

The Coffee Constituent Chlorogenic Acid Induces Cellular DNA Damage and Formation of Topoisomerase I– and II–DNA Complexes in Cells

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ABSTRACT: Chlorogenic acid (CGA) is a plant polyphenol with known antioxidant properties. Although some studies suggest that CGA has anticancer properties, others indicate that this dietary constituent may cause DNA damage and induce carcinogenic effects. Because CGA is widely consumed in the form of coffee, it is important to further evaluate the putative DNA-damaging activity of CGA. Here we have employed two standard techniques commonly used for DNA damage detection (the comet assay and the γ -H2AX focus assay) and observed that CGA (0.5–5 mM) induces DNA damage in normal and cancer cells. We report for the first time that CGA induces high levels of topoisomerase I- and topoisomerase II-DNA complexes in cells (TARDIS assay). Catalase pretreatment abolished the formation of these topoisomerase-DNA complexes and reduced the cytotoxic activity of CGA, therefore indicating that hydrogen peroxide plays an important role in these activities. Lung cancer cells (A549) were more sensitive than normal lung fibroblasts (MRC5) to the cytotoxic activity of CGA, supporting previous findings that CGA may induce selective killing of cancer cells. Taking into consideration our results and the pharmacokinetic profile of CGA, the possible cancer preventive, carcinogenic and therapeutic potential of this dietary agent are discussed.

KEYWORDS: DNA damage, DNA topoisomerases, apoptosis, hydrogen peroxide, lung cancer, leukemia

INTRODUCTION

Coffee, obtained from the roasted seeds of several *Coffea* species, is one of the most widely consumed beverages in the world. It is commonly used for its stimulating properties, which are mediated by the central nervous system stimulant caffeine. Numerous studies have been published on the health effects of coffee consumption.¹ These studies suggest that coffee consumption may help prevent several chronic diseases, including type 2 diabetes, Parkinson's disease, liver disease (e.g., cirrhosis), and some types of cancer (e.g., liver and endometrial cancers).^{1,2} Coffee consumption, however, has also been associated with increases in several cardiovascular disease risk factors (e.g., blood pressure) and with a higher risk of some types of cancer.^{1,2} According to the International Agency for Research on Cancer (IARC, Vol. 51), coffee is possibly carcinogenic to the human urinary bladder. Epidemiological data also suggest that coffee consumption may increase the risk of lung cancer³ and that maternal consumption of coffee during pregnancy may be associated with childhood leukemia.^{2,4}

Coffee is a complex mixture of chemicals and contains significant amounts of caffeine and chlorogenic acid (CGA).¹ CGA is an ester in which the acid part of caffeic acid is bound to the hydroxyl group in position 5' of quinic acid (5'-caffeoylquinic acid; Figure 1). Although CGA has been identified in many plant-derived foods, coffee is its major dietary source; the CGA content of a 200 mL cup of coffee has been reported to range from 70 to 350 mg.⁵ CGA has known antioxidant properties,^{6–8} which may be involved in the

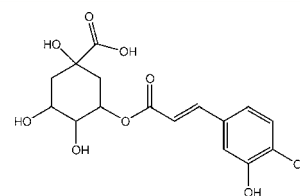


Figure 1. Chemical structure of chlorogenic acid.

antigenotoxic activity that some in vitro and in vivo studies have attributed to this polyphenol.^{9–12} Because oxidative stress plays an important role in the development of chronic diseases,¹³ the antioxidant properties of CGA may participate in the reduced risk of some chronic diseases that have been associated with coffee consumption.¹ Other studies have shown, however, that CGA induces genotoxic effects, which are accentuated by copper and may be mediated by a pro-oxidant mechanism.^{14–18} The possible genotoxic properties of CGA may be involved in the increased risk of some types of cancer that some studies have linked to coffee consumption.^{2–4}

DNA topoisomerases (topos) might play a role in the possible anticancer and genotoxic activity of CGA (see references below). Topo I and topo II are nuclear enzymes

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that introduce transient single- or double-strand breaks in the DNA to solve the topological problems associated with DNA replication, transcription, recombination, and chromatin remodeling. Topo I and topo II are the targets of several clinically useful anticancer drugs, including etoposide and the camptothecin derivatives topotecan and irinotecan. These drugs, known as topoisomerase poisons or topoisomerase inhibitors, stabilize the normally transient topo–DNA complexes formed during the catalytic cycle of the enzyme. Then, cellular processing converts these topo–DNA complexes into permanent DNA strand breaks that trigger cell death.^{19–22} The induction of topoisomerase-mediated DNA damage by these drugs may also lead to genotoxic effects; the clinical utilization of topo II poisons has indeed been associated with an increased risk of developing some types of leukemia.²³ An increased risk of childhood leukemia has also been associated with maternal ingestion of dietary flavonoids, an effect that has been associated with the ability of these polyphenols to inhibit topo II.^{24,25} Previous papers have shown that organic extracts from a CGA-containing plant inhibited topo II²⁶ and that caffeic acid (the acid part of the CGA ester) inhibited topo I.²⁷ The pro-oxidant activity of CGA^{14,16} also suggests that CGA may induce topo–DNA complexes, as the oxidant hydrogen peroxide can induce topoisomerase-mediated DNA damage.^{28–30}

Currently, it is unclear if CGA can prevent^{9–12} or induce^{14–18} DNA damage. In addition, it is unknown if DNA topoisomerases I and II are involved in the biological activities of CGA. Because CGA is widely consumed in the form of coffee, it is important to further evaluate the putative DNA-damaging activity of CGA and to test the activity of CGA on DNA topoisomerases to better understand the possible anticancer and carcinogenic properties of this dietary constituent. This paper shows that specific concentrations of CGA induce DNA damage (measured by the alkaline comet assay and by γ H2AX foci formation), high levels of topo I– and topo II–DNA complexes (TARDIS assay), and selective killing of cancer cells. These carcinogenic and anticancer effects are discussed, taking into account the pharmacokinetic profile of CGA.

MATERIALS AND METHODS

Chemicals and Cell Lines. Chlorogenic acid, etoposide, camptothecin, hydrogen peroxide, curcumin, and catalase were purchased from Sigma. Human K562 leukemia cells were maintained as a suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin (50 μ g/mL)/streptomycin (50 μ g/mL). The human A549 lung cancer cell line, the human embryo lung fibroblastic MRC-5 cell line, and the Chinese hamster ovary AA8 cell line were maintained in DMEM supplemented with 2 mM glutamine, 50 μ g/mL penicillin, 50 μ g/mL streptomycin, and 10% fetal bovine serum. All cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Cell culture reagents were obtained from Life Technologies.

Cell Proliferation Assays. The XTT and MTT assays are colorimetric techniques that allow the quantitative determination of cell viability.³⁰ They are based on the capability of viable cells to transform the XTT salt (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]bis(4-methoxy-6-nitro) or the MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into formazan dyes. Exponentially growing cells were seeded into 96-well plates, and drugs were added 24 h later. In the XTT assay, following an incubation period of 5 days, cell viability was quantified using an XTT cell proliferation kit assay (Roche, Mannheim, Germany). After drug exposure, plates were incubated for 4 h with XTT before they were

read on a Bio-Rad 550 plate reader at 450 nm. In the MTT assay, following an incubation period of 2 days, medium was removed and 125 μ L of MTT (1 mg/mL in medium) was added to each well for 5 h. Then, 80 μ L of 20% SDS in 0.02 M HCl was added, plates were incubated for 10 h at 37 °C, and optical densities were measured at 540 nm on a multiwell plate spectrophotometer reader. In both assays, cell viability was expressed as percentage in relation to controls. All data were averaged from at least three independent experiments and were expressed as the means \pm the standard deviation of the mean (SEM).

Comet Assay. The single-cell gel electrophoresis assay (comet assay) is a sensitive technique for the detection of DNA damage in cells and has been described in detail by Singh et al.³¹ We followed this protocol with minor modifications described previously.³² Briefly, standard slides were immersed in 1% normal melting agarose at 55 °C, left to allow the agarose to solidify, and kept at 4 °C until use. After cell treatments, approximately 10000 cells were mixed with 85 μ L of low-melting agarose (LMA) at 37 °C. This mixture was rapidly pipetted onto the slides with the first agarose layer, spread using a coverslip, and kept at 4 °C for 8 min to allow the LMA to solidify. The coverslips were then removed, and a third layer of 100 μ L of LMA at 37 °C was added, covered with a coverslip, and allowed to solidify at 4 °C for 8 min. After removal of the coverslips, cells were incubated in the dark for 1 h at 4 °C in a lysis solution containing 10 mM Tris-HCl, 2.5 M NaCl, 100 mM Na₂-EDTA, 0.25 M NaOH, 1% (v/v), Triton X-100, and 10% (v/v) DMSO, pH 12.0. To unwind the DNA, the slides were incubated for 20 min in an electrophoretic buffer containing 1 mM Na₂-EDTA and 300 mM NaOH, pH 12.8. Electrophoresis was carried out at 1 V/cm for 20 min. After neutralization with 3 \times 5 min washes of 0.4 M Tris-HCl, pH 7.5, to remove alkali and detergent, cells were stained with the fluorochrome 4',6-diamidino-2-phenylindole (DAPI) in Vectashield (mounting medium for fluorescence H-1000, Vector Laboratories, Peterborough, UK). Images of 50 randomly selected cells from each sample were analyzed using the "comet score" software. DNA damage was calculated for each comet and was expressed as percent of DNA in the tail and as tail moment (defined as the product of the tail length and the fraction of total DNA in the tail).

Immunofluorescence γ H2AX Focus Assay. The immunofluorescence γ H2AX focus assay is a sensitive technique to evaluate DNA damage. It is based on the ability of double-strand breaks (DSBs) to trigger phosphorylation of histone H2AX on Ser-139, which leads to the formation of nuclear foci that can be visualized with anti- γ H2AX antibodies.^{33,34} After treatments, K562 cells were pelleted at 1200 rpm for 5 min, washed two times with PBS, and resuspended and incubated in cold pure methanol for 20 min. They were then centrifuged, resuspended in 200 μ L of methanol, dropped onto coverslips, and allowed to dry. AA8 cells were seeded on coverslips and allowed to attach for 24 h. After treatments, AA8 cells were washed three times with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and washed three times with PBS. After fixation, K562 cells and AA8 cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min and then blocked three times with 0.1% Tween 20 and 1% BSA in PBS for 5 min each. Cells were then incubated for 1 h with a mouse anti- γ H2AX monoclonal antibody (Upstate; 1:800 dilution). Cells were washed three times with PBS and blocked three times prior to the incubation with a secondary anti-mouse antibody linked to Alexa Fluor 488 (Invitrogen; 1:500 dilution) for 1 h. Cells were washed with PBS, blocked, and washed again with PBS as indicated before. DNA was stained with DAPI, and immunofluorescence was observed at 40-fold magnification with an Olympus BX 61 microscope. A total of \sim 200 cells/dose were scored, and cells with 10 or more foci were scored as positive. Ionizing radiation was used as a positive control for the assay; cells were exposed to 4 Gy of ionizing radiation using an X-ray irradiator (Philips MU15F) operated at 100 kV and a dose rate of 1 Gy/min.^{35,36}

TARDIS Assay. This immunofluorescence technique employs specific antibodies to DNA topo I or topo II to detect the protein covalently bound to the DNA in individual cells. Anti-topo II polyclonal antibody α CT was raised in rabbits to recombinant topo II α (C-terminal fragment) and detected both the α and β isoforms of

topo II. For topo I, a polyclonal human antibody (2012, Topogen) was used. Antibodies were diluted in PBS containing 0.1% Tween 20 and 1% bovine serum albumin (BSA). α CT (topo II) was used at a 1:50 dilution and 2012 (topo I) at 1:1000. An anti-rabbit FITC-conjugated second antibody (1262, Sigma) was used at 1:200 dilution for topo II antibody, and a goat anti-human FITC-conjugated second antibody (F5512, Sigma) was used at 1:50 dilution for topo I.^{30,37,38} The TARDIS assay has been described in detail previously.^{30,37,38} Briefly, cells were seeded (3×10^4 cells/well) into six-well tissue culture plates. These were grown for 48 h, and drugs were added to exponentially growing cells at several concentrations. Microscope slides were pre-coated with agarose, and drug-treated or untreated cells (control) were immediately embedded in agarose and spread onto the slide. Slides were then placed in lysis buffer containing protease inhibitors for 30 min (after this stage, slides could be stored at -20°C in PBS containing 10% glycerol), followed by 30 min in 1 M NaCl plus protease inhibitors. Slides were then washed three times in PBS (5 min/wash) and exposed to primary antisera for 1–2 h. Slides were washed three times in PBS containing 0.1% Tween 20 (PBST) and subsequently exposed for 1–2 h to a secondary antibody (anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody, F(ab')₂ fragment; Sigma). Slides were washed three times in PBST followed by an overnight wash in PBS containing protease inhibitors, at 4°C . Slides were stained with Hoechst 33258 ($10\ \mu\text{M}$ in PBS; Sigma Chemical Co.) for 5 min, and coverslips were applied and secured. Images of blue (Hoechst-stained DNA) fluorescence and green (FITC-stained covalently bound topo-DNA) immunofluorescence were then captured with an epifluorescence microscope attached to a cooled slow-scan charge-coupled device camera. For each of the approximately eight randomly chosen fields of view, images of blue and green fluorescence were captured to give a total of ~ 100 cells/dose. Images were then analyzed to quantify the levels of Hoechst (blue) fluorescence and FITC (green) immunofluorescence with Imager 2 software (Astrocam, Cambridge, UK) based on Visilog 4 (Noesis, Paris, France). All images were corrected for stray light and camera background. Additionally, images were subjected to blue and green shade correction to compensate for variation in intensity of illumination and nonuniformities in light transmission.^{30,37,38}

Annexin V–FITC Apoptosis Detection Assay. Apoptosis was quantified by flow cytometric analysis (Cytomics FC 500 MPL, Beckman Coulter; CXP analysis) using annexin V–FITC apoptosis detection kit (catalog no. 556547, BD Biosciences) according to the manufacturer's instructions.

Statistical Analysis. All data were averaged from at least three independent experiments and were expressed as the mean \pm the standard error of the mean (SEM). For statistical analysis we used the *t* test (paired, two-tailed). A *P* value >0.05 is not considered to be statistically significant and is not represented by any symbol. A *P* value <0.05 is considered to correspond with statistical significance and is indicated with an asterisk (*), a *P* value <0.01 is indicated with a double asterisk (**), and a *P* value <0.001 is indicated with a triple asterisk (***) .

RESULTS AND DISCUSSION

Because it is unclear whether the dietary constituent CGA can prevent or cause genetic damage, we initially used the comet assay to evaluate if CGA could induce DNA damage in cells. Three independent experiments revealed that K562 leukemia cells exposed for 2 and 24 h to three concentrations of CGA (0.5, 1, and 5 mM) had higher levels of DNA damage than untreated cells, although no clear dose- or time-dependent relationship was observed. A representative photograph of cells exposed to each concentration of CGA, the quantification of DNA damage, and the percentage of cells within different ranges of DNA damage are illustrated in Figure 2. These experiments showed that, with the exception of 5 mM CGA for 24 h, approximately 20% of cells exposed to CGA had higher levels of DNA damage than control cells. All cells exposed to 5

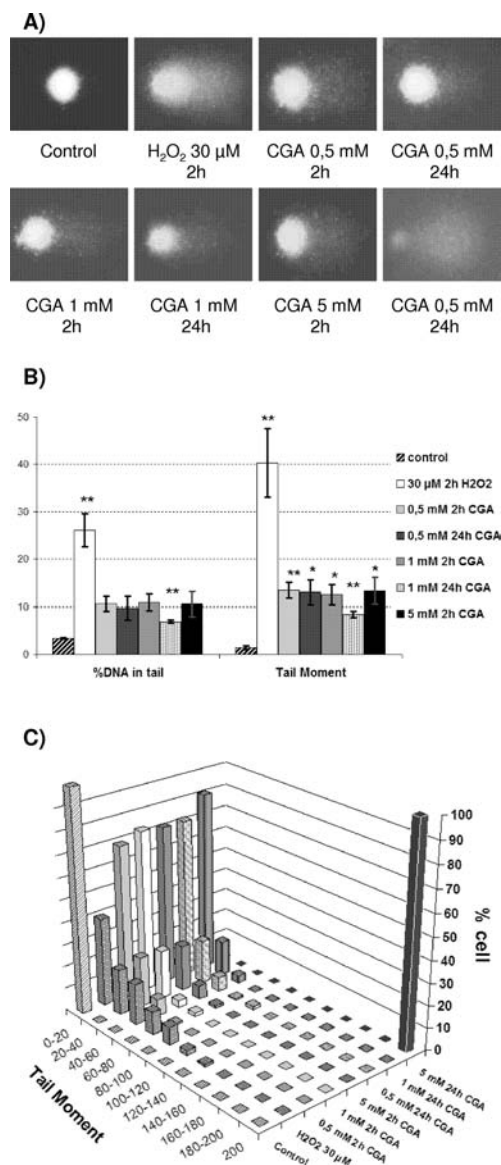


Figure 2. Assessment of chlorogenic acid (CGA)-induced DNA damage in K562 cells by the comet assay. (A) Representative photographs of untreated cells, of cells treated with the positive control hydrogen peroxide (H_2O_2), and of cells exposed for different times to several concentrations of CGA. (B) Quantification of DNA damage expressed as percent of DNA damage in tail and as tail moment (tail length \times percentage of DNA in the tail). Data are expressed as the mean \pm SEM. For statistical analysis we used the *t* test (paired, two-tailed). A *P* value >0.05 is not considered to be statistically significant and is not represented by any symbol. A *P* value <0.05 is considered to correspond with statistical significance and is indicated with an asterisk (*), and a *P* value <0.01 is indicated with a double asterisk (**). (C) Distribution of cells in the different intervals of values of tail moments (data are averaged from three independent experiments).

mM CGA for 24 h had levels of DNA damage so high that they were difficult to quantify (Figure 2). No DNA damage was detected with the comet assay in HL-60 cells exposed to lower concentrations of CGA (0.2 mM) in a previous study.³⁹

The ability of CGA to induce DNA damage was further assessed with the immunofluorescence γ H2AX focus assay. We initially used the K562 leukemia cell line and observed in three independent experiments that cells treated with CGA (0.5 mM,

2 h) had higher levels of fluorescence than untreated cells (Figure 3A, left panels). However, the background signal in

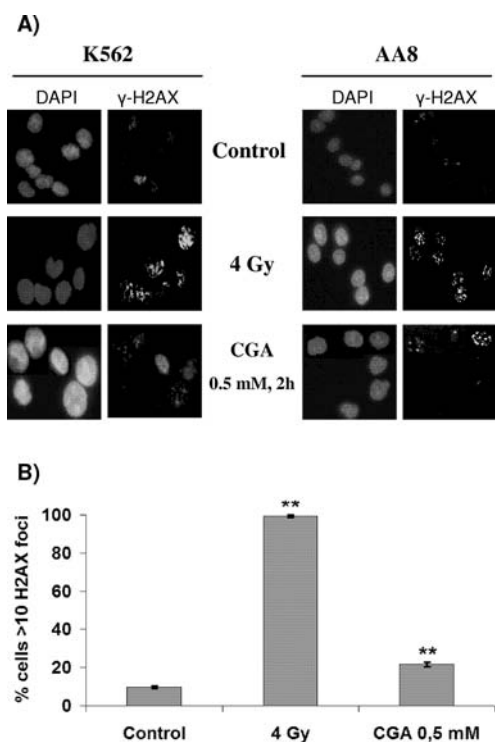


Figure 3. Assessment of chlorogenic acid (CGA)-induced DNA damage in K562 cells and AA8 cells by the immunofluorescence γ H2AX focus assay. (A) Representative photographs of control cells, of cells irradiated with an X-ray device at a dose of 4 Gy (positive control), and of cells exposed for 2 h to 0.5 mM CGA. (B) Quantification of nuclear foci in nonmalignant AA8 cells exposed to X-ray (4 Gy) and CGA (2 h, 0.5 mM). Data are expressed as the mean \pm SEM. For statistical analysis we used the *t* test (paired, two-tailed). A *P* value <0.01 is considered to correspond with statistical significance and is indicated with a double asterisk (**).

these cancer cells was high, and the number of foci could not be properly quantified. We therefore used a nonmalignant cell line (AA8) to corroborate that CGA induced DNA damage in cells and to quantify the damage. Three independent experiments confirmed that CGA (0.5 mM, 2 h) increased the formation of nuclear foci (indicative of DNA damage) in relation to control cells, although such an increase was lower than that induced by the positive control (X-rays, 4 Gy). Representative photographs of AA8 cells treated with CGA and the quantification of the DNA damage are respectively shown in Figure 3A (right panels) and Figure 3B.

Topoisomerase inhibitors are a group of antitumor drugs that kill cancer cells by inducing topoisomerase-mediated DNA damage.^{20,22,23} Because previous evidence suggested that CGA might induce topoisomerase-mediated DNA damage (discussed in the Introduction), we investigated the ability of CGA to induce topo–DNA complexes in individual cells with the TARDIS assay.^{30,37,38} We used higher concentrations of CGA because this assay requires concentrations approximately 10 times higher than those needed to induce cell growth inhibition to observe a clear effect. A 2 h exposure of CGA (1 and 5 mM) did not induce the formation of significant levels of topo I– or topo II–DNA complexes in K562 cells (Figure 4). However, high levels of both topo I– and topo II–DNA complexes were

observed when cells were exposed to 5 mM CGA for 24 h. Because previous papers suggested that CGA can generate hydrogen peroxide^{14,16,40} and because hydrogen peroxide can induce topo–DNA complexes in cells,³⁰ we evaluated if catalase pretreatment could reduce the levels of topo–DNA complexes induced by CGA. Figure 4 shows that catalase pretreatment abolished the formation of topo I– and topo II–DNA complexes induced by CGA, indicating that hydrogen peroxide mediates the formation of topo–DNA complexes in cells. A pro-oxidant mechanism is known to participate in the formation of DNA damage by other dietary polyphenols.⁴¹

Recent data suggest that topo I and topo II play a role in DNA fragmentation during the execution phase of apoptosis.^{42–45} Unlike the standard topo poisons camptothecin and etoposide, CGA did not induce significant levels of topo–DNA complexes at short exposure times (Figure 4). This made us consider the possibility that the high levels of topo–DNA complexes observed in cells after a 24 h exposure could be apoptotic topo–DNA complexes. Flow cytometry experiments using the annexin V–FITC assay revealed a relatively high percentage of cells in late apoptosis after a 24 h treatment with CGA (Figures 5A,B); this supports the idea that the topo–DNA complexes induced by CGA may be apoptotic topo–DNA complexes. The percentage of cells in apoptosis after treatment with 5 mM CGA was lower than that induced by the positive control curcumin (20 μ M), which is a potent and efficient inducer of apoptosis. It is important to mention that these experiments were conducted using a high concentration of CGA (5 mM) and a long exposure time (24 h). Under these conditions, cells may have lost membrane integrity, and it is difficult to clearly differentiate genuine late apoptosis from necrosis. The percentage of cells in each phase should therefore be interpreted with caution. In a previous paper we showed that hydrogen peroxide induced high levels of topo–DNA complexes in K562 cells.³⁰ We also observed a clear correlation between the formation of topo–DNA complexes and the induction of apoptosis, which strongly suggested that the induction of apoptosis played a major role in the formation of high levels of topo–DNA complexes at long exposure times.³⁰ Figure 4 shows that catalase pretreatment abolished the formation of topo I– and topo II–DNA complexes induced by CGA, indicating that hydrogen peroxide mediates the formation of topo–DNA complexes in cells. We therefore propose that the main pathway for the induction of topo–DNA complexes in cells by CGA is the generation of hydrogen peroxide, which in turn induces apoptotic topo–DNA complexes.

We next evaluated the possible involvement of hydrogen peroxide in the cytotoxic activity of CGA. Figure 5C shows that catalase pretreatment reduced the cytotoxic activity of CGA on K562 leukemia cells, indicating that hydrogen peroxide is involved in this activity. These results are in accordance with those of Rakshit et al.,⁴⁰ who observed that PEG–catalase and *N*-acetylcysteine prevented CGA-induced apoptosis in leukemia cells. Like other plant polyphenols,⁴¹ the anticancer activity of CGA seems to be mediated by a pro-oxidant mechanism. Cancer cells are known to produce high amounts of hydrogen peroxide.⁴⁶ The increased levels of hydrogen peroxide in cancer cells may explain why these cells are more vulnerable than normal cells to the cytotoxic activity of hydrogen peroxide.^{30,47,48} Recently, the use of hydrogen peroxide-generating systems has emerged as an attractive anticancer strategy to selectively kill cancer cells.^{48–51} Previous findings showed that

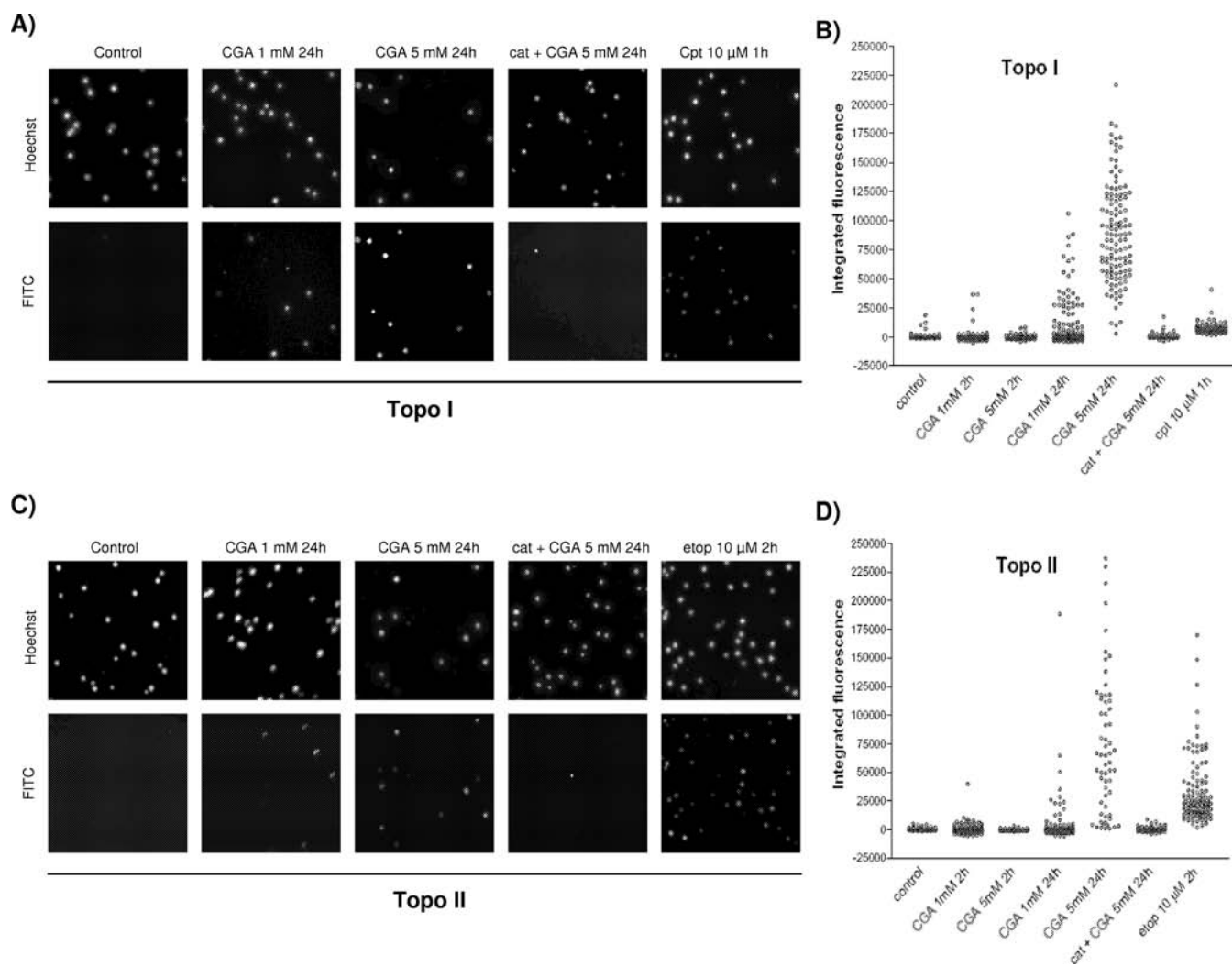


Figure 4. Chlorogenic acid (CGA) induces topo I- and topo II-DNA complexes in K562 leukemia cells mediated by hydrogen peroxide (TARDIS assay). (A, C) Representative immunofluorescence images. FITC fluorescence represents covalently bound topo-DNA. (B, D) Integrated fluorescence values (arbitrary units), indicating levels of topo I-DNA complexes (B) and topo II-DNA complexes (D), have been quantified and shown in individual cells. Catalase (cat, 1000 U/mL) was added 0.5 h before CGA. Camptothecin (cpt) and etoposide (etop) were used as positive controls.

CGA selectively induced apoptosis in Bcr-Abl positive chronic myelogenous leukemia (CML) cell lines and primary cells from CML patients in vitro and reduced xenografts of Bcr-Abl positive CML cells in nude mice.^{40,52} To test if CGA could also induce selective cytotoxicity against cancer cells from solid tumors, we evaluated the cytotoxic activity of this compound against A549 human lung cancer cells and MRC5 human nonmalignant lung fibroblasts using the MTT assay under the same experimental conditions. Our results indicate that the viability of lung cancer cells treated with specific concentrations of CGA (i.e., 1 mM) was significantly lower than that of normal cells (Figure 5D,E); the IC_{50} values were 0.47 ± 0.06 mM for A549 lung cancer cells and 1.97 ± 0.11 mM for MRC5 nonmalignant lung cells. Several compounds were screened against these two cell lines and showed no selective cytotoxicity.³⁰ These and previous findings^{40,52} suggest that CGA has potential for cancer therapy.

Although CGA induces cellular DNA damage and topo-DNA complexes in cells, we cannot conclude that these effects are responsible for CGA-induced selective killing of cancer cells. Our data suggest that such a selective effect could be

mediated by the generation of hydrogen peroxide. Indeed, catalase pretreatment extensively reduced the cytotoxic activity of CGA (Figure 5C), indicating that hydrogen peroxide plays a major role in CGA-induced cell death. In addition, we have recently shown (using the same experimental approach) that specific concentrations of hydrogen peroxide also killed A549 lung cancer cells without affecting MRC5 nonmalignant lung fibroblasts; the cytotoxic profile of 100 μ M hydrogen peroxide on these two cell lines was similar to that of 1 mM CGA.³⁰

The in vivo relevance of the anticancer and carcinogenic effects of CGA reported in this and previous studies depends on its pharmacokinetic profile. Because CGA is a common dietary constituent and because the oral route is the preferred route of administration for most drugs, it is important to understand the fate of this polyphenol in the body when taken orally. Human studies suggest that about one-third of the CGA ingested is absorbed from the small intestine. Hydrolysis of CGA in the stomach or small intestine is not very important, and about two-thirds of the CGA ingested reaches the colon.⁵³ There, the colon microflora can first hydrolyze CGA into caffeic acid and quinic acid, which are then converted into cinnamic

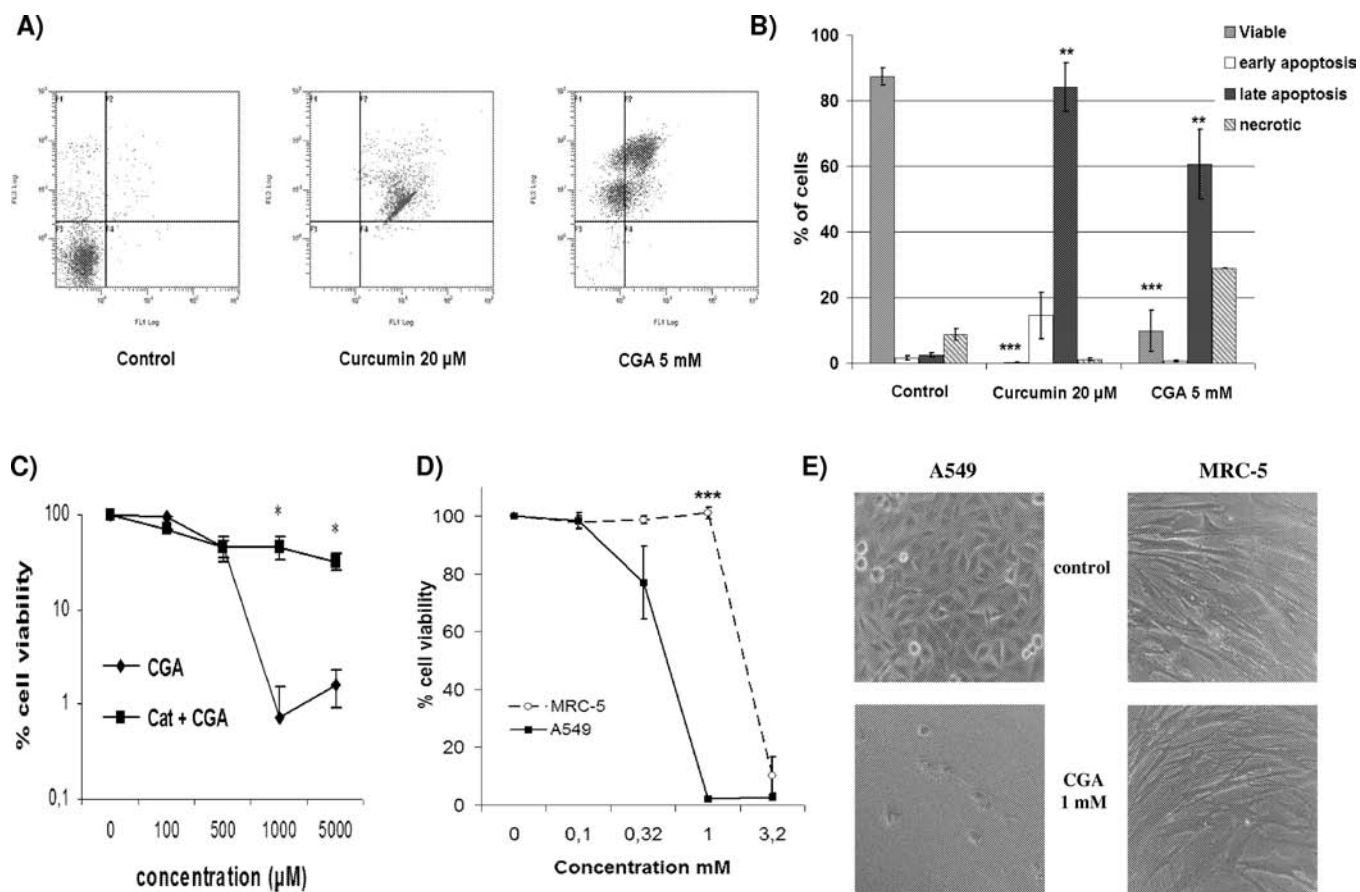


Figure 5. Induction of cancer cell death by chlorogenic acid (CGA). (A, B) K562 cells were treated for 24 h with CGA or the positive control curcumin, and the percentage of viable cells, apoptotic cells, and necrotic cells was assessed by flow cytometry with the annexin V–FITC assay. (C) Catalase (cat, 1000 U/mL) prevents CGA-induced cell growth inhibition in K562 leukemia cells (XTT assay). (D) A549 human lung cancer cells are more sensitive than MRC5 human nonmalignant lung fibroblasts to the cytotoxic activity of CGA (MTT assay). (E) Representative photographs of A549 and MRC5 cells cultured in 96-well plates for 72 h in the absence (control) or presence of CGA (1 mM, 48 h exposure). Data are expressed as the mean \pm SEM. For statistical analysis we used the *t* test (paired, two-tailed). A *P* value >0.05 is not considered to be statistically significant and is not represented by any symbol. A *P* value <0.05 is considered to correspond with statistical significance and is indicated with an asterisk (*), a *P* value <0.01 is indicated with a double asterisk (**), and a *P* value <0.001 is indicated with a triple asterisk (***)

acids and benzoic acids that are absorbed from the colon. The CGA absorbed from the small intestine is also metabolized, first into caffeic and quinic acids, then into cinnamic and benzoic acids, and finally into hippuric acid. After the oral ingestion of CGA by humans, CGA, caffeic acid, several benzoic and cinnamic acids, and hippuric acid can reach systemic circulation and are excreted in the urine.^{53–57} Several studies have measured the plasma levels of CGA after the oral administration of coffee or coffee extracts. Following coffee brew administration (one cup; 478.9 ± 23.2 µg/mL CGA), Nardini et al. did not detect CGA in the plasma of 10 volunteers.⁵⁵ Monteiro et al., however, detected CGA in the plasma of 6 healthy adults ($C_{\max} = 3.14 \pm 1.64$ µM; $t_{\max} = 2.33 \pm 1.17$ h) after consumption of brewed coffee containing 1068 ± 49 µmol of CGA (378.40 mg).⁵⁶ The same research group detected CGA in the plasma of 10 healthy adults ($C_{\max} = 5.9 \pm 4.2$ µM; $t_{\max} = 3.3 \pm 2.4$ h) following the administration of two capsules of a decaffeinated green coffee extract containing 119.8 ± 0.23 µmol of CGA (42.4 mg), although the levels varied considerably among subjects.⁵⁷ The concentrations of CGA detected in the urine after ingestion of CGA are also low.⁵³ These data indicate that CGA does not reach the plasma and tissues (other than those of the gastrointestinal tract) at high concentrations after its oral administration.

Taking into account the pharmacokinetic profile of CGA, several considerations can be made regarding the possible cancer preventive, carcinogenic, and therapeutic potential of this dietary agent. This study has demonstrated that CGA induces DNA damage, topoisomerase–DNA complexes, and selective cancer cell death at concentrations in the 0.5–5 mM range. However, it is unlikely that moderate dietary consumption of CGA leads to an accumulation of CGA in the body high enough to reach such concentrations. At low concentrations, the antioxidant and chemopreventive properties of CGA reported in previous studies may prevail.

A high intake of CGA (heavy coffee consumption, intake of CGA supplements) is unlikely to directly cause genotoxic effects outside the gastrointestinal tract, as millimolar concentrations of CGA cannot be achieved outside the gastrointestinal tract after the oral administration of this polyphenol. This suggests that CGA is not directly involved in the increased risk of bladder cancer, lung cancer, and leukemia that some studies have linked to coffee consumption.^{2–4} However, because CGA is extensively metabolized in the body, we cannot exclude the possibility that the intake of high concentrations of CGA leads to genotoxic effects outside the gastrointestinal tract through the formation of active metabolites. Indeed, caffeic acid is a major metabolite of CGA

able to induce DNA breakage in cells at micromolar concentrations.⁵⁸ In addition, high intake of CGA may lead to high concentrations of CGA in the gastrointestinal tract that, according to our results, may induce DNA damage.

With regard to the therapeutic potential of CGA, it is important to mention that CGA induces selective anticancer effects in leukemia cells,^{40,52} and in lung cancer cells (Figure SD,E). The low oral bioavailability of CGA suggests, however, that these effects cannot be achieved through the oral route. A previous study showed that, after intravenous injection of CGA in rats, only 9% was recovered in the urine,⁵⁹ therefore indicating that CGA is metabolized extensively in the body. The low oral bioavailability of CGA and its extensive metabolism could be overcome with a sustained intravenous infusion of CGA. This strategy could be used to obtain and maintain millimolar concentrations of CGA in plasma and tissues, which would maximize the therapeutic potential of this dietary compound. In vivo animal models might be useful to evaluate the possible toxicity and anticancer activity of a sustained intravenous infusion of CGA.

In conclusion, this study has demonstrated that CGA induces cellular DNA damage in the low millimolar range. In addition, it is shown for the first time that CGA induces DNA complexes with both topoisomerases I and II. A possible mechanism for the induction of such complexes is provided, in which the generation of hydrogen peroxide and the induction of apoptosis may play a major role. This paper also shows that specific concentrations of CGA kill lung cancer cells without affecting normal lung fibroblasts, supporting previous findings that CGA should be more thoroughly investigated as an anticancer agent.

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