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Curcumin as a DNA Topoisomerase II Poison

CARMEN MARTÍN-CORDERO, MIGUEL LÓPEZ-LÁZARO, MARINA GÁLVEZ and MARIA JESÚS AYUSO*

Laboratorio de Farmacognosia, Facultad de Farmacia, C/P. García González No. 2, 41012 Sevilla, Spain

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Curcumin, the major active component of the spice turmeric, is recognised as a safe compound with great potential for cancer chemoprevention and cancer therapy. It induces apoptosis, but its initiation mechanism remains poorly understood. Curcumin has been assessed on the human cancer cell lines, TK-10, MCF-7 and UACC-62, and their IC_{50} values were 12.16, 3.63, 4.28 μM respectively. The possibility of this compound being a topoisomerase II poison has also been studied and it was found that 50 μM of curcumin is active in a similar fashion to the antineoplastic agent etoposide. These results point to DNA damage induced by topoisomerase II poisoning as a possible mechanism by which curcumin initiates apoptosis, and increase the evidence suggesting its possible use in cancer therapy.

Keywords: curcumin; SRB assay; cytotoxicity; topoisomerase poison; topoisomerase II

INTRODUCTION

Turmeric is a bright yellow aromatic powder obtained from the rhizome of a plant of the ginger family (*Curcuma longa*, Zingiberaceae). It was formerly used as a fabric dye for flavouring and colouring in Asian cookery, but now is widely used as a dietary additive in a variety of foods including curries, saffron, mustard, gelatines, puddings, sorbets, ice creams, soups, meats, pickles, margarine, and both alcoholic and non-alcoholic beverages.[1] Curcumin, a yellow pigment isolated as the major active component of turmeric, has recently acquired the official status of a promising chemopreventive agent[2] after showing safety and chemopreventive efficacy in preclinical studies. Curcumin possesses different pharmacological activities, such as anti-inflammatory and antioxidative properties, but its anticancer propensity seems to attract the most interest.[3] Many in vitro experiments have shown that curcumin modulates many targets and pathways involved in cancer. This anticancer activity has been confirmed in many animal models of carcinogenesis. Several human trials have shown that curcumin is well tolerated and antitumor activity has also been observed (see Ref. [4] for a review). Although the exact mechanism underlying the antiproliferative effect of curcumin is not clear, many recent reports suggest that its anticancer activity may be mediated by an apoptotic effect (see Refs. [5–10] as examples). However, the mechanism by which curcumin initiates apoptosis remains poorly understood.

DNA topoisomerases (topos) are essential enzymes that govern DNA topology through transient DNA cleavage, strand passing and religation during fundamental nuclear metabolic processes, such as replication and transcription. Topo I acts by forming a transient single strand break through which the other DNA strand passes to achieve relaxation and topo II is able to do so with the two strands that make up duplex DNA, creating a DNA-linked protein gate through which another intact duplex passes.[11] Topoisomerase II poisons allow the enzyme to cut and covalently bind to DNA, but abort the subsequent rejoining of the molecule after relieving the torsional stress producing stabilization of covalent attachments of the topoisomerase II subunits to DNA at sites of DNA strand breaks. Stabilization of the cleavage complexes may not be directly cytotoxic. One attractive model that has experimental support suggests that collision of DNA replication forks with cleavage complexes causes the complex to fall apart without rejoining DNA, thereby generating lethal double strands breaks.[12,13] Therefore, exposure to topoisomerase poisons can convert

*Corresponding author. Fax: +34-5-4-23-37-65. E-mail: ayuso@us.es

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these enzymes into DNA-breaking nucleases resulting in cell death, which may be mediated by an apoptotic process. Despite the efficacy of topoisomerase poisons in the clinic, these drugs induce severe toxic side effects such as myelosuppression, nausea, hair loss, congestive heart failure and, in some instances, increases in the risk of secondary malignances.[14] Therefore, non-toxic novel bioactive compounds able to interfere with topoisomerases and with the ability to spare normal cells from their cytotoxic effects are required. In this way, it is worth nothing that curcumin and some other polyphenols has been reported to induce apoptosis in various cancer cell lines but not in normal cells.[15–17] With these precedents, and as part of our research concerning cytotoxic natural products being topoisomerase poisons,[18–21] we have studied the cytotoxicity of curcumin on three human cancer cell lines and have evaluated its topoisomerase II poisoning activity. Our results show that curcumin was able to produce topoisomerase II poisoning in a similar fashion to that obtained for the antineoplastic agent etoposide, being topoisomerase II-mediated DNA damage a possible apoptosis-mediated mechanism by which curcumin exerts its antitumor effect.

MATERIALS AND METHODS

Enzymes, Nucleic Acids and Chemicals

Purified enzyme human topoisomerase II, pRYG DNA and the positive control etoposide were purchased from TopoGen, Inc (Columbus, OH, USA). Proteinase K and curcumin were from Sigma Chemical Co.

Assay for Cytotoxic Activity on Human Cancer Cell Lines

The following three human cancer cell lines were used in these experiments: the human renal adenocarcinoma (TK-10), the human breast adenocarcinoma (MCF-7) and the human melanoma (UACC-62) cell lines. They were kindly provided by Dr. G, Gragg, Department of NCI, Maryland, USA. The human tumor cytotoxicities were determined following protocols established by the National Cancer Institute, National Institute of Health.[22] TK-10, MCF-7 and UACC-62 cell lines were cultured in RPMI 1640 medium (Bio whittaker) containing 20% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week. According to their growth profiles, the optimal plating densities of each cell line was determined (15×10^3, 5×10^3 and 100×10^3 cell/well for TK-10, MCF-7 and UACC-62 respectively) to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analysed by the SRB assay.

Testing procedure and data processing: The sulphorhodamine B (SRB) assay was used in this study to assess growth inhibition. This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB.[22] For the assay, cells were detached with 0.1% trypsin–EDTA (Sigma) to make single-cell suspensions, and viable cells were counted using a Coulter counter and diluted with medium to give final concentrations of 15×10^4, 5×10^4 and 100×10^4 cells/ml for TK-10, MCF-7 and UACC-62 respectively. Cell suspensions (100 μl/well) were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24 h the cells were treated with serial concentrations of the compounds. The compounds were initially dissolved in 100% DMSO (10 mM) and further diluted in medium to produce 5 concentrations. 100 μl/well of each concentration was added to the plates to obtain final concentration of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M. The DMSO concentration for the tested dilutions was not greater than 0.25% (v/v), the same as in the solvent control wells. The final volume in each well was 200 μl. The plates were incubated for 48 h.

Sulphorhodamine B method: After incubating for 48 h, adherent cell cultures were fixed in situ by adding 50μl of cold 50% (w/v) trichloroacetic acid (TCA) and incubating for 60 min at 4°C. The supernatant was then discarded, and the plates are washed five times with deionized water and dried. 100 μl of SRB solution (0.4% w/v in 1% acetic acid) was added to each microtiter well and the culture was incubated for 30 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid. The plates were then air-dried. Bound stain was solubilized with Tris buffer, and the optical densities measured on an automated spectrophotometric plate reader at a single wavelength of 492 nm. Finally, IC_{50} values (concentrations required to inhibit cell growth by 50%), TGI (concentration resulting in total growth inhibition) and LC_{50} (concentration causing 50% of net cell killing) were calculated in accord with previously described protocols.[22] At least three independent experiments were conducted for each compound. Data are given as the mean ± SEM.

DNA Cleavage Reactions with Topoisomerase II

Cleavage topo II buffer contained 30 mM Tris–HCl (pH 7.6), 60 mM NaCl, 15 mM mercaptoethanol, 8 mM $MgCl_2$ and 3 mM ATP. The cleavage reaction

TABLE I Cytotoxic effects of curcumin and etoposide on selected human cancer cell lines

		TK-10	MCF-7	UACC-62
Curcumin	IC_{50}	12.16 ± 0.31*	3.63 ± 0.05*	4.28 ± 0.35*
	TGI	36.01 ± 3.65	17.66 ± 0.60	21.73 ± 1.10
	LC_{50}	76.09 ± 8.80	97.83 ± 0.80	>100
Etoposide	IC_{50}	9.95 ± 0.08	0.87 ± 0.21	1.13 ± 0.21

*Compound concentration (expressed as μM ± SEM) required to inhibit cell growth by 50% (IC_{50}) to produce total growth inhibition (TGI) and to cause 50% of net cell killing (LC_{50}).

(20 μl) contained cleavage buffer (2μl), the tested drugs dissolved in 2 μl dimethylsulfoxide/H_2O (2.5%), pRYG DNA (0.25 μg in 1 μl of buffer), and 2 μl (4 units) of human topoisomerase II, and water up to 20 μl. Reactions were incubated at 37°C for 30 min, terminated by the addition of 2 μl SDS 10% and 1 μl proteinase K 2 mg/ml and followed by an additional 15 min incubation at 37°C. Subsequently, the samples were extracted with chloroform: isoamyl alcohol, and 2 μl bromophenol blue was added. Samples were loaded on 1% agarose gels and electroforesed at 6 V/cm for 3 h in Tris–acetate–EDTA buffer. Gels were stained with ethidium bromide and washed in water. For the quantitative determination, videoimpresion was densitometrically measured using PCBAS software. After integration of the bands, the linear and nicked open circle (OC) DNA forms induced by the tested drugs were expressed as percentage of total DNA in relation to these DNA forms obtained in the absence of drug.

RESULTS

Five concentrations of curcumin and the positive control etoposide, in the range 0.01–100 μM, were assessed on the human cancer cell lines TK-10, MCF-7 and UACC-62. Cytotoxic results are presented in Table I. The IC_{50} values of curcumin were 3.63–12.16 μM and etoposide from 0.87–9.95 μM.

Curcumin was studied to see if it was able to stabilise covalent attachment of the topoisomerase II subunits to DNA at sites of DNA strand breaks, so generating cleavage complexes intermediates that could be detected in agarose gels. The compound was assayed at concentrations of 50 and 100 μM. Etoposide was used as a positive control at a concentration of 100 μM. The gel presented as Figure 1 shows that curcumin was able to induced linear and open circular DNA. Then, after staining the gels with ethidium bromide, the linear and nicked open circle (OC) DNA bands were densitometrically measured using PCBAS software. Linear and nicked OC DNA forms were expressed as a percentage of total DNA (Figure 2) and it could be

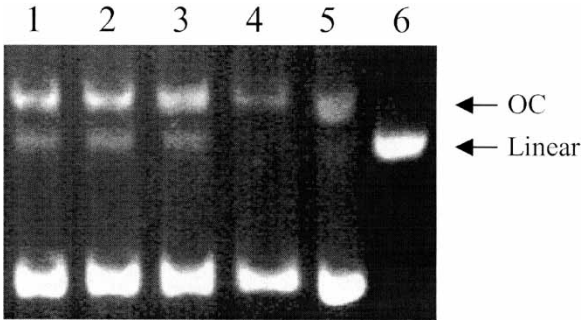


FIGURE 1 DNA-topoisomerase II mediated DNA cleavage: 1. Topo II + pRYG DNA + 50 μM curcumin. 2. Topo II + pRYG DNA + 100 μM curcumin. 3. Topo II + pRYG DNA + 100 μM etoposide. 4. Topo II + pRYG DNA. 5. pRYG DNA. 6. Linear DNA.

observed that curcumin induced formation of both DNA cleavage complexes at the two tested concentrations in a similar fashion to that observed for etoposide, acting as a DNA topoisomerase II poison.

DISCUSSION

Curcumin (Figure 3), a widely consumed dietary compound, is recognized as a safe compound with great cancer chemoprevention and cancer therapy potential.[23–25] Although its anticancer activity has been reported to be mediated by many different mechanisms of action involved in cancer initiation, promotion, and progression,[4] the ability of curcumin to induce apoptosis might be considered as one of the most important mechanisms mediating its anticancer effect. Likewise, evidence shows that suppression of apoptosis by tumor-promoting agents in preneoplastic cells is thought to be an important mechanism in tumor promotion and,

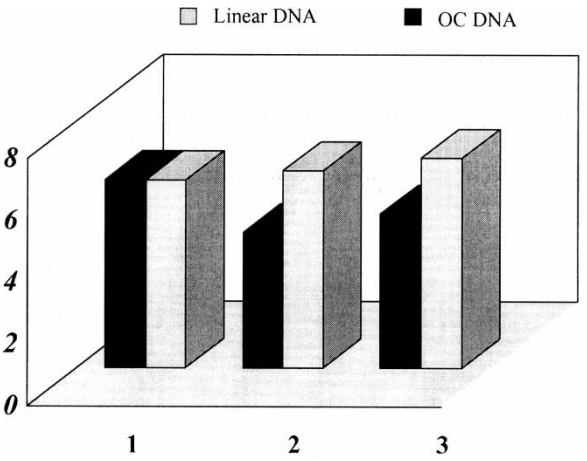


FIGURE 2 Percentage of topoisomerase II-mediated DNA cleavage complexes (linear and open circular DNA) induced by etoposide 100 μM (1), curcumin 50 μM (2) and curcumin 100 μM (3). The percentage of DNA cleavages of topoisomerase II in presence of these compounds was determined by gel scanning (OC: open circular).

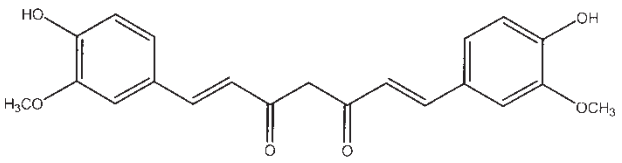
therefore, apoptosis-inducing ability seems to have become a primary factor in considering the efficiency of chemopreventive agents. Therefore, it is hardly surprising that there is an intense search for the underlying mechanism by which curcumin induces apoptosis. Many recent reports show that curcumin can modulate different proteins involved in apoptosis,[5–10,26–30] but the data published about its mode of action seems to be controversial, suggesting that curcumin, depending on the cell fate, can influence different pathways of cell death through different mechanisms. Alternatively, the mechanism by which curcumin initiates apoptosis remains poorly understood.

Topoisomerase II, during fundamental nuclear metabolic processes such as replication and transcription, acts by forming a transient single or double-strand break that, in absence of a topoisomerasse II poison, would be subsequent rejoined after relieving the torsional DNA stress. But in the presence of a topoisomerase II poison these cleavage complexes are stabilized generating simple and double strand breaks that might trigger apoptosis. There are several facts that support the view that topo II cleavable complexes may be involved in the commitment step of apoptotic cell death.[31]

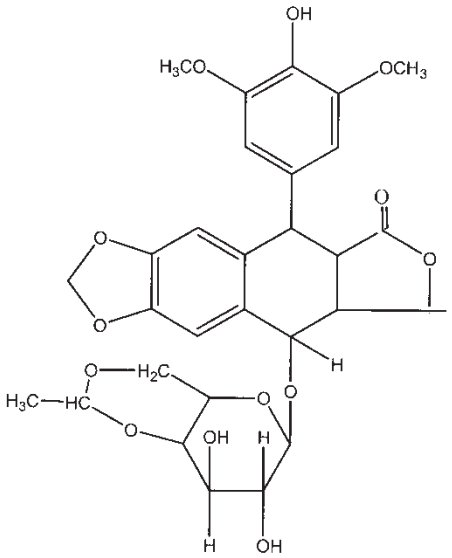
Three facts suggested that curcumin might be a topoisomerase II poison. First, topoisomerase II-mediated DNA damage can initiate apoptosis and many reports have shown the ability of curcumin to induce apoptosis. Second, curcumin is an intercalating agent with unknown clastogenic mechanism,[32,33] and clastogenicity can arise as a result of topo II poisoning via non-covalent chemical intercalation into DNA. In this way, the topo II poisons etoposide and doxorubicin are clastogenic agents.[33] Finally, it is well known that catalytic inhibitors of topo II prevent the formation of topo II-induced DNA double strand breaks induced by topo II poisons, therefore reducing their clastogenicity. Therefore, the fact that the clastogenicity of curcumin is prevented by three topo II catalytic inhibitors, also suggest that curcumin is likely to be a topoisomerase poison.[33]

Based on the above discussion, and after evaluating curcumin cytotoxic activity, we observed that curcumin and etoposide had a similar cytotoxic profile in the three human cancer cell lines tested. Knowing that a compound is considered cytotoxic by the National Cancer Institute (USA) (NCI) when its IC_{50} value is less than 4 μM, and bearing in mind the safety profile of curcumin, the growth inhibition by 50% produced by this dietary occuring compound in the tested cell lines could be considered as an interesting result. Curcumin was able to produce total growth inhibition at concentration less than 40 μM and caused 50% of net cell killing at concentrations around 100 μM. The possibility was considered of the involvement of topo II in its antiproliferative response and selected concentrations of 50 and 100 μM were used to evaluate curcumin as a possible topoisomerase II poison. After completion of the experiments, the presence and levels of linear and OC cleavage complexes observed, suggested that curcumin is a topoisomerase II poison in vitro in a similar fashion to that obtained for the antineoplastic agent etoposide since both compounds induced a similar percent of linear DNA. At this point it should be said that curcumin was previously assayed by Snyder and Arnone as a topo II poison,[33] on the basis of the same evidence mentioned above which suggested that curcumin was a topo II poison, but could not prove such activity. They did not understand this failure to confirm this activity, suggesting that the specific assay conditions employed may not be suitable for this agent.[33]

Since curcumin is described as a non-toxic anticancer compound, it could be useful in combination with toxic drugs that nowadays are being used in cancer therapy so as to reduce their toxic effects and also because of the possible usefulness of the combination of curcumin with other topo I poisons since it showed a synergistic effect between them.[34] This synergism has been recently applied by Kancherla and coworkers, who have reported Phase



CURCUMIN



ETOPOSIDE

FIGURE 3 Chemical structure of curcumin and etoposide.

I data for a topotecan (topo I poison) and etoposide combination for patients with recurrent or refractory non-Hodgkin lymphoma.[35] Likewise, the synergistic effect between topo poisons and cisplatin is well known.[36,37]

In conclusion, our results suggest that DNA damage induced by topoisomerase II poisoning is a possible mechanism by which curcumin initiates apoptosis. The present results, when integrated with the broad literature available concerning curcumin as an anticancer safe compound, suggest that it should be borne in mind as a possible candidate not only for cancer prevention but also for cancer therapy. As far as the latter is concerned, we suggest that a combination of curcumin with specific currently used anticancer drugs might be considered for further research.

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