Curcumin Induces High Levels of Topoisomerase I– and II–DNA Complexes in K562 Leukemia Cells

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Received July 10, 2007

Recent data suggest that curcumin, a phytochemical with cancer chemopreventive potential, might be useful in the treatment of several solid and hematological malignancies. DNA topoisomerases (topos) are the target of several drugs commonly used in cancer chemotherapy. These drugs induce topo–DNA complexes with either topo I or topo II; then cellular processing converts these complexes into permanent DNA strand breaks that trigger cell death. Using the TARDIS in vivo assay, this study shows for the first time that curcumin induces topo I and topo II (α and β)–DNA complexes in K562 leukemia cells. A comparative analysis revealed that the levels of these complexes were higher than those induced by several standard topo I and topo II inhibitors at equitoxic doses. Curcumin-induced topo I and topo II–DNA complexes were prevented by the antioxidant N-acetylcysteine; this suggests that, unlike the standard topo inhibitors, reactive oxygen species may mediate the formation of these complexes. Overall, this work shows that curcumin is capable of inducing topo–DNA complexes in cells with both topo I and topo II and increases the evidence suggesting that this dietary agent has potential to be tested in cancer chemotherapy.

Turmeric is a ground powder obtained from the root of the plant Curcuma longa (Zingiberaceae); it is often used as a spice, food preservative, and flavoring or coloring agent, and it is an essential component in the preparation of curries. This botanical product has also been used in Asian medicine for generations in the treatment of many disorders including certain tumors. It is recognized that the major active constituent of turmeric is curcumin (diferuloylmethane), a phenolic compound with a diarylheptanoid structure. Accumulating epidemiological, preclinical, and clinical evidence suggests that curcumin has potential cancer chemopreventive properties.1–3 Epidemiological data have revealed that the incidence of several common cancers is higher in Western countries than in countries such as India, where curcumin is widely consumed.1,4 A number of studies in rodents have shown that curcumin can prevent several types of cancer (e.g., colon, lung, breast, liver, stomach, esophagus, or skin) induced by different carcinogens.1–3 In addition, a phase I clinical trial in which people with cancer predisposition were given curcumin orally for 3 months showed histological improvement of precancerous lesions in seven out of 25 patients.5 The outcome of this and other phase I clinical trials has led to the development of phase II trials that are currently enrolling patients.6 On the other hand, accumulating preclinical data suggest that curcumin is a potential therapy for different types of cancer. It has been reported extensively that curcumin is an efficient inducer of apoptosis in cancer cells,7 and evidence indicates that malignant cells are more susceptible to curcumin-induced apoptosis than nonmalignant cells.7–11 Experimental data have also shown that the therapeutic effects of radiation and some anticancer drugs are potentiated by curcumin, therefore suggesting that curcumin might be used in the clinic to sensitize cancer cells to radiotherapy and chemotherapy.12–14

DNA topoisomerases (topos) are essential enzymes that govern DNA topology during cellular processes such as replication, transcription, recombination, and chromatin remodeling. Topo I and topo II are the targets of clinically important anticancer drugs, including the camptothecin derivatives topotecan and irinotecan and the podophyllotoxin derivatives etoposide and teniposide, respectively. These drugs induce topo–DNA complexes with either topo I or topo II; then cellular processing converts these complexes into permanent DNA strand breaks that trigger cell death.15–18 Despite their efficiency in the clinic, topoisomerase inhibitors induce severe toxic effects that limit their use.17,19 Resistance to these drugs also limits their clinical utilization, and it has been suggested that such resistance may be caused, at least in part, by their inability to target both topo I and topo II simultaneously.20 Several drugs are capable of inducing DNA complexes with both topo I and II in vitro, but none of them have shown a clear activity in cells.21–22

Several lines of evidence suggest that curcumin may induce DNA complexes with both topo I and II in cells. First, topo poisons induce DNA strand breaks as their underlying mechanism of action, and it is known that curcumin can induce DNA strand breaks.23–26 Second, 8-oxoguanosine (8-oxoG) has been shown to increase topo I binding to DNA and induce a 3–7-fold increase in topo I–DNA complexes;27 curcumin can induce 8-oxoG in isolated DNA and in cell systems.25,28 Third, curcumin is known to react with thiol groups,29 and thiol-reactive drugs can induce topo II–DNA complexes;30 indeed, curcumin has been shown to induce topo II–DNA complexes in vitro.31 Finally, using mutant yeast strains resistant to topo I and topo II poisons, it has been suggested that curcumin can inhibit both topo I and II.32 Using the TARDIS assay (an immunofluorescence technique that uses specific antibodies to DNA topo I or topo II to detect the protein covalently bound to the DNA in cells), the present communication reports that curcumin induces both topo I– and topo II–DNA complexes in K562 leukemia cells.

Results and Discussion

The ability of curcumin to induce topo I– and topo II–DNA complexes in K562 leukemia cells was evaluated using the TARDIS assay. In this assay, control or drug-treated cells are embedded in agarose on microscope slides. The cells are then lysed to disrupt

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the cellular membranes and remove soluble proteins, and salt extraction is used to remove nuclear proteins and any noncovalently bound topoisomerase from the DNA matrix. Drug-stabilized topo–DNA complexes remain and are detected by staining with isoform-specific antisera followed by an FITC-conjugated secondary antibody. Digital images of Hoechst fluorescence (DNA) and FITC immunofluorescence (drug-stabilized topo–DNA complexes) are captured, and the levels of fluorescence are quantified. To obtain an appropriate concentration range, K562 cells were treated with curcumin for different times, and cell growth inhibition was estimated using the XTT assay. Figure 1 shows that curcumin reduced the viability of K562 leukemia cells in a dose- and time-dependent manner. K562 cells were then exposed to several concentrations of curcumin for different times, and the levels of topo I and topo II (α and β)–DNA complexes were analyzed using the TARDIS assay. Figure 2 (A–H) shows that curcumin induced topo I– and topo II–DNA complexes in K562 cells in a dose- and time-dependent manner. As shown in Figure 2G,H, curcumin induced topo II–DNA complexes with both topo Iα and topo IIβ.

In order to directly compare the levels of topo I− and topo II−DNA complexes induced by curcumin with those induced by standard topo inhibitors, K562 cells were treated for different times with equitoxic concentrations of the standard topo I inhibitor (camptothecin) and four representative topo II inhibitors (etoposide, idarubicin, amsacrine (m-AMSA), and mitoxantrone). K562 cells were first treated with these drugs at different concentrations and the IC50 values were calculated (µM ± SEM, XTT assay, 5 days exposure): 6.81 ± 0.23 (curcumin), 0.04 ± 0.01 (camptothecin), 0.32 ± 0.03 (etoposide), 0.012 ± 0.004 (idarubicin), 0.023 ± 0.003 (m-AMSA), and 0.0025 ± 0.0007 (mitoxantrone). Then, K562 cells were treated for 2, 8, and 24 h with these drugs at concentrations 10 times higher than their IC50 value determined after a 5-day exposure to drug, and the levels of topo−DNA complexes were evaluated using the TARDIS assay. Figures 2I and 2K show the results obtained for camptothecin and the four representative topo II inhibitors. Figures 2I and 2L compare the activity of the standard topo inhibitors with that of curcumin (note that panels 2J and 2L are on a different scale than panels 2I and 2K). It can be observed that, at short exposure times, the levels of complexes induced by curcumin were comparable to those induced by these five inhibitors. At longer exposure times (8 and 24 h), however, curcumin induced higher levels of complexes than these five standard drugs; after 8 h the activity of curcumin was significantly higher than for camptothecin, and after 24 h, than for all the tested standard drugs (p < 0.05, paired and two-tailed t test).

Previous experiments showed that the cellular levels of topo−DNA complexes induced by etoposide and camptothecin decreased dramatically after drug removal.\textsuperscript{33,34} but remained stable in cells treated with idarubicin after removing the drug.\textsuperscript{36} To evaluate the stability of the topo−DNA complexes induced by curcumin, K562 cells were treated with this drug for 24 h. After drug removal, cells were grown in fresh medium for 0, 2, 8, 24, and 48 h; then the levels of topo−DNA complexes were evaluated using the TARDIS assay. Figure 3 shows that curcumin-induced topo I− and topo II−DNA complexes remained stable after drug removal for up to 48 h. Therefore, the high levels of topo−DNA complexes observed in cells exposed to curcumin for 24 h (Figure 2) might be due to their longevity. Apoptotic changes (reduction of pro-caspases 8 and 9 and induction of cleavage of PARP) have been observed in K562 cells exposed to curcumin for 24 h or longer.\textsuperscript{35} Evidence suggests that topo−DNA complexes can be formed during the execution phase of apoptosis;\textsuperscript{18,26,37} these stable apoptotic topo−DNA complexes may therefore account for the high levels of topo−DNA complexes observed in cells exposed to curcumin for 24 h.

In vitro experiments have shown that curcumin can directly interact with purified topo II and DNA to induce topo II−DNA complexes.\textsuperscript{38} Indeed, curcumin is a thiol-reactive agent, and these agents can induce topo II−DNA complexes in vitro.\textsuperscript{39} Experiments were conducted to evaluate if curcumin could also induce topo I−DNA complexes in vitro, and it was found that it could not (results not shown). This indicates that the topo I−DNA complexes observed in cells treated with curcumin are not directly induced by this agent. It is recognized that curcumin can increase the cellular levels of reactive oxygen species (ROS)\textsuperscript{9,25,38–41} and that ROS can induce topo I−DNA complexes in vitro and in cells.\textsuperscript{27,42} To evaluate if curcumin-induced topo I−DNA complexes were mediated by ROS, the levels of these complexes were measured in cells pretreated with the antioxidant N-acetylcysteine (NAC). Figure 4 shows that NAC completely prevented the formation of topo I complexes induced by curcumin, therefore suggesting that these complexes are mediated by an increase in the cellular levels of ROS. Recent findings indicate that several inducers of apoptosis that were inactive on purified topo I induced topo I−DNA complexes in cells. These apoptotic topo I−DNA complexes result from oxidative DNA lesions generated by ROS during apoptosis. It has been proposed that the functional role of these topo I complexes could be to directly fragment chromatin and to further activate (amplify) apoptotic pathways.\textsuperscript{37} Figure 4 shows that NAC also prevented curcumin-induced topo II−DNA complexes; this seems to indicate that, although curcumin can directly form topo II−DNA complexes in vitro, ROS may play a role in the formation of these complexes in cells. Overall, these results suggest that curcumin does not act as a classical topoisomerase poison. Several drugs have already been shown to interact with topoisomerases through nontypical mechanisms.\textsuperscript{43–45}

Curcumin-induced growth inhibition activity was also prevented in K562 cells pretreated with NAC. The IC50 of curcumin alone was 8.4-fold lower than for curcumin previously pretreated with 10 mM NAC (XTT assay, 5 days exposure, NAC added 0.5 h before curcumin, results not shown). These results are in agreement with those obtained by Scott et al., who observed that curcumin-induced DNA damage and apoptosis was prevented by NAC pretreatment.\textsuperscript{25}
Ferulic acid and dibenzoylmethane are two phenolic compounds structurally related to curcumin. Like curcumin, dibenzoylmethane contains a \( \beta \)-diketone moiety, and ferulic acid contains a phenol ring with the same substitution pattern as that of curcumin. Both compounds were evaluated for their ability to induce topo I and II-DNA complexes in K562 cells. These compounds were first evaluated for cell growth inhibition activity in K562 cells; the IC_{50} values were determined.

**Figure 2.** Induction of topo I- and topo II-DNA complexes in K562 cells by curcumin and standard topo I and II inhibitors. All y-axes show integrated fluorescence values (indicating levels of topo-DNA complexes, arbitrary units). In panels A, C, G, H, J, and L, cells were treated with curcumin (70 \( \mu \)M) for different times. In panels B, D, E, and F, cells were treated for 24 h with different concentrations of curcumin. In panels A–D, one representative experiment is represented, where the distribution of topo-DNA complexes in individual cells can be observed. The rest of the plots show mean values \( \pm \) SEM of at least three independent experiments. In panels I–L, the curcumin-induced topo I- and topo II-DNA complexes are compared with those induced by the standard topo I inhibitor (camptothecin) and four representative topo II inhibitors (etoposide, idarubicin, mitoxantrone, and m-AMSA) under similar assay conditions (see text for details).

**Figure 3.** Stability of topo I- and topo II-DNA complexes induced by curcumin. K562 cells were treated with 70 \( \mu \)M curcumin for 24 h. After compound removal, cells were grown in fresh medium for 0, 2, 8, 24, and 48 h before evaluating the levels of topo I- and topo II-DNA complexes in the TARDIS assay. y-Axes show means of integrated fluorescence values (arbitrary units) of three independent experiments \( \pm \) SEM.

Ferulic acid and dibenzoylmethane are two phenolic compounds structurally related to curcumin. Like curcumin, dibenzoylmethane contains a \( \beta \)-diketone moiety, and ferulic acid contains a phenol ring with the same substitution pattern as that of curcumin. Both compounds were evaluated for their ability to induce topo I and II-DNA complexes in K562 cells. These compounds were first evaluated for cell growth inhibition activity in K562 cells; the IC_{50} values were determined.
Inhibitors of topo II inhibit the formation of topo II complexes. Curcumin induces topoisomerase II activity (XTT assay, 5 days exposure) was 30.9 absorbance and the first compound capable of inducing topoisomerase I and II (pretreatment could reduce the levels of topo II). Conversely, curcumin pretreatment increased the levels of topo II, a combination of both compounds (curcumin being administered 22 h prior to etoposide). The levels of topo II–DNA complexes were evaluated using the TARDIS assay and expressed as means of integrated fluorescence values (arbitrary units) ± SEM. It can be observed that the effect obtained when both drugs are administered in combination is higher than the additive effect (AE), which is calculated as the sum of the effects produced when the compounds are administered alone.

The value (XTT assay, 5 days exposure) was 30.9 ± 9.4 μM for dibenzoylmethane and >1000 μM for ferulic acid. K562 cells were then treated for 2 and 24 h with 300 μM dibenzoylmethane or 4 mM ferulic acid, and the cellular levels of topo I and topo II–DNA complexes were evaluated using the TARDIS assay. However, neither drug induced topo–DNA complexes under these experimental conditions (results not shown).

As discussed elsewhere, it is recognized that catalytic inhibitors of topos inhibit the formation of topo II–DNA complexes. To test whether or not curcumin could inhibit the catalytic activity of topo I and topo II in intact cells, we evaluated if curcumin pretreatment could reduce the levels of topo II–DNA complexes induced by camptothecin and etoposide. The levels of topo–DNA complexes induced by camptothecin (10 μM, 1 h) and etoposide (10 μM, 2 h) in K562 cells were measured in the presence and absence of curcumin (20, 35, or 70 μM, 24 h). Curcumin did not reduce camptothecin-induced topo I–DNA complexes at any of the tested concentrations, therefore suggesting that curcumin does not inhibit the catalytic activity of topo I in cells. The effect observed when curcumin and camptothecin were administered in combination was higher than those observed when both drugs were tested individually, but lower than the sum of their separate effects (results not shown).

Curcumin did not reduce etoposide-induced topo II–DNA complexes either, therefore suggesting that this compound does not inhibit the catalytic activity of topo II in cells. Conversely, curcumin pretreatment increased the levels of topo II–DNA complexes induced by etoposide; the combined effect of curcumin and etoposide was clearly greater than the sum of their separate effects (Figure 5). This is in agreement with a recent report that has shown that curcumin pretreatment increased etoposide-induced DNA fragmentation and cell growth inhibition in glioblastoma cells. This suggests that curcumin might sensitize cancer cells to the activity of etoposide, a topo II poison currently used in the treatment of solid and hematological cancers.

In summary, the present communication shows that curcumin is the first compound capable of inducing topoisomerase I and II (α and β)–DNA complexes in intact cells and increases evidence suggesting that this phytochemical has potential to be more thoroughly tested in cancer chemotherapy. Pharmacokinetic preclinical and clinical studies have established clearly that curcumin has low oral bioavailability and undergoes extensive metabolism; this suggests that other routes of administration (e.g., i.v. infusion) or other formulations (e.g., liposomal curcumin) should be explored to achieve cytotoxic levels of curcumin in tissues and maximize its potential chemotherapeutic activity. Finally, since curcumin is currently being developed as a cancer chemopreventive agent, it is important to note that high oral concentrations of curcumin may induce nonlethal levels of topo–DNA complexes that may lead to carcinogenic effects. Indeed, other dietary agents such as the flavonoid genistein are known to induce topo II–DNA complexes in cells and this effect has been associated with a higher risk of breast cancer and leukemia. Based on short-term studies, the doses of curcumin recommended for future chemopreventive studies largely exceed those estimated in people who consume high amounts of curcumin in their diet. The results presented in this report suggest that it would be sensible to test the potential carcinogenic activity of such high doses of curcumin in long-term studies before large cancer chemoprevention clinical trials are implemented.

Experimental Section

General Experimental Procedures. Human K562 leukemia cells were maintained as a suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin (50 µg/mL)/streptomycin (50 µg/mL). This cell line was maintained at 37 °C (5% CO2). Reagents were obtained from Life Technologies. Curcumin, etoposide, camptothecin, amssarcine (m-AMSA), mitoxantrone, and N-acetylcycteine were purchased from Sigma. Idarubicin was kindly provided by Pharmacia-Upjohn (London, UK). Stock solutions were prepared in DMSO (except for etoposide, which was in MeOH) and were stored at −20 °C.

XTT Assay. This is a colorimetric assay that allows the quantitative determination of cell viability. It is based on the capability of viable cells to transform the tetrazolium salt XTT (sodium 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium-bromide) into a formazan dye. Exponentially growing cells were seeded (2 × 104/well in 100 µL) into 96-well plates. Drugs were added to plates 24 h later. Following the incubation period indicated in figure or table legends, 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 reading on a Bio-Rad 550 plate reader at 450 nm. Cell viability was expressed as percentage in relation to controls. All data were averaged from at least three independent experiments ± SEM.

Antibodies. Antitopo II polyclonal antibodies were raised in rabbits, with 18511 raised to recombinant human topo IIα, 18513 to recombinant human topo IIβ C-terminal fragment, and αCT to recombinant topo IIα C-terminal fragment. Antibody 18511 detected the α isoform specifically and 18513 detected the β isoform specifically. Western blots confirmed that αCT detected both isoforms of topo II (results not shown). This was the antibody used for all the experiments with topo II in which the isozyme is not specified. For topo I a polyclonal human antibody from Topogen (2012) was used. Antibodies were diluted in PBS containing 0.1% Tween 20 and 1% BSA. Antibody 18511 (topo IIα) and αCT (topo II αβ) were used at a 1:50 dilution, 18513 (topo IIβ) at 1:200, and 2012 (topo I) at 1:1000. For topo II (α, β, and αβ), the anti-rabbit FITC-conjugated secondary antibody (1262), from Sigma, was used at 1:200 dilution. For topo I, the goat anti-human FITC-conjugated second antibody (F5512), from Sigma, was used at 1:50 dilution.

Preparation of Slides. The slide preparation method is described in detail by Willmore et al. Briefly, cells were seeded (3 × 104 cells/well) into six-well tissue culture plates. These were grown for approximately 48 h, and drug was added to exponentially growing cells at appropriate concentrations. Microscope slides were precoated with agarose. Then, 50 µL of cell suspension was warmed to 37 °C and immediately mixed with an equal volume of agarose solution (2% w/v in PBS), which had been melted and kept at 37 °C. The mixture was immediately spread evenly across a slide, and the agarose was quickly gelled by placing the slides on a cold surface. Slides were then placed for 30 min in lysis buffer, which contained 1% (w/v) sodium dodecyl sulfate, 80 mM phosphate buffer, pH 6.8, 10 mM EDTA, and a protease inhibitor mixture (2 µg/mL peptatin A, 2 µg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 mM

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**Figure 5.** Curcumin pretreatment increases etoposide-induced topo II–DNA complexes. K562 cells were treated with curcumin (24 h, concentrations shown below in µM), etoposide (2 h, 10 µM), or a combination of both compounds (curcumin being administered 22 h prior to etoposide). The levels of topo II–DNA complexes were evaluated using the TARDIS assay and expressed as means of integrated fluorescence values (arbitrary units) ± SEM. It can be observed that the effect obtained when both drugs are administered in combination is higher than the additive effect (AE), which is calculated as the sum of the effects produced when the compounds are administered alone.
dithiothreitol, final concentrations). This was 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 antiserum for 1 to 2 h. Slides were washed three times in PBS containing 0.1% Tween 20 (PBST) and subsequently exposed for 1 to 2 h to a secondary antibody (anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody, F(ab') 2 fragment; Sigma) diluted in PBST containing 1% w/v BSA. Slides were washed three times in PBST followed by an overnight wash in PBS containing protease inhibitors, at 4°C.

Quantification of Complexes. An epifluorescence microscope (Olympus BH2-RFCA, 10× objective) was used to separately visualize the blue (Hoechst-stained DNA) fluorescence and the green (FITC-stained top II) immunofluorescence. Images were captured using a cooled slow-scan charge-coupled device camera (Astrocam, Cambridge, UK). DNA in a particular field of view was focused only under blue light to minimize photobleaching of FITC. An image was then captured of green fluorescence (20 s exposure) by using specific filter sets. An average of eight pairs of images per drug dose were captured from replicate slides for each antibody (this gave a total of approximately 100 cells per dose). Images were then analyzed using Imager 2 software (Astrocam Ltd., Cambridge, UK) based on Visilog 4 (Neosis, Crolles, France). Background correction procedures were carried out on each image to correct for any stray light and camera background. Additionally, all images were subjected to blue and green shade correction procedures to compensate for variation in intensity of illumination and nonuniformities in light transmission. Corrected images of blue fluorescence were used to define the areas containing the DNA in each cell, through the creation of a separate binary image. A standardized sequence of image processing functions was applied to the images of each field of view to eliminate from the analysis small particles and objects adjoinning the edge of the image and subtract a background intensity value calculated for each image. The blue and green fluorescence intensities of all pixels in each object (i.e., the area occupied by DNA from an individual cell) were integrated.3,33

Acknowledgment. This work was supported by a Marie Curie Individual Fellowship (MCFI-2002-01661), the Leukaemia Research BSA. Slides were washed three times in PBST followed by an overnight wash in PBS containing protease inhibitors, at 4°C.

References and Notes

(14) López-Lázaro, et al.