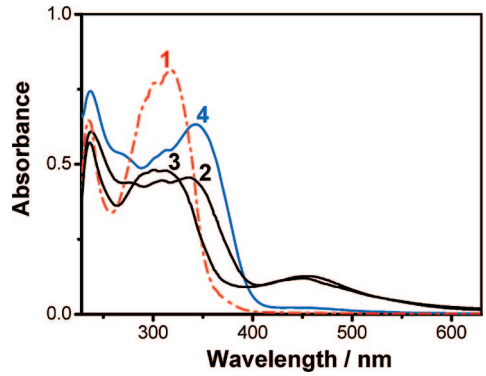


Figure 7. Absorption spectral changes of 3,4-DHS: (trace 1) 3,4-DHS (50 μM); (trace 2) 3,4-DHS (50 μM) plus Cu(II) (25 μM) for 10 min under air; (trace 3) same as 2 but adding EDTA (75 μM) under air; (trace 4) same as 2 but recorded under argon atmosphere.

somal size fragments that form a typical DNA ladder during gel electrophoresis.[18] Therefore, nuclear DNA was isolated from treated as well as untreated HL-60 cells and subjected to agarose gel electrophoresis. As illustrated in Figure 9, electrophoresis of DNA extracted from HL-60 cells treated with ROHs at certain concentrations for 48 h exhibited a progressive increase of the nonrandom fragmentation of the DNA into a ladder of 180−200 bp nucleosomes.

In order to quantify the apoptotic activity of ROHs flow cytometric analysis was performed to detect the apoptotic cells, i.e., cells with sub-G1 content of DNA.[19] HL-60 cells were treated with ROHs at certain concentrations for 48 h, then fixed and stained with propidium iodide (PI) for flow cytometric analysis. The results are shown in the DNA frequency distribution histograms (Figure 10), in which the M1 marker shows the sub-G1 cell fraction that corresponds to the percentage of apoptotic cells. It is seen from the figure that these compounds at certain concentrations exhibit significant apoptotic activity with the sub-G1 cell fraction ranging from 17.6% to 56.15%. Especially, 50 μM 3,4,5-THS shows very high apoptotic activity (Figure 10). Again, the molecules bearing the *o*-dihydroxyl functionality exhibited remarkably higher activity than resveratrol and molecules bearing no such functionality.

Discussion

Chemoprevention is an active cancer preventive strategy to intervene in the progress of carcinogenesis, using naturally occurring or synthetic substances.[20] There is considerable evidence that ROS or free radical-induced oxidative DNA damage contributes to human tumorigenesis[7] and that fruits and vegetables contain substantial amounts of various natural compounds with antioxidant properties that are associated with a lower incidence of various cancers.[21] From this has developed the idea that antioxidants in these foods are the effective cancer chemopreventive agents.[21] In the past year of the past century the Chemoprevention Working Group to the American Association for Cancer Research[22] published its report "Prevention of Cancer in the Next Millennium", demonstrating that chemoprevention by using antioxidants has become a viable alternative means in cancer control. On the other hand, however, there is no doubt that free radical biology and medicine and antioxidant therapy are still in their infancy. Many mechanisms on the action of antioxidants in cancer chemoprevention are still unclear, which are called "antioxidant conundrum",[23] and many questions are still under debate.[24] The cancer chemopreventive properties of antioxidants are generally believed to be due to their ability to scavenge endogenous ROS. However, just as ROS has pros and cons for human health, an antioxidant can switch to a prooxidant under certain conditions and the prooxidant action might not always be adverse for humans. As a matter of fact, cancer cell apoptosis induced by polyphenolic antioxidant are considered to be mediated by ROS,[25] and these antioxidants are also nontoxic to the normal cells.[26] It is indeed quite puzzling as to how these antioxidants can differentiate between "normal" versus "abnormal tumor" cells in terms of signaling, gene expression, and pharmacological effects. The present work focused on the antioxidant and prooxidant activity and the apoptosis-inducing activity against HL-60 cells of resveratrol analogues with the different structure features that enable us to deduce a clear picture of the structure determinants for the activity and a structure−activity relationship. Furthermore, an interesting correlation among the antioxidant activity, prooxidant activity, cytotoxicity, and apoptosis-inducing activity has emerged. The present paper is intended to provide information for understanding the chemopreventive mechanism of polyphenolic antioxidants and an idea for designing antioxidant-based cancer chemoprevention agents.

Structure−Activity Relationship and Mechanism for Antioxidant Reaction. Resveratrol and its analogues were found to be able to protect DNA from AAPH-induced strand breakage as illustrated in Figure 1. Similarly, we found that these compounds could inhibit hydrogen peroxide induced apoptotic death via oxidative stress in thymocytes (Figure 2). Furthermore, it was found from Figure 3 that these compounds could inhibit hydrogen peroxide induced DNA damage of human lymphocytes. It is worth noting that the activities of 3,4-DHS, 3,4,5-THS, and 3,4,4′-THS, that is, the molecules bearing *o*-dihydroxyl groups, are appreciably higher than those of resveratrol and molecules bearing no such groups in the above-mentioned experimental systems. This can be understood because the *o*-hydroxylphenoxyl radical, the oxidation intermediate for these three more active species, is more stable because of the

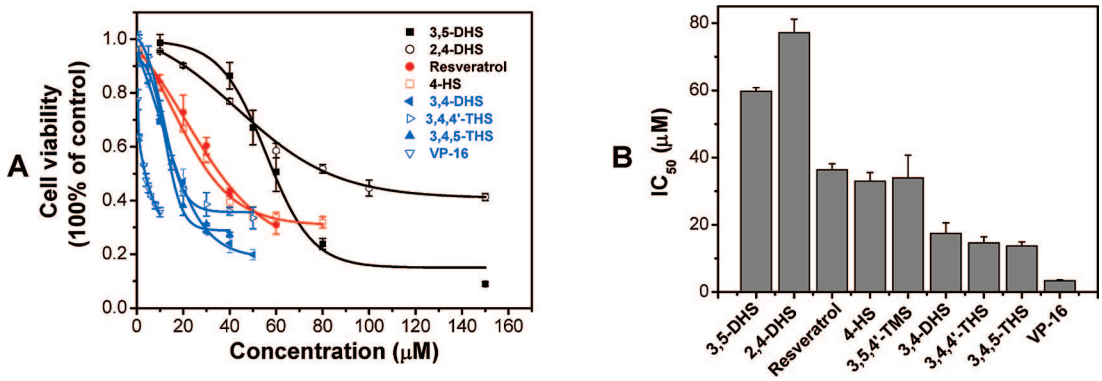


Figure 8. (A) Inhibitory effects of ROHs on HL-60 cell viability. HL-60 cells were treated with tested compounds for 48 h, and the percent viability was determined using the MTT method as described in the section Materials and Methods. Values are the mean ± SD of four independent experiments. (B) In vitro cytotoxicity of ROHs for HL-60 cells. Cytotoxicity is expressed as IC_{50}, the concentration of the compound to cause 50% inhibition of the cell viability. Data are expressed as the mean ± SD of four determinations.

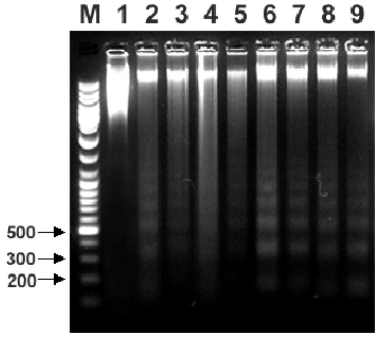


Figure 9. DNA fragmentation of HL-60 cells by ROHs for 48 h. The nuclear DNA was extracted from treated cells and analyzed by conventional agarose gel electrophoresis followed by ethidium bromide staining as described in the section Materials and Methods: (lane M) DNA molecular weight markers; (lane 1) control (cells treated with medium); (lane 2) treated with 3,4-DHS (50 μM); (lane 3) treated with 3,4,4′-THS (50 μM); (lane 4) treated with resveratrol (150 μM); (lane 5) treated with 2,4,-DHS (200 μM); (lane 6) treated with 3,5-DHS (200 μM); (lane 7) treated with 3,5,4′-TMS (50 μM); (lane 8) treated with 3,4,5-THS (50 μM); (lane 9) treated with VP-16 (25 μM).

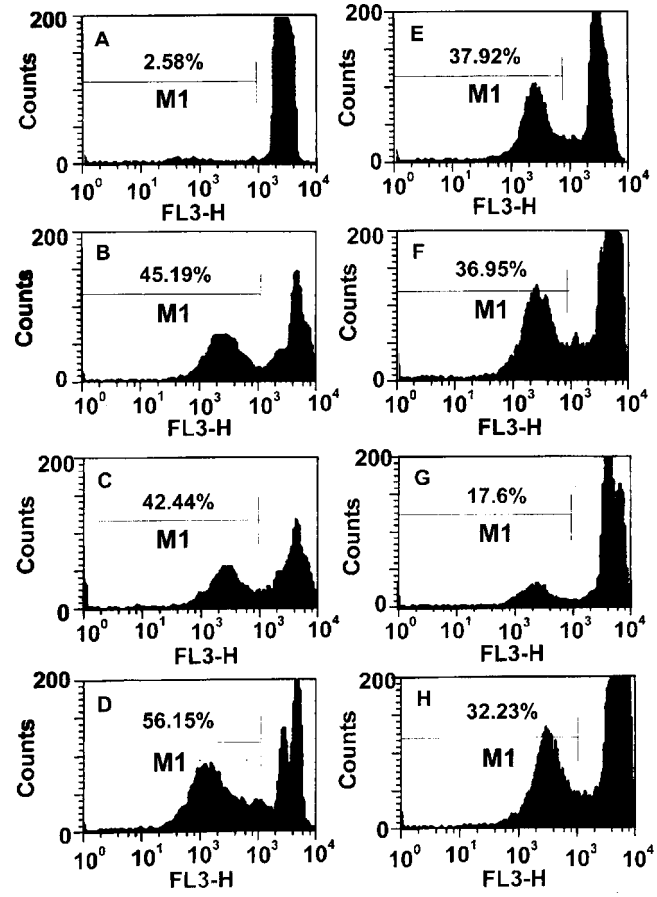
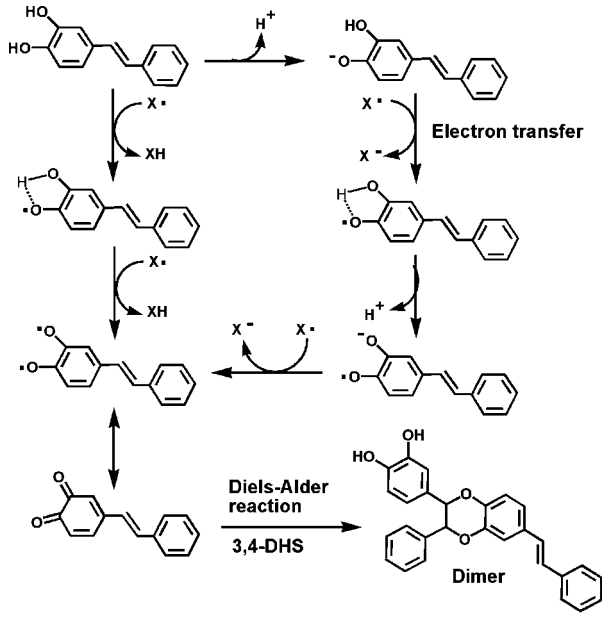


Figure 10. Flow cytometric analysis for apoptosis of HL-60 cells. Cells were treated with ROHs with certain concentration for 48 h, then washed and harvested. DNA fragmentation was determined by flow cytometry after PI staining. The M1 marker shows the sub-G1 cell fraction that corresponds to the apoptotic cell: (A) control (cells treated with medium); (B) treated with 3,4-DHS (50 μM); (C) treated with 3,4,4′-THS (50 μM); (D) treated with 3,4,5-THS (50 μM); (E) treated with resveratrol (150 μM); (F) treated with 3,5-DHS (200 μM); (G) treated with 2,4,-DHS (200 μM); (H) treated with 3,5,4′-TMS (50 μM).

intramolecular hydrogen bonding interaction, as evidenced recently from experiments by spectrophotometric measurement[27] and theoretical calculations.[28] The theoretical calculation showed that the hydrogen bond in the *o*-OH phenoxyl radical is approximately 4 kcal/mol stronger than that in the parent catechol and that the bond dissociation energy (BDE) of catechol is 9.1 kcal/mol lower than that of phenol and 8.8 kcal/mol lower than that of resorcinol.[28] In addition, it should be easier to further oxidize the *o*-OH phenoxyl radical and/or *o*-semiquinone anion to form the final *o*-quinone (Scheme 1) as evidenced from the formation of the dioxane-like dimer of 3,4-DHS (Figure 4) in the presence of galvinoxyl radical in ethanol at room temperature. Obviously, this dimer is the [4 + 2] Diels−Alder adduct of the *o*-quinone intermediate and another molecule of 3,4-DHS. Sugumaran reported that the formation of 1,2-dehydro-*N*-acetyldopamine quinone is favorable in acidic media. But the quinone is highly unstable in the neutral media.[29] Davies also pointed out that caffeoquinone is unstable at temperatures as low as −20 °C.[30] The 3,4-DHS *o*-quinone may be unstable under our experimental conditions (pH 7.4), but it can be trapped by 3,4-DHS via the Diels−Alder reaction, similar to the formation of α-tocopherol dimer from the Ag_2O oxidation of α-tocopherol.[31]

It is worth noting that the antioxidant activity is correlated with the electrochemical behavior of the molecule. Molecules with lower oxidation potentials and reversible cyclic voltammograms, that is, 3,4-DHS, 3,4,4′-THS, and 3,4,5-THS (0.36, 0.34, and 0.23 V vs SCE, respectively), exhibit higher activity, while molecules with higher oxidation potentials and irreversible cyclic voltammograms, that is, 3,5-DHS and resveratrol (0.79 and 0.67 V vs SCE, respectively), are less active.[12a] These facts

Scheme 1. Antioxidative Mechanism of 3,4-DHS[a]



[a] X• denotes free radical.

suggest that electron-transfer antioxidation might take place simultaneously with the direct hydrogen-abstraction reaction, as exemplified in Scheme 1. It is well-known that the phenoxide (ArO^-) undergoes electron transfer oxidation more easily to produce the relatively stable phenoxyl radical ($ArO^•$) in alkaline media. Resveratrol, with a pK_{a1} of 8.8,[32] partially dissociates under our experimental conditions (pH 7.4); this makes the electron-transfer reaction feasible. Cooperation between hydrogen-abstraction and electron-transfer processes in an antioxidation reaction by phenolic antioxidants has recently been discussed theoretically.[28] It was also proved by the oxidative product of resveratrol that the hydroxyl group at the 4′-position is much easier to subject to oxidation than other hydroxyl groups. Therefore, the oxidation reactions take place at the 4′-OH position in the case of resveratrol, resulting in the formation of phenoxyl radicals or semiquinone ("A", "B", and "C"). Successively, the coupling of one radical "B" and one radical "C", followed by tautomeric rearrangement and intramolecular nucleophilic attack to the intermediate quinone, gave the dihydrofuran dimer as shown in Scheme 2.

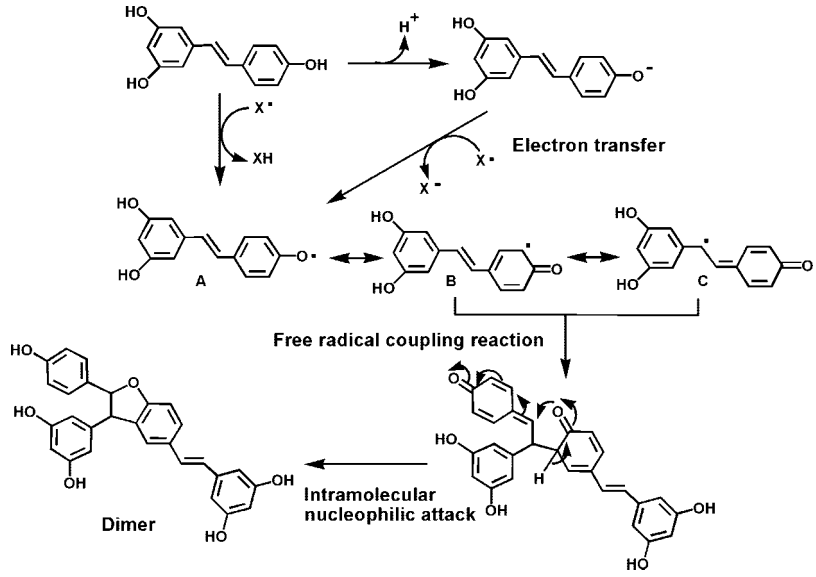
Structure−Activity Relationship and Mechanism for Prooxidant Reaction. Resveatrol is recognized as a naturally occurring polyphenol antioxidant but also acts as a prooxidant, inducing DNA damage in the presence of Cu(II).[10] Copper is an important metal ion present in chromatin and is closely associated with DNA bases, in particular, guanine.[33] It is also one of the most redox active metal ions present in cells.[34] Furthermore, evidence suggests that the concentration of copper is greatly increased in various malignancies.[11b,35] For example, it was shown that copper concentrations in serum and cells of leukemic patients (328 μg/mL and 52 μg/10^{10} cells in serum and cells, respectively) were significantly higher than those of health donors (114 μg/mL and 15 μg/10^{10} cells in serum and cells, respectively).[35] Copper ions from chromatin can be mobilized by metal-chelating agents, leading to internucleosomal DNA breakage, a property that is the hallmark of a cell undergoing apoptosis.[11b] Much evidence suggests that antioxidant properties of polyphenolic compounds may not fully account for their chemopreventive effects.[11b] Therefore, it has been proposed that it is the prooxidant action of plant-derived polyphenols rather than their antioxidant activity that may play a critical role in their anticancer and apoptosis-inducing properties, as ROS can mediate apoptotic DNA fragmentation.[25] Recently, Hadi and co-workers proposed a mechanism for the cytotoxic action of plant polyphenols against cancer cells that involves mobilization of endogenous copper and the consequent prooxidant action.[10d,11b] Indeed such a common mechanism better explains the anticancer effects of polyphenols with diverse chemical structures. Therefore, it is of important to see how an antioxidant can switch to a prooxidant and its reaction mechanism.

It is seen from Figure 5 that ROHs can show prooxidative action to induce the calf thymus DNA damage in the presence of Cu(II). It is clearly seen that the compounds bearing *o*-dihydroxyl groups (3,4-DHS, 3,4,4′-THS, and 3,4,5-THS) are the most active ones in inducing DNA damage, followed by compounds bearing 4-hydroxyl groups (2,4-DHS and resveratrol), while those bearing no such groups (3,5-DHS and 3,5,4′-TMS) are inactive. This activity sequence is similar to that observed in the inhibition of DNA damage of ROHs.

The high activity of 3,4-DHS, 3,4,4′-THS, and 3,4,5-THS is obviously due to their *o*-dihydroxyl groups, which can chelate with Cu(II) to form the ArOH−Cu(II) complex, as evidenced from Figures 6. The ArOH−Cu(II) complex can undergo intramolecular electron transfer to form semiquinone anion and Cu(I). It has been pointed out that the protonated phenolic group is not a particularly good ligand for metal cations, but once deprotonated, an oxygen center is generated that possesses a high charge density, the so-called "hard" ligand.[36] Although the pK_{a1} value of catechol is 9.25,[37] the hydroxyl proton could dissociate at much lower pH values, e.g., 5.0−8.0 in the presence of Cu(II).[36] Therefore, 3,4-DHS should dissociate to form a phenoxide, which chelates Cu(II) as a bidentate ligand and undergoes intramolecular electron transfer to form *o*-OH phenoxyl radicals. The acidity dissociation constant of the latter is much lower ($pK_{a1} = 4.1$)[38] than that of catechol ($pK_{a1} = 9.25$).[37] Thus, the *o*-OH phenoxyl radical should easily dissociate to form the *o*-semiquinone anion, which chelates with Cu(I). The new peak appearing at 459 nm in the case of 3,4-DHS demonstrates the formation of the *o*-semiquinone anion or *o*-quinone (Figure 6A). This is similar to the previous observation of the absorbance of *o*-semiquinone anion at 440 nm appearing during the oxidation of resveratrol analogues bearing *o*-dihydroxyl groups,[8b] of chlorogenic acid quinone at 408 nm,[39] and of 1,2-dehyo-*N*-acetyldopamine quinone at 390 nm.[29] In addition, the *o*-semiquinone anion should be more easily further oxidized to form the final products *o*-quinone as evidenced from the dimer product of 3,4-DHS in the presence of Cu(II) (Scheme 3).

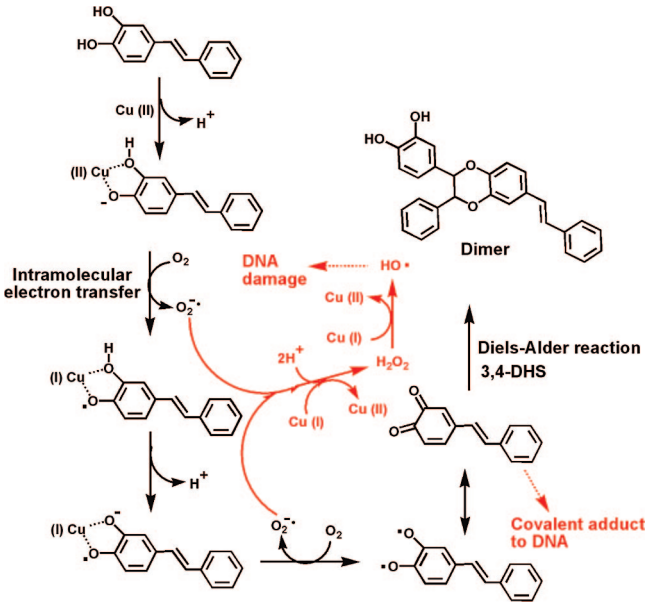
The fact that Cu(II) could not effectively oxidize 3,4-DHS to *o*-semiquinone anion intermediates in the absence of oxygen (Figure 7) demonstrates unambiguously the involvement of O_2 in the process. It is well-known that phenolics and Cu(II)-mediated DNA damage is due to the involvement of ROS and Cu(I) in the process.[10] Therefore, possible mechanisms of DNA damage induced by 3,4-DHS and resveratrol in the presence of Cu(II) can be clarified as shown in Schemes 3 and 4, respectively. The initial electron transfer oxidation of 3,4-DHS or resveratrol with O_2 generates the corresponding semiquinone and superoxide radical anion. The semiquinone in the case of 3,4-DHS undergoes second electron transfer with O_2 to form *o*-quinone and the superoxide radical anion and subsequent Diels−Alder reaction with another 3,4-DHS molecule leading to dioxane-like dimer as the ultimate product as shown in Scheme 3. In comparison, the semiquinone in the case of resveratrol undergoes a radical coupling and intramolecular

Scheme 2. Antioxidative Mechanism of Resveratrol[a]

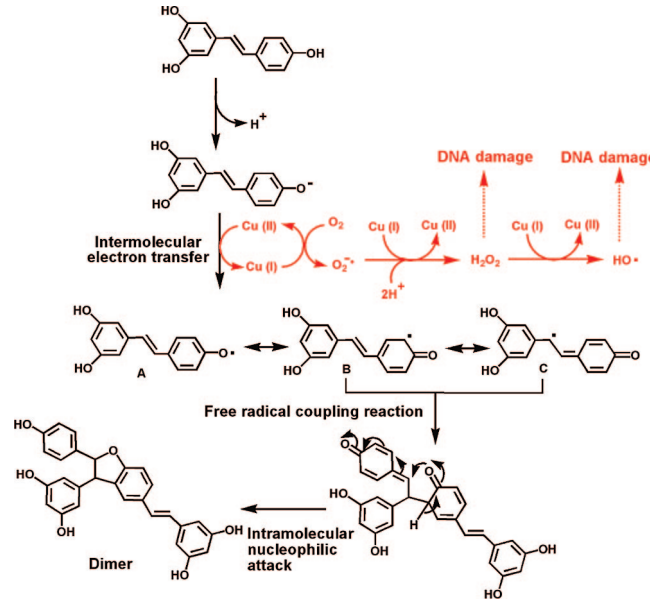


[a] X• denotes free radical.

Scheme 3. Prooxidative Mechanism of 3,4-DHS



Scheme 4. Prooxidative Mechanism of Resveratrol

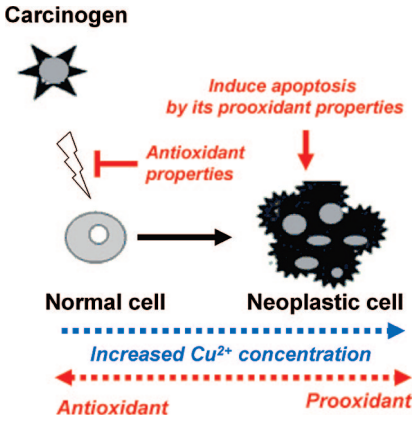


nucleophilic attack, resulting in the formation of the dihydrofuran dimer as shown in Scheme 4. The superoxide radical anion reacts with Cu(I), giving hydrogen peroxide which is readily converted by a Fenton-type reaction to a hydroxyl radical to induce the oxidative DNA damage. In addition, *o*-quinones have also been suggested to be involved in DNA damage by forming covalent adducts with DNA.[40]

Correlations among Antioxidant, Prooxidant, Cytotoxic, and Apoptosis-Inducing Activities. The present work demonstrates that resveratrol analogues bearing *o*-dihydroxyl groups (3,4-DHS, 3,4,4′-THS, and 3,4,5-THS) exhibit signicantly higher antioxidant and prooxidant activities than resveratrol and other analogues bearing no such a groups. It is worth noting that these compounds exhibit enhanced cytotoxicity (Figure 8) and apoptosis-inducing activity (Figures 9 and 10) on HL-60 cells. It was also recently reported that 3,4-DHS, 3,4,4′-THS, and 3,4,5-THS had lower $ApoEC_{50}$ values than resveratrol and other analogues against human leukemia (HL-60 and Jurkat) cell lines.[41] Of interest, intriguing results were recently described that treatment of resveratrol-resistant Jurkat cells with 3,4,5-THS rapidly induced extensive apoptosis.[42]

Comparison of the antioxidant activities and prooxidant activities, cytotoxicities, and apoptosis-inducing activities against HL-60 cells, as well as the oxidation potentials of those molecules, reveals a very interesting correlation between these activities and a very clear structure−activity relationship. That is, the antioxidant activities of these molecules correlate well with their prooxidant activities in the presence of Cu(II) and with their cytotoxicities and apoptosis-inducing activities against HL-60 cells. These activities also correlate well with the oxidation potentials of the molecules, i.e., the lower is the oxidation potential, the higher is the activity. The latter correlation suggests clearly that electron transfer plays a critical role in the antioxidative and prooxidative reactions, apoptosis-inducing events against HL-60 cells of resveratrol analogues. The finding appears to corroborate the observation from other

Scheme 5. Possible Mechanism for Chemopreventive Effects of Phenolic Antioxidant



authors that electron-transfer capacity of catechin derivatives correlate well with arrest activity of the cell cycle and apoptosis-inducing activity in HT29 cells.[43] The electron-transfer capacity of exogenous plant phenolics and its influence on the delicate balance between the antioxidant and prooxidant events governing cell functions may help to explain the putative cancer chemopreventive properties of resveratrol analogues.

How polyphenolic antioxidant mediates their cancer chemoprevention effects from the view of free radical biology is not fully understood. Antioxidants counteract ROS production and inhibit the latter-induced oxidative DNA damage and therefore reduce the risk of cancer. On the other hand, growing experimental evidence suggests that polyphenolic antioxidant-mediated production of ROS (prooxidant action) may be responsible for the ability to induce apoptosis of cancer cells.[25] Therefore two main questions rise from this paradox: (i) "When" will the antioxidant and prooxidant activities of polyphenolics take place to show efficacy? (ii) "Which" antioxidant and prooxidant activities of polyphenolics are responsible for the ability of cancer chemopreventive properties? Antioxidants such as resveratrol were shown to induce apoptoic cell death in HL-60 cell lines but not in normal peripheral blood lymphocytes.[26b] If so, how can these antioxidants differentiate between "normal" versus "abnormal tumor" cells? The present work (correlation and structure−activity relationship), combined with the observation from other authors, can supply an explanation to this paradox and these questions. As a matter of fact, antioxidants induce a multitude of effects that depend on the cell type ("normal" and cancer cells), cellular condition (normal, stressed, or malignant), and concentration, and it can have opposing activities. In the case of normal cells with low levels of copper, antioxidants can counteract excess ROS production (oxidative stress) and inhibit ROS-induced oxidative DNA damage, retaining the delicate redox balance, and therefore reduce the risk of cancer (Scheme 5). Compared with normal cells, preneoplastic cells and neoplastic cells have been shown to contain elevated levels of copper[35] and may be more sensitive to electron transfer with polyphenolic antioxidants to generate ROS (strong prooxidant properties), resulting in strong but "good" oxidative stress and DNA damage. It was also reported that copper could enhance cytotoxic and apoptosis-inducing activities of phenolic antioxidants against human leukemia and breast cancer cells.[44] Recently, Levine and co-workers demonstrated that ascorbate (a famous antioxidant) at pharmacologic concentrations was a prooxidant, generating hydrogen peroxide dependent cytotoxicity toward a variety of cancer cells in vitro without adversely affecting normal cells.[45] Therefore, DNA damage induced by polyphenolic antioxidants in the presence of Cu(II) may be an important pathway through which preneoplatic cells and neoplastic cells can be killed while normal cells survived (Scheme 5).

It is also understood that 3,4-DHS, 3,4,4′-THS, and 3,4,5-THS with low oxidation potentials (strong electron-transfer capacity) and high antioxidant and prooxidant activities exhibit high cytotoxic and apoptosis-inducing activities against HL-60 cells. In addition, the oxidative products of the compounds bearing *o*-dihydroxyl groups (3,4-DHS, 3,4,4′-THS, and 3,4,5-THS), i.e., *o*-dihydroxyphenoxyl radical and *o*-quinone, may be also responsible for the apoptosis-inducing activities. It has been reported that the *o*-quinone products from oxidation of catecholic estrogen[40a] and dopamine[40b] are responsible for the observed apoptotic effects of these chemicals in mutagenic and neuroblastoma cells. Furthermore, their oxidative oligomer products of resveratrol might also be responsible for the cytotoxic and apoptosis-inducing activities against cancer cells.[46]

Conclusions

Resveratrol-directed compounds such as 3,4-DHS, 3,4,4′-THS, and 3,4,5-THS bearing *o*-dihydroxyl groups exhibit remarkably higher activity in the experiments of inhibiting ROS-induced DNA damage and accelerating DNA damage in the presence of copper than the ones bearing no such groups, and the correlations among the antioxidant activities and prooxidant activities, cytotoxicities, and apoptosis-inducing activities against HL-60 cells are obtained. Although the precise mechanism is still unclear, these results support the free radical theory of cancer and give us useful information for antioxidant and chemoprevention drug design.

Materials and Methods

Materials. Resveratrol and its analogues 3,4-DHS, 3,4,4′-THS, 3,4,5-THS, 2,4-DHS, 3,5-DHS, and 3,5,4′-TMS were synthesized as previously described.[47] This method produced exclusively the trans-isomer. Their structures were fully identified using ^{1}H NMR and EI-MS (see Supporting Information), and the data were consistent with those reported in the literature.[47] The purity (≥95%) of each compound was checked using a HPLC on a Waters 600 instrument with a photodiode array detector (see Supporting Information).

Galvinoxyl radical, AAPH, EB, pBR322 DNA, calf thymus DNA, agarose, low-melting point agarose, PI, RNase, MTT, and sodium dodecyl sulfate (SDS) were purchased from Aldrich-Sigma. RPMI medium 1640 was from GIBCO. All other chemicals were of the highest quality available.

Assay for Oxidative DNA Strand Breakage Induced by AAPH. The inhibition of AAPH-induced DNA strand breakage by ROHs was assessed by measuring the conversion of the supercoiled pBR322 plasmid DNA to open circular and linear forms by gel electrophoresis.[13a] pBR322 DNA (0.1 μg) was incubated with the indicated concentration of resveratrol, its analogues, and AAPH in phosphate-buffered saline (PBS) at pH 7.4 and 37 °C for 1 h. After incubation, the samples were mixed with 5 μL of gel loading buffer (0.13% bromophenol blue and 30% (w/v) sucrosel) and immediately loaded in 1% agarose gels containing 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA and subjected to electrophoresis in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer for 1 h. The gels were stained with 0.6 μg/mL ethidium bromide for 30 min, followed by destaining in water for 30 min, and photographed under a transilluminator with UV light. Gel_Pro Analyzer (version 3.0 from Media Cybernetics) was used to quantify the density of supercoiled DNA form.

Thymocytes Preparation. Thymocytes were prepared as described previously.[48] Briefly, whole thymuses from young healthy BALB/c mice were finely minced and pressed through stainless

steel sieves in cold RPMI-1640 under aseptic conditions. After centrifugation at 500*g* for 10 min, the cells were suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL).

Assessment of Thymocyte Viability. The thymocyte viability 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.[14] Briefly, 100 μL aliquots of thymocytes containing 5×10^5 cells/mL were added to each well of a 96-well flat microtiter plate and incubated with various amounts of hydrogen peroxides and/or ROHs dissolved in 0.1% dimethyl sulfoxide (DMSO). Six replicate wells were used at each point in the experiments. After 24 h of incubation at 37 °C, MTT solution (5 mg/mL in PBS) was added and incubated for another 4 h at 37 °C in a 5% CO_2 incubator. The resulting MTT-formazan product was dissolved by the same volume of lysis buffer (10% SDS and 0.1 M HCl), and the incubation was continued overnight at 37 °C. The amount of formazan was determined by measuring the absorbance at 570 nm using a Bio-Rad 550 ELISA microplate reader.

Analysis of DNA Fragmentation of Thymocytes by Agarose Gel Electrophoresis. DNA fragmentation was assessed by agarose gel electrophoresis as reported previously.[18] Thymocytes were suspended at a density of 1×10^5 cells/mL in RAPI 1640 medium and incubated with 100 μM ROHs and 50 μM hydrogen peroxides for 6 h. The cells were harvested by centrifugation at 1000*g* for 10 min, then washed with PBS and incubated with lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA, 0.5% SDS, and 0.5 mg/mL proteinase K) at 37 °C for 2 h. The DNA was extracted successively with phenol/chloroform (1:1, v/v) and chloroform/isoamyl alcohol (24:1, v/v) and precipitated overnight in −20 °C with ethanol containing 0.3 M sodium acetate (pH 5.2), then centrifuged at 13000*g* at −4 °C for 15 min. The pellets were dried in the air and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNase-free RNase (100 μg/mL) was added to each sample and incubated at 37 °C for 1 h. The DNA samples were mixed with loading buffer and subjected to electrophoresis on 1.5% agarose gel at 5 V/cm and visualized under UV light after staining with ethidium bromide. The occurrence of apoptosis was indicated by the appearance of a ladder of oligonucleosomal DNA fragments that were approximately 180−200 bp multiples.

Isolation of Human Peripheral Blood Lymphocytes. Human lymphocytes were isolated from fresh blood obtained from healthy volunteers as described previously.[49] Briefly, human peripheral blood was collected and immediately mixed with heparin to avoid coagulation and then diluted with PBS, layered onto an equal volume of LymphoPrep LSM, and centrifuged at 700*g* for 30 min. The lymphocytes were removed into a fresh tube and washed once with PBS and then suspended in PBS for comet assay.

Assay for Cellular DNA Damage by Single-Cell Alkaline Electrophoresis (Comet Assay). Cellular DNA damage was assessed by comet assay.[15] Normal melting point agarose at 0.5% in PBS was dropped onto a microscope glass slides, and a coverslip was applied immediately to cover it. The human lymphocytes (2×10^6 cells) treated with hydrogen peroxide and/or ROHs were washed with PBS and then mixed thoroughly with 1% low melting point agarose dissolved in PBS at 39 °C. Next, the mixture was transferred onto the microscope slide that had been precoated with normal melting point agarose and a glass coverslip was applied on the top of the liquid agarose to spread the agarose across the surface of the slides, which was placed on top of ice for 10 min. After removing the coverslips, the slides were immersed slowly in ice-cold alkaline cell lysis solution (2.5 M NaCl, 0.1 M EDTA, 1% *N*-lauroylsarcosine, 1% Triton X-100, 10% DMSO, 10 mM Tris, pH 10) and allowed to stay for 1 h. Slides then were denatured in an eletrophoresis tank containing 0.3 M NaOH/1 mM EDTA for 20 min. Next, electrophoresis was carried out at 25 V/300 mA for 20 min. Afterward, the slides were gently immersed in neutralization buffer (0.4 mM Tris-HCl, pH 7.5) for 5 min after applying ethidium bromide solution to stain and covering with coverslips. The slides were viewed with fluorescence microscope with camera attachment. The representative views of slides were photographed, and 50 randomly selected cell nucleoids were scored on a slide and gave an overall comet score (A), in which the tail is longer than half of length of head. The percentage DNA damage was calculated from the ratio of the measurements as follows: $(A/50) \times 100$. The scoring method is consistent with computer imaging analysis.[50]

Oxidation Product Analysis of Resveratrol or 3,4-DHS in the Presence of Galvinoxyl Radical in Ethanol. The 60 mg of resveratrol (0.26 mmol) and 220 mg of galvinoxyl (0.52 mmol) were mixed in 30 mL of ethanol and stirred for 8 h. The products were purified by silica gel chromatography and eluted with chloroform−methanol (5:1, v:v) to afford 24 mg of dimer, recovering unreacted resveratrol (29 mg) with the conversion, and yields of 78% and 41%, respectively. The dehydrodimer of resveratrol: ^{1}H NMR (400 MHz, $(CD_3)_2CO$, δ): 4.62 (d, $J = 8.0$ Hz, 1H, H-8), 5.48 (d, $J = 8.0$ Hz, 1H, H-7), 6.19 (d, $J = 2.0$ Hz, 2H, H-10 and H-14), 6.25 (d, $J = 2.0$ Hz, 2H, H-12), 6.53 (d, $J = 2.0$ Hz, 2H, H-10′ and H-14′), 6.84 (d, $J = 8.0$ Hz, 2H, H-3 and H-5), 6.88 (d, $J = 8.0$ Hz, 1H, H-3′), 6.92 and 7.08 (AB system, d, $J = 16.0$ Hz, 2H, H-7′ and H-8′), 7.24 (d, $J = 8.0$ Hz, 2H, H-2 and H-6), 7.25 (d, $J = 8.0$ Hz, 1H, H-2′), 7.45 (d, $J = 2.0$ Hz, 1H, H-6′). ^{13}C NMR (100 MHz, $(CD_3)_2CO$, δ): 57.7 (1C, C-8), 94.2 (1C, C-7), 102.4 (1C, C-12), 102.7 (1C, C-12′), 105.7 (2C, C-10′ and C-14′), 107.4 (2C, C-10′ and C-14′), 110.1 (1C, C-3′), 116.1 (2C, C-3 and C-5), 123.9 (1C, C-2′), 127.2 (1C, C-7′), 128.5 (3C, C-6′, C-2 and C-6), 129.1 (1C, C-8′), 131.7 (1C, C-1), 132.1 (1C, C-5′), 132.5 (1C, C-1′), 140.7 (1C, C-9′), 145.1(1C, C-9), 158.4 (1C, C-4), 159.5 (2C, C-11′ and C-13′), 159.7 (2C, C-11 and C-13), 160.5 (1C, C-4′). HRMS (ESI): calcd *m/z* 455.1489, found *m/z* 455.1481 $[M + H]^+$, error = 1.8 ppm.

The 54 mg of 3,4-DHS (0.25 mmol) and 107 mg of galvinoxyl radical (0.25 mmol) were mixed in 30 mL ethanol and stirred for 3 h. The products were purified by silica gel chromatography and eluted with chloroform−methanol (10:1, v:v) to afford 47 mg of dimer with an isolated yield of 87%. The dimer of 3,4-DHS: ^{1}H NMR (400 MHz, $(CD_3)_2CO$, δ): 4.92 (d, $J = 8$ Hz, 1H, H-7), 5.05 (d, $J = 8$, 2 Hz, 1H, H-8), 6.51 (d, $J = 8$ Hz, 1H, H-6), 6.68 (d, $J = 8$ Hz, 1H, H-5), 6.77 (d, $J = 8$ Hz, 1H, H-2), 6.97 (d, $J = 8$ Hz, 1H, H-3′), 7.13 (d, $J = 16$ Hz, 1H, H-7′), 7.17 (d, 1H, $J = 8$ Hz, H-2′), 7.19 (d, $J = 16$ Hz, 1H, H-8′), 7.23 (m, 2H, H-11 and H-13), 7.24 (d, $J = 7.8$ Hz, 1H, H-12′), 7.25 (m, 1H, H-12), 7.26 (m, 2H, H-10 and H-14), 7.27 (d, $J = 2.4$ Hz, 1H, H-6′), 7.35 (d, $J = 7.8$ Hz, 2H, H-11′ and H-13′), 7.57 (d, $J = 7.8$ Hz, 2H, H-10′ and H-14′). ^{13}C NMR (100 MHz, $(CD_3)_2CO$, δ): 81.8 (1C, C-7), 81.3 (1C, C-8), 115.4 (1C, C-2), 115.5 (1C, C-3′), 115.6 (1C, C-6′), 117.9 (1C, C-5), 120.6 (1C, C-6), 120.9 (1C, C-2′), 127.1 (2C, C-10′ and C-14′), 127.9 (1C, C-7′), 128.1 (2C, C-1 and C-8′), 128.8 (2C, C-10 and C-14), 128.9 (2C, C-11 and C-13), 129.2 (1C, C-12), 129.4 (1C, C-12′), 129.5 (2C, C-11′ and C-13′), 132.1 (1C, C-1′), 137.7 (1C, C-9), 138.6 (1C, C-9′), 144.8 (1C, C-4′), 145.2 (1C, C-5′), 145.6 (1C, C-3), 146.2 (1C, C-4). HRMS (ESI): calcd *m/z* 423.1591, found *m/z* 423.1597 $[M + H]^+$, error = 1.4 ppm.

Determination of Calf Thymus DNA Damage. Calf thymus DNA damage was determined by using the EB-binding assay.[16] A total 3 mL of PBS (pH 7.4) containing calf thymus (80 μg/mL) with different concentrations of ROHs and $CuSO_4$ (250 μM) was incubated at 37 °C for 1 h under aerobic conditions. After incubation, 50 μL of 0.75 mg/mL EB was added and the fluorescence was measured using a Shimadzu RF-540 spectrofluorimeter with excitation at 510 nm and emission at 590 nm. A solution containing all reagents and DNA except ROHs was used as the control for 100% fluorescence, and zero fluorescence was assessed in a solution identical to the control except DNA. The loss of the fluorescence was used as a measure of DNA damage.

UV−Visible Spectral Measurement. UV−visible spectra were measured at room temperature with a Hitachi 557 spectrophotometer. PBS containing 50 μM ROH was kept at room

temperature, and the spectral tracing was started by addition of 25 μM $CuSO_4$.

Oxidation Product Analysis of Resveratrol or 3,4-DHS in the Presence of Cu(II). A solution of resveratrol or 3,4-DHS (0.5mmol) and anhydrous copper sulfate (96 mg, 0.55mmol) were mixed in anhydrous acetonitrile (50 mL) and stirred for 24 h at room temperature, as monitored by silica gel (petroleum ether/acetone, 20/1). The dimers of resveratrol and 3,4-DHS were isolated by silica gel chromatography with isolated yields of 15.7 and 22%, respectively. Their structures were characterized with HRMS (ESI) and 1D and 2D NMR.

Cell Culture. HL-60 cell lines were originally obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat inactivated fetal bovine serum, penicillin (100 U/mL), streptomycin (100 U/mL), and 2 mM of glutamine. The cell cultures were maintained at 37 °C in a humidified CO_2 (5%) incubator. Exponentially growing cells were used throughout.

Assessment of HL-60 Cell Viability. The HL-60 cell viability was also assessed using the MTT colorimetric assay,[14] and the experimental procedure was the similar to that in the section of assessment of thymocytes viability with different cell concentration (5×10^4 cells/mL) and incubation time (48 h).

Analysis of DNA Fragmentation of HL-60 Cells by Agarose Gel Electrophoresis. DNA fragmentation of HL-60 cells was also assessed by agarose gel electrophoresis, and the experimental procedure[18] was the similar to that in the section of analysis of DNA fragmentation of thymocytes by agarose gel electrophoresis with different incubation time (48 h), centrifugation rate (500*g* for 10 min), and lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% SDS, and 1 mg/mL proteinase K).

Dermination of Apoptosis Analysis by Flow Cytometry. Flow cytometric DNA analysis was performed by an available method[51] with minor modifications. Briefly, an amount of 10 mL of exponentially growing HL-60 cells at a density of 1×10^5 cells/mL was untreated or treated with various concentrations of ROHs. The cells were then prepared as a single cell suspension in 200 μL of PBS, fixed with 2 mL of ice-cold 70% ethanol, and maintained at 4 °C overnight. The cells were harvested by 500*g* centrifugation for 10 min, resuspended in 500 μL of PBS with 0.1% Triton-X100 and DNase-free RNase (100 μg/mL), incubated at 37 °C for 30 min, and stained with 50 mg/L PI in the dark at 4 °C for 30 min. The red fluorescence of individual cells was determined with a flow cytometer (FACS Calibur, Becton-Dickinson, CA). All data were analyzed by CellQuest, version 3.3, software.

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Supporting Information Available: Experimental details for the synthesis and characterization of all compounds; HPLC, HRMS, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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