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Hydroxycinnamic Acids as DNA-Cleaving Agents in the Presence of Cu^{II} Ions: Mechanism, Structure–Activity Relationship, and Biological Implications

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Abstract: The effectiveness of hydroxycinnamic acids (HCAs), that is, caffeic acid (CaA), chlorogenic acid (ChA), sinapic acid (SA), ferulic acid (FA), 3-hydroxycinnamic acid (3-HCA), and 4-hydroxycinnamic acid (4-HCA), as pBR322 plasmid DNA-cleaving agents in the presence of Cu^{II} ions was investigated. Compounds bearing *o*-hydroxy or 3,5-dimethoxy groups on phenolic rings (CaA, SA, and ChA) were remarkably more effective at causing DNA damage than the compounds bearing no such groups; furthermore, CaA was the most active among the HCAs examined. The involvement of reactive oxygen species (ROS) and Cu^{I} ions in the DNA damage was affirmed by the inhibition of the DNA breakage by using specific scavengers of ROS and a Cu^{I} chelator. The interaction between CaA and Cu^{II} ions and the influence of ethylenediaminetetraacetic acid (EDTA), the solvent, and pH value on the interaction were also studied to help elucidate the detailed prooxidant mechanism by using UV/Vis spectroscopic analysis. On the basis of these observations, it is proposed that it is the CaA phenolate anion, instead of the parent molecule, that chelates with the Cu^{II} ion as a bidentate ligand, hence facilitating the intramolecular electron transfer to form the corresponding CaA semiquinone radical intermediate. The latter undergoes a second electron transfer with oxygen to form the corresponding *o*-quinone and a superoxide, which play a pivotal role in the DNA damage. The intermediacy of the semiquinone radical was supported by isolation of its dimer from the Cu^{II}-mediated oxidation products. Intriguingly, CaA was also the most cytotoxic compound among the HCAs toward human promyelocytic leukemia (HL-60) cell proliferation. Addition of exogenous Cu^{II} ions resulted in an effect dichotomy on cell viability depending on the concentration of CaA; that is, low concentrations of CaA enhanced the cell viability and, conversely, high concentrations of CaA almost completely inhibited the cell proliferation. On the other hand, when superoxide dismutase was added before, the two stimulation effects of exogenous Cu^{II} ions were significantly ameliorated, thus clearly indicating that the oxidative-stress level regulates cell proliferation and death. These findings provide direct evidence for the antioxidant/prooxidant mechanism of cancer chemoprevention.

Keywords: caffeic acid · cancer · chemoprevention · DNA damage · reaction mechanisms · reactive oxygen species

Introduction

Chemoprevention by using naturally occurring or synthetic antioxidants has recently emerged as a viable alternative strategy for preventing carcinogenesis.[1] Growing evidence has revealed that oxidative DNA damage induced by reactive oxygen species (ROS) contributes to human tumorigenesis[2] and that regular consumption of certain fruits and vegetables containing substantial amounts of various polyphenolics with antioxidative properties can decrease the risk of suffering from cancer.[3] From this evidence, the idea that antioxidants in these foods that are effective cancer chemo-

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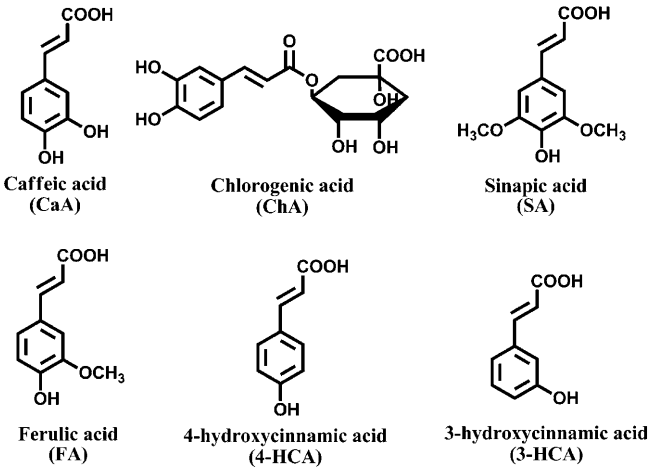
preventive agents has developed.[3] Generally speaking, antioxidants can counteract ROS production and inhibit ROS-induced oxidative DNA damage, hence decreasing the risk of cancer.[4] However, every antioxidant is in fact a redox agent and thus might become a prooxidant to induce DNA damage under special conditions. Antioxidant vitamins, such as vitamins E[5] and C[6] and other polyphenolic antioxidants, including quercetin,[7] curcumin,[8] tea catechins,[9] salsolinol,[10] resveratrol,[11] and phenolic acids,[12] have been reported to act as DNA-cleaving agents in the presence of cupric ions by producing ROS. Over the past few years, increasing experimental evidence has indicated that antioxidant-mediated production of ROS (prooxidant action) may be responsible for the induction of apoptosis of cancer cells and cancer chemopreventive.[13] Recently, Levine and coworkers demonstrated that ascorbate (a well-known antioxidant) at pharmacological concentrations is a prooxidant, thus generating hydrogen peroxide-dependent cytotoxicity toward a variety of cancer cells in vitro without adversely affecting normal cells.[13b,c] Therefore, it is desirable to know how an antioxidant molecule can switch from an antioxidant to a prooxidant and from an inhibitor of DNA damage to an inducer of DNA damage as well as its implication in cancer chemoprevention.

Copper is an important metal ion present in chromatin and is closely associated with DNA bases, in particular guanine.[14] It is also one of the most redox-active metal ions present in cells.[15] Several studies have shown that serum and tumor copper levels are significantly elevated in various types of cancers, such as breast cancer,[16] lung cancer,[17] and leukemia.[18] For example, it was shown that copper concentrations in the serum and cells of leukemic patients (328 $\mu g\,mL^{-1}$ and 52 $\mu g\,10^{10}$ $cells^{-1}$ in the serum and cells, respectively) were significantly higher than in healthy donors (114 $\mu g\,mL^{-1}$ and 15 $\mu g\,10^{10}$ $cells^{-1}$ in the serum and cells, respectively).[18a] Thus, compared with normal cells, preneoplastic and neoplastic cells might be more sensitive to copper-related redox reactions that switch an antioxidant to a prooxidant to generate ROS, thus resulting in DNA damage. DNA damage induced by antioxidants in the presence of copper might be an important pathway through which preneoplastic and neoplastic cells can be killed while normal cells survive.[19] This behavior could be one reason for the ability of antioxidants to differentiate between normal and abnormal tumor cells. Indeed, many antioxidants are nontoxic to normal cells.[20] Resveratrol, a well-known antioxidant and cancer chemopreventive agent found in a wide variety of dietary sources including grapes, plums, and peanuts, has been shown to induce apoptosis in human leukemia (HL-60) cells but not in normal peripheral blood lymphocytes.[20b] In our ongoing research on bioantioxidants,[4a,11f,21] we recently found an interesting correlation between antioxidant activity, prooxidant activity, cytotoxicity, and apoptosis-inducing activity of resveratrol and its analogues.[21a] However, little is known about the detailed prooxidative mechanism of polyphenolic antioxidants in the presence of copper from a chemical point of view and about the biological implications of the copper-dependent prooxidative action of polyphenolic antioxidants. Therefore, we were motivated to see whether the correlation between the prooxidative activity on DNA damage and cytotoxicity against cancer cells exists in other polyphenolic antioxidants and whether the addition of cupric ions can result in the enhancement of the cytotoxicity of polyphenolic antioxidants.

Phenolic acids, especially hydroxycinnamic acid derivatives, are widely distributed in plants and are present in considerable amounts in fruits, vegetables, and beverages in the human diet.[22] The daily uptake of caffeic acid (3,4-dihydroxycinnamic acid) has been estimated to be 206 mg in subjects drinking coffee.[23] The antioxidative properties of hydroxycinnamic acids have been extensively studied,[24] but their prooxidative properties, especially the detailed mechanism, have not been well understood. Recently, we conducted a preliminary investigation into the prooxidative action of hydroxycinnamic acids,[12] but the detailed prooxidative mechanism and biological implications have not been well exploited. Therefore, in the present study hydroxycinnamic acids (HCAs), including caffeic acid (CaA), chlorogenic acid (ChA), sinapic acid (SA), ferulic acid (FA), 3-hydroxycinnamic acid (3-HCA), and 4-hydroxycinnamic acid (4-HCA; Scheme 1) were chosen to probe their abilities to act as DNA-cleaving agents in the presence of Cu^{II} ions. The interaction between HCAs and Cu^{II} ions and the oxidation products of CaA and SA obtained in the presence of Cu^{II} ions were also studied to help elucidate the prooxidative mechanism. To verify the biological implications of the prooxidative action, the antiproliferative effects of HCAs on HL-60 cells in the absence and presence of exogenous Cu^{II} ions were assessed with a trypan-blue dye-exclusion test.



Scheme 1. Molecular structures of hydroxycinnamic acids (HCAs).

Results

Strand breakage of plasmid pBR322 DNA induced by HCAs and Cu^{II} ions and the involvement of ROS and Cu^{I} ions: The single-strand breakage of supercoiled plasmid

DNA with a relatively high electrophoretic mobility leads to the formation of an open circular conformation with a decreased electrophoretic mobility in agarose, whereas the formation of linear DNA is indicative of a double-strand breakage and has a mobility intermediate between that of the supercoiled and open circular conformation.[25] We examined the effect of HCAs in combination with Cu^{II} ions on the plasmid DNA breakage by using agarose gel electrophoresis (Figure 1 A). Neither Cu^{II} ions (0.2 mM) nor HCAs

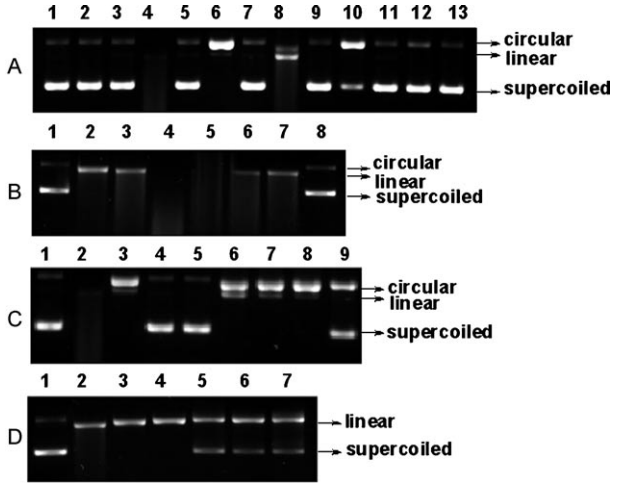


Figure 1. A) Effect of HCAs on pBR322 DNA strand breakage in the presence of Cu^{II} ions. Supercoiled pBR322 DNA (125 ng) was incubated with 0.2 mM Cu^{II} and 0.1 mM HCAs at 37°C for 1 h in 10 mM PBS (pH 7.4). Lanes: 1: control; 2: Cu^{II} alone; 3: CaA alone; 4: CaA and Cu^{II}; 5: ChA alone; 6: ChA and Cu^{II}; 7: SA alone; 8: SA and Cu^{II}; 9: FA alone; 10: FA and Cu^{II}; 11: 4-HCA alone; 12: 4-HCA and Cu^{II}; 13: 3-HCA and Cu^{II}. B) Effect of CaA on pBR322 DNA strand breakage in the presence of Cu^{II} ions. The conditions were the same as for (A), except that the total concentration of CaA plus Cu^{II} ions was maintained constant (0.3 mM). Lanes: 1: control; 2: 0.225 mM CaA and 0.075 mM Cu^{II}; 3: 0.2 mM CaA and 0.1 mM Cu^{II}; 4: 0.15 mM CaA and 0.15 mM Cu^{II}; 5: 0.1 mM CaA and 0.2 mM Cu^{II}; 6: 0.075 mM CaA and 0.225 mM Cu^{II}; 7: 0.06 mM CaA and 0.24 mM Cu^{II}; 8: 0.24 mM Cu^{II} alone. C) Effect of GSH and BCDS on CaA and Cu^{II}-mediated DNA strand breakage. The experimental conditions were the same as for (A): 1: control; 2: 0.1 mM CaA plus 0.2 mM Cu^{II}; 3–5: 0.25, 0.5, and 1 mM GSH, respectively; 7–9: 0.05, 0.1, 0.2, and 0.4 mM BCDS, respectively. D) Effect of catalase on CaA and Cu^{II}-mediated DNA strand breakage. The experimental conditions were the same as for (A). Lanes: 1: control; 2: 0.1 mM CaA and 0.1 mM Cu^{II}; 3–7: 12.5, 25, 50, 75, and 100 $\mu g\,mL^{-1}$ CAT, respectively.

(0.1 mM) alone caused detectable DNA damage (lanes 2, 3, 5, 7, 9, and 11, Figure 1 A). However, CaA (0.1 mM) could work cooperatively with Cu^{II} ions (0.2 mM) to produce a smear of fragments, which is indicative of extensive DNA damage (lane 4, Figure 1 A). When the total concentration of CaA and Cu^{II} ions was maintained at a constant (0.3 mM) and the CaA/Cu^{II} molar ratio was varied (Figure 1 B), ratios of 1:1 and 1:2 were the most active in inducing DNA damage. Therefore, concentrations of 0.1 mM HCAs and 0.2 or 0.1 mM Cu^{II} ions were chosen for the following experiments. SA and Cu^{II} ions resulted in the conversion of the substrate DNA almost completely into open circular, linear, and fragmental forms (lane 8, Figure 1 A). ChA completely converted the supercoiled DNA into a relaxed circular form in the presence of the Cu^{II} ions and FA showed a similar but somewhat weaker effect (lanes 6 and 10, respectively, Figure 1 A). On the other hand, 3-HCA and 4-HCA were completely ineffective even in the presence of Cu^{II} ions (lanes 12 and 13, respectively, Figure 1 A). The order of DNA-cleaving effectiveness is CaA > SA > ChA > FA > 4-HCA and 3-HCA. It is worth noting that the compounds bearing *o*-hydroxy or 3,5-dimethoxy groups on the phenolic rings (i.e., CaA, SA, and ChA) are most active followed by a compound bearing *o*-methoxyhydroxy groups (i.e., FA), whereas the monohydroxy-substituted substrates (4-HCA and 3-HCA) are inactive.

To analyze the role of ROS in the HCAs/Cu^{II}-dependent DNA breakage, we used specific scavengers of activated oxygen and a Cu^{I} chelator to define the nature of the reactive species. Glutathione (GSH), a ROS scavenger, and bathocuproinedisulfonic acid disodium salt (BCDS), a specific Cu^{I} chelator, both provided protection against DNA strand breakage induced by CaA in the presence of Cu^{II} ions (Figure 1 C). GSH and BCDS at concentrations of 0.5 and 0.4 mM, respectively, completely inhibited the CaA/Cu^{II}-mediated stand breakage. Catalase (CAT; 50 $\mu g\,mL^{-1}$) effectively protected plasmid DNA from DNA strand breakage induced by CaA (0.1 mM) and Cu^{II} ions (0.1 mM), thus clearly indicating the involvement of hydrogen peroxide in the process (Figure 1 D). These findings suggest that both ROS and the Cu^{II}/Cu^{I} redox cycle are critical to the DNA damage.

UV/Vis spectral changes of HCAs in the presence of Cu^{II} ions and influence of EDTA, solvent, and pH value on the interaction of CaA and Cu^{II} ions: To clarify the mechanism of the DNA damage, the UV/Vis absorption changes of HCAs upon the addition of Cu^{II} ions were examined. Figure 2 A was obtained when Cu^{II} ions were added to CaA in phosphate-buffered saline (PBS; pH 7.4). The rapid disappearance of the absorption bands of CaA centered at $\lambda =$ 287 and 311 nm was accompanied by the appearance of bathochromic-shifted peaks at $\lambda = 304$ and 349 nm and the hypsochromic-shifted peak at $\lambda = 259$ nm, which is characteristic for the formation of a chelate complex of a CaA phenolate anion with a Cu^{II} ion (see below). The intensity of the former two peaks ($\lambda = 304$ and 349 nm) decreased with an increase in time, and the absorption bands in the range $\lambda =$ 259–270 nm and at about $\lambda = 400$ nm were enhanced due to the formation of CaA *o*-quinone (see below). Two isosbestic points at $\lambda = 288$ and 402 nm suggested a direct transition from one form (CaA phenoxide/Cu^{II} chelate) to another one (CaA *o*-quinone). A similar bathochromic shift was also observed in the case of ChA, but its decay was remarkably slower than that of CaA (spectrum not shown). A comparison of Figure 2 A with Figure 2 B indicates clearly that the decay of SA is significantly faster than the decay of CaA in the presence of Cu^{II} ions. The addition of Cu^{II} ions to SA resulted in the decrease and gradual redshift of the maximal absorption at $\lambda = 306$ nm accompanied by the increased ab-

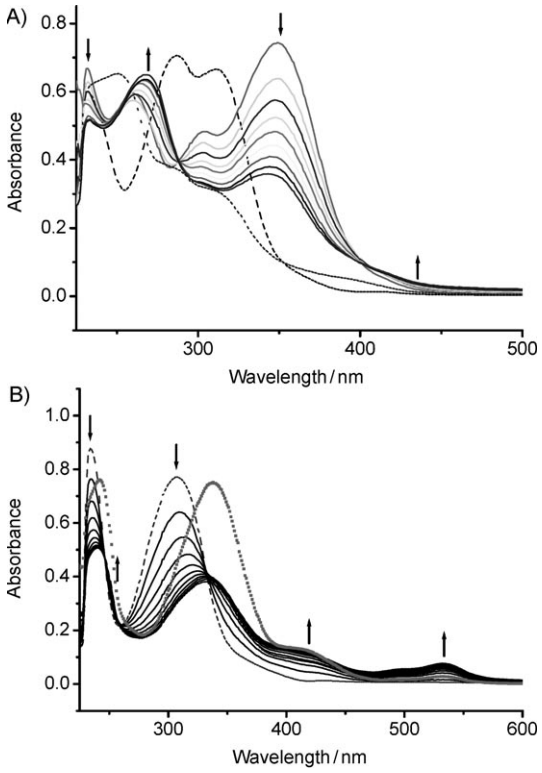


Figure 2. Absorption spectral changes of HCAs (50 μM) in the absence (dashed line) and in the presence (solid line) of 100 μM Cu^{II} ions in PBS buffer (pH 7.4) in air. A) CaA (interval = 15 min), the dotted line shows the effect of 300 μM EDTA on the interaction of CaA and Cu^{II} ions for 120 min. B) SA (interval = 5 min), the square line shows the UV/Vis spectrum of the SA dimer (50 μM). The arrows show the time-related absorbance changes.

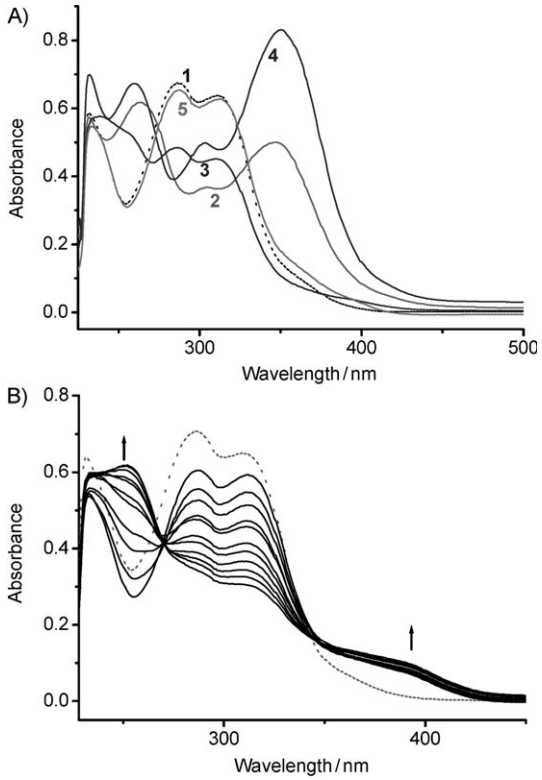


Figure 3. A) The absorption spectral changes of CaA. Lines: 1: CaA (50 μM); 2: CaA (50 μM) and Cu^{II} (100 μM) for 60 min in air; 3: the same as (2) but with EDTA (300 μM) in air; 4: the same as (2) but in argon; 5: the same as (4) but with EDTA (300 μM). B) The absorption spectrum of CaA (dashed line) and the effect of EDTA (300 μM) on the absorption spectrum of the CaA/Cu^{II} system (solid line) at different time intervals (interval = 15 min). All the spectra were recorded in 10 mM PBS (pH 7.4) at room temperature. The arrows show the time-related absorbance changes.

sorption in the range $\lambda = 400$–550 nm, and the appearance of three isosbestic points at $\lambda = 246$, 259, and 333 nm, respectively. This type of absorption between $\lambda = 400$ and 550 nm could be due to the formation of dimerization products.[26] At this point, we identified the oxidative product (furofuran bislactone) of SA in the presence of Cu^{II} ions (see below) and recorded its UV/Vis spectrum to substantiate the formation of a dimer. The formation of a dimer corresponds to the observed UV/Vis spectral changes of SA (square line, Figure 2B). There was no clear change of absorption spectrum for FA, 4-HCA, and 3-HCA in the presence of Cu^{II} ions. The decay rate of HCA or the HCA/Cu^{II} chelate complex follows the sequence of SA > CaA > ChA > FA, 4-HCA, and 3-HCA, which is similar to the activity sequence of the DNA damage mentioned above.

The formation of the CaA phenolate anion/Cu^{II} chelate complex was confirmed by reaction with ethylenediaminetetraacetic acid (EDTA), a well-known chelating agent for metal ions (Figure 3A). EDTA was added after the Cu^{II} ions underwent a reaction with CaA for 60 minutes. Upon the addition of 300 μM EDTA, the redshifted bands ($\lambda = 304$ and 349 nm) returned back to their initial position with a decrease in absorbance (line 3, Figure 3A). This outcome indicates unambiguously that CaA phenoxide can chelate with Cu^{II} ions as a bidentate ligand, hence facilitating intramolecular electron transfer to form *o*-quinone (see below). The formation of *o*-quinone became clear by the addition of EDTA at different reaction times (Figure 3B). Two new bands ($\lambda_{max} = 252$ and 392 nm) observed in the range $\lambda =$ 232–270 nm and at around $\lambda = 400$ nm can be assigned to CaA *o*-quinone (see below; Figure 3B). The spectral changes showed two isosbestic points at $\lambda = 270$ and 347 nm, thus suggesting that a simple equilibrium between two species (CaA and CaA *o*-quinone) was operating. The participation of oxygen in the reaction of CaA with Cu^{II} ions was studied by mixing CaA with Cu^{II} ions in an inert atmosphere. Although the Cu^{II} chelate still formed, the bands at $\lambda = 304$ and 349 nm did not decrease after the Cu^{II} ions had undergone a reaction with CaA for 60 minutes in the inert atmosphere (line 4, Figure 3A). When EDTA was added at this point, the redshifted bands diminished further and the absorption bands of CaA recovered almost to their initial intensities (line 5, Figure 3A). Therefore, Cu^{II} ions could not induce the reaction of CaA in the absence of O_2.

In solvents that support ionization, such as water and alcohols, phenol (ArOH) may equilibrate with the corre-

sponding phenolate anion (ArO^-) [Eq. (1)], which is a much stronger electron donor relative to the parent ArOH. Ethyl acetate has a much lower dielectric constant than ethanol ($\varepsilon = 6.02$ versus 24.30, respectively)[27] and hence is less able to support ionization of the substrate. To rationalize the mechanism and actual electron donor, the effect of the solvent (ethanol and ethyl acetate) on the decay rate of CaA was examined (see Figures S1A and S1B in the Supporting Information for the results). In ethanol, the absorbance of CaA at $\lambda_{max} = 325$ nm was indeed slightly redshifted to $\lambda_{max} = 329$ nm on the addition of Cu^{II} ions, but the decay of the latter was significantly slower than in PBS (pH 7.4; see Figure S1A in the Supporting Information). However, in ethyl acetate, no redshifted peak was observed and the Cu^{II} ions could not induce the reaction of CaA (see Figure S1B in the Supporting Information). This outcome clearly suggests that the actual electron donor is the CaA phenolate anion (ArO^-) instead of the parent molecule (ArOH).

$$ArOH \rightleftharpoons ArO^- + H^+ \quad (1)$$

It has been reported that the oxidation rate of phenol increases remarkably with the increasing anionic character of phenol in alkaline media.[28] Therefore, the influence of the pH value on the decay rate of CaA in the presence of Cu^{II} ions and the spectral change of CaA was investigated. In an NH_4Cl/NH_4OH buffer solution (pH 10.0), the maximum absorption of the CaA phenolate anion at $\lambda_{max} = 344$ nm was slightly redshifted to $\lambda_{max} = 349$ nm on the addition of Cu^{II} ions (Figure 4A). This latter peak is the same as the absorption observed in PBS (pH 7.4), thus indicating unambiguously that it is the CaA phenolate anion, instead of the parent molecule, that chelates with the Cu^{II} ions in PBS (pH 7.4). This spectral evolution in the NH_4Cl/NH_4OH buffer solution (pH 10.0) was characterized by the decrease in the absorption bands at $\lambda = 255$ and 349 nm, the enhancement of the absorption bands in the range $\lambda = 267$–297 nm and above $\lambda = 400$ nm, and the presence of three isosbestic points at $\lambda = 267$, 297, and 391 nm. Notably, the decay of the CaA phenolate anion/Cu^{II} chelate complex was much faster in the NH_4Cl/NH_4OH buffer solution (pH 10.0) than that in PBS (pH 7.4). On the other hand, no redshifted peak was observed in a NaAc/HAc buffer solution (pH 6.0) and the decay of CaA is very slow (Figure 4B), thus further confirming the obligatory role of the phenolate anion in the reaction process.

Oxidative products of CaA and SA in the presence of Cu^{II} ions in a mixed solvent: To further verify the prooxidative mechanism, we subsequently isolated and identified the oxidative products of CaA and SA in the presence of Cu^{II} ions in a mixed solvent (water/acetonitrile = 2:1 (v/v)) at room temperature. The major products (Figure 5) were the dimers (furofuran bislactone) 2,6-bis(3′,4′-dihydroxyphenyl)-3,7-dioxabicyclo[3.3.0]octane-4,8-dione and 2,6-bis(4′-hydroxy-3′,5′-dimethoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane-4,8-dione as characterized with HRMS (ESI) and ^1H and ^{13}C NMR spectroscopic analysis (see the Supporting Information). The CaA dimer has been isolated previously from the cultured mushroom *Inonotus sp.* K-1410[29] and obtained by oxidizing CaA with $NaIO_4$ in water.[30]

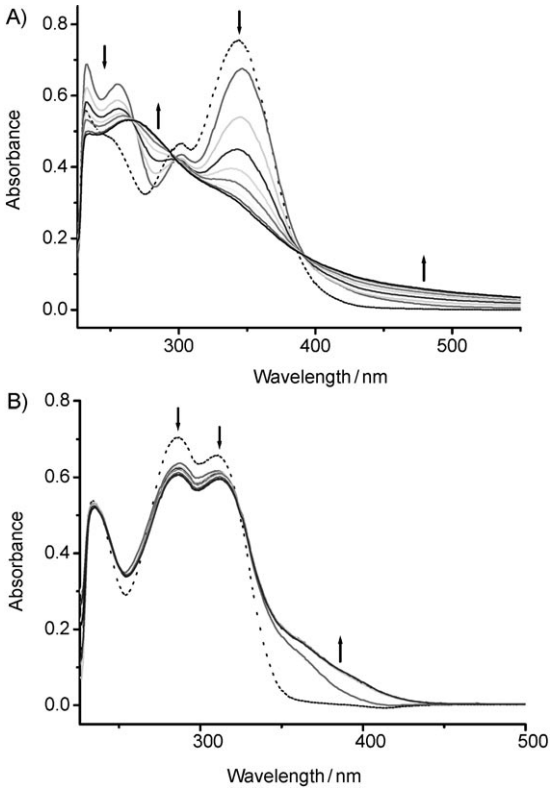


Figure 4. Absorption spectral changes of CaA (50 μM) in the absence (dashed line) and in the presence (solid line) of 100 μM Cu^{II} ions in A) NH_4Cl/NH_4OH buffer solution (pH 10.0) and B) NaAc/HAc buffer solution (pH 6.0) in air (interval = 15 min). The arrows show the time-related absorbance changes.

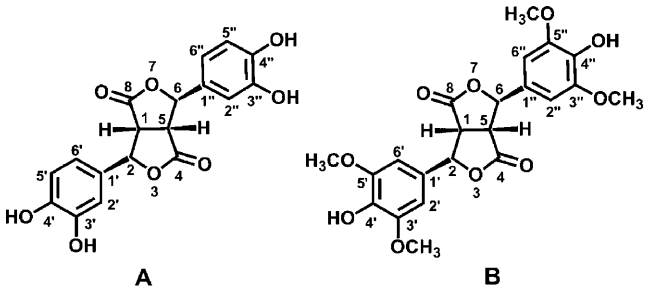


Figure 5. Oxidative products of A) CaA and B) SA in the presence of Cu^{II} ions.

Effect of HCAs on HL-60 cell proliferation in the absence and presence of exogenous Cu^{II} ions: The antiproliferative effect of HCAs on HL-60 cells (seeded density = 2×10^5 cells mL^{-1}) was assessed by using a trypan-blue dye-exclusion test[31] (the results are exemplified in Figure 6 and summarized in Table 1). CaA exhibited dose-dependent inhibitory effects on HL-60 cell proliferation (Figure 6), and the cell viability was decreased to 67, 50, and 35% after

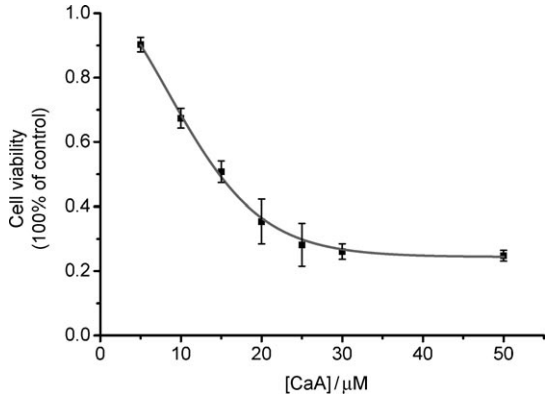


Figure 6. Inhibitory effects of CaA on HL-60 cell viability (seeded density = 2×10^5 cells mL^{-1}). Values are the mean ± S.D. for three independent experiments.

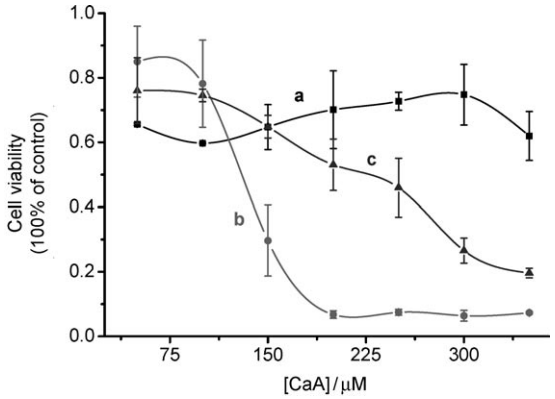


Figure 7. Inhibitory effects of CaA in the a) absence and b) presence of 250 μM exogenous Cu^{II} ions on HL-60 cell viability. Line (c) is the same as (b) but with SOD (0.5 mg mL^{-1}). Cells (seeded density = 5×10^5 cells mL^{-1}) were treated continuously with CaA for 24 h. Values are the mean ± S.D. for three independent experiments.

Table 1. Cytotoxicity against HL-60 cells of HCAs in vitro.[a]

HCAs	IC_{50} [μM]
CaA	14.9 ± 0.3
ChA	18.8 ± 0.8
SA	>250
FA	>250
4-HCA	>250
3-HCA	>250
VP-16	1.4 ± 0.4

[a] Cytotoxicity is expressed as IC_{50}, that is, the concentration of the compound that causes 50% inhibition of the cell viability. Cells (seeded density = 2×10^5 cells mL^{-1}) were treated continuously with HCAs for 48 h. The data are expressed as the mean ± S.D. for three determinations.

treatment with 10, 15, and 20 μM CaA, respectively, for 48 h. The IC_{50} values for all of these compounds are listed in Table 1. Interestingly, the molecules bearing *o*-dihydroxy groups (CaA and ChA) exhibited remarkably higher cytotoxicity than other analogues, and CaA was the most reactive among the HCAs.

Furthermore, to investigate whether Cu^{II} ions can act synergistically with HCAs against cancer-cell proliferation, the antiproliferative effect of HCAs in the presence of exogenous Cu^{II} ions on HL-60 cells was also measured (Figures 7 and 8). In contrast to the experiments in the absence of exogenous Cu^{II} ions, the relatively high cell-seeded density (5×10^5 cells mL^{-1}) and short exposure time (24 h) were chosen for the following experiments to investigate clearly the effect of exogenous Cu^{II} ions on the cytotoxicity of HCAs against HL-60 cells. At this cell-seeded density and exposure time, the presence of CaA increased the cell viability slightly from 50 to 300 μM (line a, Figure 7). A concentration of 250 μM exogenous Cu^{II} ions was selected because Cu^{II} ions alone do not influence the cell proliferation. The addition of exogenous Cu^{II} ions enhanced the cell viability in the presence of a low concentration of CaA (50 μM). However, the cell viability was sharply decreased with an increase in concentration of CaA (line b, Figure 7). At a concentration of CaA that approached 150 μM, the cell viability was decreased to 30%, and at concentrations higher than 150 μM CaA almost completely inhibited the cell proliferation (line b, Figure 7). On the other hand, when superoxide dismutase (SOD; 0.5 mg mL^{-1}, a noncytotoxic concentration) was added before biphasic stimulation effects of exogenous Cu^{II} ions (pro-proliferative and antiproliferative effects in the presence of low and high concentrations of CaA, respectively) were significantly ameliorated (line c, Figure 7), thus suggesting that superoxide produced by CaA in the presence of exogenous Cu^{II} ions might play a pivotal role in the stimulation effects and the level of this superoxide modulates cell proliferation and death.[19a,b,c] Figure 8 shows the

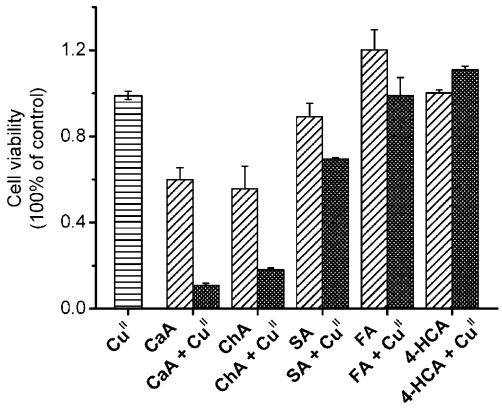


Figure 8. Inhibitory effects of HCAs (250 μM) and exogenous Cu^{II} (250 μM) on HL-60 cell viability. Cells (seeded density = 5×10^5 cells mL^{-1}) were treated continuously with HCAs for 24 h. Values are the mean ± S.D. for three independent experiments.

synergistic effect against cancer-cell proliferation of HCAs with exogenous Cu^{II} ions (250 and 250 μM, respectively), thus indicating clearly that the synergism is much more pronounced in the case of CaA and ChA than in the other cases. Specifically, the cell viability in the presence of CaA and Cu^{II} ions (250 and 250 μM, respectively) was 10%, which is much lower than those produced by CaA and Cu^{II}

ions (60 and 99%, respectively) when they were used individually under the same experimental conditions. Again, the molecules bearing *o*-dihydroxy groups exhibited remarkably higher activities than those bearing no such groups.

Discussion

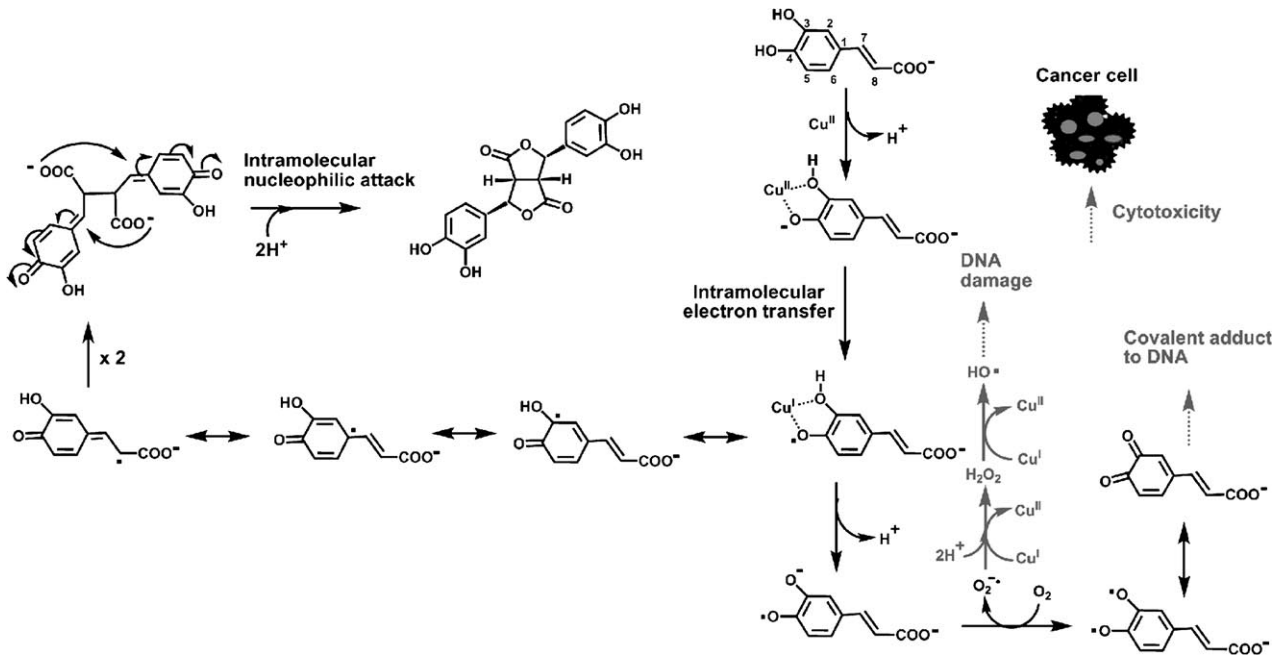
The notion of cancer chemoprevention through antioxidant intervention arises from the fact that fruits and vegetables contain antioxidants that are linked to low cancer rates in those who consume them. In contrast, a recent meta-analysis of the clinical data from 68 randomized human trials with 232606 participants revealed that an increased risk of mortality was associated with the regular use of certain putative antioxidants, such as vitamins A and E and β-carotene.[32] In several large-scale interventions with disease or death as the endpoint, supplementation with β-carotene resulted in no effect or an increase in cancer incidence.[33] These mixed results have made people reconsider the role of antioxidants.[34] As a matter fact, in addition to the counteraction of ROS production, antioxidants induce a multitude of effects on many other cellular functions including cell signaling, apoptosis, production of phase-II enzymes, and so forth. Recently, it has been proposed in some comprehensive reviews that the prooxidant action of plant-derived phenolics rather than their antioxidant action may play an important role in their cancer-chemopreventive properties, as ROS produced by prooxidant action can mediate apoptotic DNA fragmentation.[19] In the present study, HCAs with different structural features were selected as the representative phenolic antioxidants to investigate their prooxidant effect on DNA damage in the presence of Cu^{II} ions and the related mechanisms and biological implications. We not only explored the detailed prooxidative mechanism for HCAs in the presence of cupric ions from a chemical point of view but also provided direct evidence for prooxidant cancer-chemopreventive action.

Mechanism and structure–activity relationship: HCAs, which are generally effective antioxidants, can switch to being prooxidants in the presence of Cu^{II} ions to induce DNA damage, except for monohydroxy-substituted HCAs (4-HCA and 3-HCA; Figure 1A). It is clearly seen that the compounds bearing *o*-dihydroxy and *o*-dimethoxyhydroxy groups (i.e., CaA, SA, and ChA) are most active in inducing plasmid pBR322 DNA strand breakage in the presence of Cu^{II} ions.

The high activity of CaA and ChA is obviously due to their *o*-dihydroxy groups, which can lose a proton to produce the corresponding phenolate anion (ArO^-) followed by chelation with Cu^{II} ions to form ArO^-/Cu^{II} complexes (see Figures 2–4 and Figure S1 in the Supporting Information). Although the formation of metal complexes of polyphenols bearing *o*-dihydroxy groups has been well documented,[10] the present work provides unambiguous evidence, by means of the effects of EDTA, the solvent, and pH value on the spectral changes of CaA in the presence of Cu^{II} ions, that it is the ArO^- ion instead of the parent molecule (ArOH) that chelates with the Cu^{II} ions. The ArO^-/Cu^{II} complex can undergo intramolecular electron transfer to form the corresponding semiquinone radical and Cu^{I} ion. It has been pointed out that a 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.[35] Although the pK_{a2} value of CaA is 8.48,[36] in the presence of Cu^{II} ions, the proton is displaced at much lower pH values, for example, pH 5.0–8.0.[35] Therefore, CaA should dissociate to form a phenoxide, which chelates Cu^{II} ions as a bidentate ligand and undergoes intramolecular electron transfer to form an *o*-hydroxyphenoxyl radical (semiquinone radical). The radical intermediate was also proved by the formation of the dimer (furofuran bislactone) (Figure 5) of CaA in the presence of Cu^{II} ions. The dimer must be formed by the 8,8′-coupling of the two radicals followed by the intramolecular nucleophilic addition of the carboxylate groups onto the quinone methide moieties (Scheme 2).

The higher activity of the compounds bearing *o*-dihydroxyl groups in the DNA damage can be understood because the oxidation intermediate, the *o*-hydroxyphenoxyl radical, is more stable due to the intramolecular hydrogen-bonding interaction, as evidenced recently from both spectrophotometric measurements[37] and theoretical calculations.[38] The acidity dissociation constant of the *o*-hydroxyphenoxyl radical is much lower ($pK_{a1}=4.1$)[39] than that of catechol ($pK_{a1}=9.25$).[40] Thus, the *o*-hydroxyphenoxyl radical should dissociate and form *o*-semiquinone radical anions. The *o*-semiquinone radical anion will be more easily further oxidized to form the final product *o*-quinone (Scheme 2). Two new peaks that appeared at $\lambda=252$ and 392 nm in the case of CaA demonstrates the formation of CaA *o*-quinone. This outcome is in accordance with the previous observation of the absorbances of CaA *o*-quinone at $\lambda=248$ and 400 nm, which appeared during the chemical and enzymatic oxidation of CaA by *o*-chloranil[41] and polyphenol oxidase,[42] respectively.

The fact that Cu^{II} ions could not effectively oxidize CaA in the absence of oxygen (line 4, Figure 3B) demonstrates unambiguously the involvement of O_2 in the process. The CaA/Cu^{II}-mediated DNA damage was ameliorated by CAT, GSH, and BCDS, thus suggesting the involvement of ROS and Cu^{I} ions in the process. Therefore, a possible mechanism of DNA damage induced by CaA in the presence of Cu^{II} ions can be clarified as shown in Scheme 2. The initial electron-transfer oxidation of CaA by Cu^{II} ions generates the corresponding semiquinone radical, which undergoes a second electron transfer with O_2 to form *o*-quinone and $O_2^{-\bullet}$ radicals. The $O_2^{-\bullet}$ radicals react with Cu^{I} ions to give hydrogen peroxide, which is readily converted by a Fenton-type reaction into hydroxyl radicals to induce the oxidative DNA damage. In addition, Cu^{I} ions can be oxidized back to Cu^{II} ions by oxygen. This Cu^{II}/Cu^{I} redox cycle renders CaA be catalytically oxidized.



Scheme 2. Proposed mechanism for CaA/Cu^{II}-mediated DNA damage.

It is also noticeable that SA bearing *o*-dimethoxyhydroxy groups exhibits the fastest reaction rate with Cu^{II} ions among the examined HCAs (Figure 2B), which is in line with the previous observation in the electron-transfer reaction of HCAs with 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), with SA as the most reactive.[24c] The possible reason for this behavior is related to the structure of the ArO^{-}(SA)/Cu^{II} complex, the formation of which was also elucidated by the gradual redshift of the maximal absorption of SA (Figure 2B). In the complex, there are two electron-donating (ED) methoxy groups in the *ortho* position of the ArO^{-} ion, which could further enhance the electron density of the ArO^{-} ion and hence result in the fastest intramolecular electron-transfer rate. By comparing the DNA-cleaving effectiveness of the three compounds (CaA, ChA, and SA), we found the active sequence CaA > SA > ChA and can conclude that in addition to the electron-transfer reaction rate (i.e., the formation rate of ROS), the formation of *o*-quinone is the other important factor to influence the DNA-cleaving effectiveness. It has been well documented that *o*-quinones are involved in DNA damage by forming covalent adducts with DNA.[10,43] Although SA exhibited the fastest electron-transfer reaction rate, it could not form *o*-quinone in its reaction with Cu^{II} ions. Therefore, SA was the second most effective at DNA cleavage.

The oxidative potential was reported to be $E_{pa} = 0.212$, 0.261, 0.314, 0.430, and 0.583 V (versus Ag/AgCl) for CaA, ChA, SA, FA, and 4-HCA respectively.[44] It should be noted that the ranking of the oxidation potentials does not corroborate with the fact that SA was the most reactive species in the electron-transfer reaction. The reason for this inconsistency could be the formation of the ArO^{-}/Cu^{II} complex, which could shift the oxidation potential of SA to the lowest value. It has been well documented that complexation of metal ions such as Cu, Fe, and Mn with phenolic compounds results in the changes in their redox potentials;[45] for example, the reduction potential of a catechol/Cu^{II} complex is about $E = 0.277$ V (versus NHE), whereas it is $E = 0.442$ V (versus NHE) for catechol.[45]

Biological implications: Intriguingly, the compounds bearing *o*-dihydroxy groups (i.e., CaA and ChA) exhibited remarkably higher cytotoxicity toward HL-60 cells than those compounds bearing no such groups (Table 1). Notably, these compounds also exhibited enhanced activity in inducing DNA damage. A similar structure–activity relationship was also observed in resveratrol and its analogues.[21a] In contrast to ChA, SA had a relatively high activity in inducing DNA damage, but exhibited low cytotoxicity toward HL-60 cells. This behavior highlights the involvement of *o*-quinone in the cytotoxicity, in line with reports of *o*-quinone products from the oxidation of catecholic estrogen[43a] and dopamine[43b] that are responsible for the observed apoptotic effects of these chemicals in mutagenic and neuroblastoma cells.

In the absence of exogenous Cu^{II} ions, CaA alone exhibited cytotoxicity toward HL-60 cells (Figure 6), thus implying the existence of endogenous Cu^{II} ions. 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 pro-oxidant action.[11c,d,19e] It was also reported that exogenous copper could enhance cytotoxic and apoptosis-inducing activities of phenolic antioxidants against human leukemia and breast cancer cells.[46] In the present study, the incorporation exogenous Cu^{II} ions into the cells at a concentration

of 250 μM demonstrated that the biphasic pattern of cell viability depended on the concentration of CaA (Figure 7). A concentration of 50 μM CaA enhanced cell viability and, conversely, CaA at high concentrations (i.e., >150 μM) almost completely inhibited cell proliferation. Furthermore, the addition of SOD significantly inhibited the two stimulation effects of exogenous Cu^{II} ions, thus clearly indicating that the ROS level modulates cell proliferation and death and the two stimulation effects of exogenous Cu^{II} ions come from weak and strong oxidative stress (prooxidant action), respectively. It is now well recognized that ROS might function as a doubled-edged sword.[19a,b,c] A moderate increase in ROS may promote cell proliferation and survival; however, when the increase in ROS reaches a certain level (the toxic threshold), it may trigger cell death.[19a,b,c] In contrast to normal cells, cancer cells exhibit increased intrinsic ROS stress and copper levels.[19a,b,c,47] Therefore, a further increase in ROS stress in cancer cells by using an exogenous ROS-generating agent (prooxidant) is likely to cause elevation of ROS above the threshold level, thus leading to death of these cells.[19a,b,c] However, the same concentration of the exogenous ROS-generating agent (prooxidant) in normal cells could not induce the ROS level to reach the toxic threshold, and thus the normal cells survive. As a whole, in the case of antioxidant-based cancer chemoprevention, strong oxidative stress acts as a "good" stress and may play a crucial role in the cytotoxicity of antioxidants against cancer cells (Scheme 2). Additionally, it is worth noting that CaA and ChA clearly exhibit a synergistic effect with Cu^{II} ions against HL-60 cell proliferation (Figure 8), thus further highlighting the importance of *o*-dihydroxy groups in phenolic antioxidants in the cancer chemoprevention.

Conclusions

In conclusion, our data substantiate the important role of HCAs as DNA-cleaving agents in the presence of Cu^{II} ions. The observation that compounds bearing *o*-dihydroxy moieties exhibit remarkably high activities in DNA damage and an antiproliferative effect on HL-60 cells in the absence and presence of exogenous Cu^{II} ions, provides useful information for the identification and development of more potent cancer-chemopreventive agents. The detailed prooxidative mechanism also provides the necessary groundwork for understanding antioxidant-based cancer chemoprevention.

Experimental Section

Materials: Caffeic acid (CaA, Acros), chlorogenic acid (ChA, Aldrich), sinapic acid (SA, Acros), ferulic acid (FA, Aldrich), 3-hydroxycinnamic acid (3-HCA, Aldrich), 4-hydroxycinnamic acid (4-HCA, Fluka), bathocuproinedisulfonic acid disodium salt (BCDS, Fluka), etoposide (VP-16, Sigma), trypan blue (Sigma), ethidium bromide (Sigma), catalase (CAT, 3000 U mg protein^{-1}, Sigma), and pBR322 DNA (Sigma) were purchased in the highest purity available and used as received. Superoxide dismutase (SOD, 6500 U mg protein^{-1}) was purchased from Beyotime Biotechnology. HL-60 cell lines were originally obtained from the Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences). All the other chemicals were of the highest quality available.

Assay for oxidative DNA strand breakage: The induction of DNA strand breakage by HCAs was assessed by measuring the conversion of the supercoiled pBR322 plasmid DNA into open circular and linear forms or into further fragmentation by gel electrophoresis.[25] pBR322 DNA (125 ng) was incubated with HCAs and/or Cu^{II} ions in PBS at pH 7.4 and 37 °C for 1 h in 1.5-mL microcentrifuge tubes. The total volume was 20 μL, that is, DNA (5 μL), HCAs (5 μL), Cu^{II} (5 μL), and PBS (5 μL). In the inhibition experiments, specific scavengers of ROS and the Cu^{I} chelator were preincubated before addition of the Cu^{II} ions. After incubation, the samples (10 μL) were mixed with gel loading buffer (2 μL; 0.25% bromophenol blue and 30% (w/v) glycerol) and immediately loaded in a 1% agarose gel containing 40 mM tris(hydroxymethyl)aminomethane (Tris), 20 mM sodium acetate, and 2 mM EDTA and subjected electrophoresis in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer for 1 h. The gels were stained with 0.5 μg mL^{-1} ethidium bromide for 1 h followed by destaining in water for 0.5 h, and photographed under UV light.

UV/Vis spectral measurements: The UV/Vis spectra were measured at room temperature with a Hitachi 557 spectrophotometer. PBS containing 50 μM HCAs was kept at room temperature, and the spectral tracing was started by the addition of 100 μM $CuSO_4$. All the spectra were run against blanks containing the buffer and the metal ions. The spectra were recorded every appointed time after addition of 100 μM Cu^{II} ions.

Oxidative product analysis of CaA and SA in the presence of Cu^{II} ions: CaA (500 mg, 2.8 mmol) or SA (672 mg, 2.8 mmol) was dissolved in acetonitrile (100 mL) and an aqueous solution of $CuSO_4{\cdot}5H_2O$ (2.8 mM, 200 mL) was added. The reaction mixture was stirred for 7 h at room temperature and evaporated to dryness at 40 °C under reduced pressure. The residue was redissolved in water and extracted with ethyl acetate (3×150 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and taken to dryness to give a residue. The residues of CaA and SA were purified by column chromatography on silica gel and eluted with chloroform/acetone/acetic acid (4:1:0.5, v/v/v) and petroleum ether/acetone (4:1, v/v), respectively. The CaA dimer (170 mg) and SA dimer (520 mg) were obtained as a white and yellow powders, respectively.

CaA dimer: ^{1}H NMR (400 MHz, $(CD_3)_2CO$): $\delta = 3.99$ (br s, 2H; H-1 and H-5), 5.72 (br s, 2H; H-2 and H-6), 6.81 (dd, $J = 8.0$, 2.0 Hz, 2H; H-6′and H-6″), 6.92 (d, $J = 2$ Hz, 2H; H-2' and H-2″), 6.87 ppm (d, $J = 8.0$ Hz, 2H; H-5' and H-5″); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): $\delta = 49.3$ (C-1, C-5), 83.2 (C-2, C-6), 113.9 (C-6′, C-6″), 116.5 (C-5′, C-5″), 118.4 (C-2′, C-2″), 131.3 (C-1′, C-1″), 146.5 (C-4′, C-4″), 146.8 (C-3′, C-3″), 176.1 ppm (C-4, C-8); HRMS (ESI): *m/z*: calcd for $C_{18}H_{14}O_8 + NH_4^+$: 376.1027 [$M + NH_4$]$^+$; found: 376.1029, error = 0.5 ppm.

SA dimer: ^{1}H NMR (400 MHz, $(CD_3)_2CO$): $\delta = 7.45$ (s, 2H; OH), 6.74 (s, 4H; H-2′, H-6′, H-2″, and H-6″), 5.76 (s, 2H; H-2 and H-6), 4.13 (s, 2H; H-1 and H-5), 3.84 ppm (s, 12H; H-3′, H-5′, H-3″ and H-5″-OCH_3); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): $\delta = 176.2$ (C-4, C-8), 149.2 (C-3′, C-5′, C-3″ and C-5″), 137.6 (C-4′, C-4″), 130.0 (C-1′, C-1″), 104.4 (C-2′, C-6′, C-2″ and C-6″), 83.5 (C-2, C-6), 56.9 (C-3′, C-5′, C-3″, C-5″-OCH_3), 49.2 ppm (C-1, C-5); ESI-MS: *m/z*: 446.9 [$M + H$]$^+$.

Assessment of cell viability: The numbers of viable cells were counted using a trypan-blue dye-exclusion test.[31] Trypan-blue dye-exclusion test is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. The viable cell number was counted. In the experiment, HL-60 cells were seeded at a density of 2×10^5 cells mL^{-1} in 24-well multiwell plates and treated with various concentrations of HCAs. After incubation in a humidified CO_2 (5%) incubator at 37 °C for 48 h, cells were harvested and stained with trypan blue and observed under a microscope for cell-number counting. For the cell-viability assay of HCAs in the presence of cupric ions, 5×10^5 cells were plated onto 24-well multiwell plates and treated with various concentrations of CaA and cupric ions (250 μM) for 24 h. For the effect of SOD on the cell viability of CaA in the present of

cupric ions, SOD (0.5 mg mL^{-1}) was incubated for 5 h before addition of CaA and cupric ions.

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