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L-929 Cells Harboring Ectopically Expressed RelA Resist Curcumin-induced Apoptosis*

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Curcumin (diferuloyl methane), the yellow pigment in turmeric (*Curcuma longa*), is a potent chemopreventive agent. Curcumin induces apoptosis of several, but not all, cancer cells. Many cancer cells protect themselves against apoptosis by activating nuclear factor-κB (NF-κB)/Rel, a transcription factor that helps in cell survival. Signal-induced activation of NF-κB is known to be inhibited by curcumin. To understand the role of NF-κB in curcumin-induced apoptosis, we stably transfected *relA* gene encoding the p65/RelA subunit of NF-κB, into L-929 cells (mouse fibrosarcoma) and the *relA*-transfected cells were resistant to varying doses of curcumin (10^{-6}–10^{-4} M), whereas the parental cells underwent apoptosis in a time- and dose-dependent manner. The *relA*-transfected cells showed constitutive NF-κB DNA binding activity that could not be inhibited by curcumin and did not show nuclear condensation and DNA fragmentation upon treatment with curcumin. When a super-repressor form of IκB-α (known to inhibit NF-κB) was transfected transiently into *relA*-transfected cells, the cells were no longer resistant to curcumin. Our results highlight a critical anti-apoptotic role for NF-κB in curcumin-induced apoptosis.

Curcumin (diferuloyl methane), the yellow pigment in turmeric (*Curcuma longa*), possesses anti-inflammatory and antioxidant activities (1, 2). It is also a potent chemopreventive agent inhibiting tumor promotion against skin, oral, intestinal, and colon carcinogenesis (3–5). Many chemopreventive agents induce programmed cell death or apoptosis, a potent mechanism by which they eliminate preneoplastic or cancer cells. Curcumin induces several characteristics of apoptosis such as cell shrinkage, chromatin condensation, and DNA fragmentation in cells from colon, kidney, blood, and liver of human origin and NIH3T3, *erbB2*-transformed NIH3T3, and fibrosarcoma cells of murine origin (6, 7). However, curcumin fails to induce apoptosis in some fibroblastic and leukemic cell lines, and inhibition of cell proliferation is not always associated with apoptosis (8–10). Although the molecular mechanisms of antiproliferative and apoptotic effects of curcumin remain elusive, the resistance of various cell types to apoptosis following treatment with curcumin may be attributed to the abnormal expression of specific proteins that regulate apoptosis. Many cancer cells protect themselves against therapy (tumor necrosis factor, ionizing radiation, and chemotherapeutic compounds) by activating NF-κB[1]/Rel, a family of dimeric transcription factors, that helps in cell survival through unknown anti-apoptotic mechanisms (11–14). Interestingly, constitutive expression of NF-κB has been reported in breast and pancreatic cancer cells and tumors (but not in their normal counterparts), contributing to their apoptosis resistance/survival and tumor progression (15, 16). The typically active NF-κB/Rel is a heterodimer composed of a 50-kDa (p50) and a 65-kDa (p65 or RelA) subunit, although other homo/heterodimers of the mammalian Rel family (c-Rel, p52, and RelB) also exist. Knockout mice lacking the RelA subunit of NF-κB die before birth and show massive degeneration of liver cells caused by apoptosis. NF-κB proteins are sequestered in cytoplasm as inactive forms by associating with inhibitory proteins known as IκBs. IκB-α represents a prototype of several IκB proteins and IκB kinases, which phosphorylate them on specific serine residues upon getting a signal, have been cloned and characterized recently (17). Phosphorylation of IκB-α leads to its ubiquitination and subsequent degradation eventually releasing the heterodimers of NF-κB to translocate to the nucleus (18).

Curcumin is known to inhibit the activation of NF-κB induced by tumor necrosis factor-α, phorbol esters, and hydrogen peroxide (19). Curcumin inhibits cytokine-mediated NF-κB activation by blocking a signal leading to IκB kinase activity (20). Presumably, curcumin-induced apoptosis is regulated, in part, by the extent of NF-κB inactivation by curcumin and the level of expression of NF-κB in cells. To understand the role of NF-κB in curcumin-induced apoptosis, we stably transfected *relA* gene into L-929 cells (mouse fibrosarcoma), and upon treatment with curcumin, the *relA*-transfected cells were resistant even to higher doses of curcumin, whereas the parental cells underwent apoptosis in a time- and dose-dependent manner highlighting a critical anti-apoptotic role for NF-κB.

MATERIALS AND METHODS

Cell Lines and Culture—L-929 cell line (murine lung fibrosarcoma) was obtained from the National Center for Cell Science, Pune, India. The cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Life Technologies, Inc.) and antibiotics. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Reagents and Antibodies—Curcumin, TPA, and MTT were obtained from Sigma. Antibodies to p65 and FLAG were from Santa Cruz Biotechnology. Stock concentrations of curcumin and TPA were made in Me_2SO. All other chemicals were procured from Sigma unless otherwise stated.

In Vitro Growth Rate Analysis—The *in vitro* growth rate of cells was assessed by counting the cell number using hemocytometer. For MTT assay (21), the cells (3×10^3/well) were cultured in 96-well plates treated with or without curcumin for 24 h, and MTT was added to each well (5 mg/ml final concentration). Cells were incubated for an addi-

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[1] The abbreviations used are: NF-κB, nuclear factor-κB; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RelA, a p65 subunit of NF-κB; PBS, phosphate-buffered saline; TPA, tetradecanoylphorbol-13-acetate; DTT, dithiothreitol.

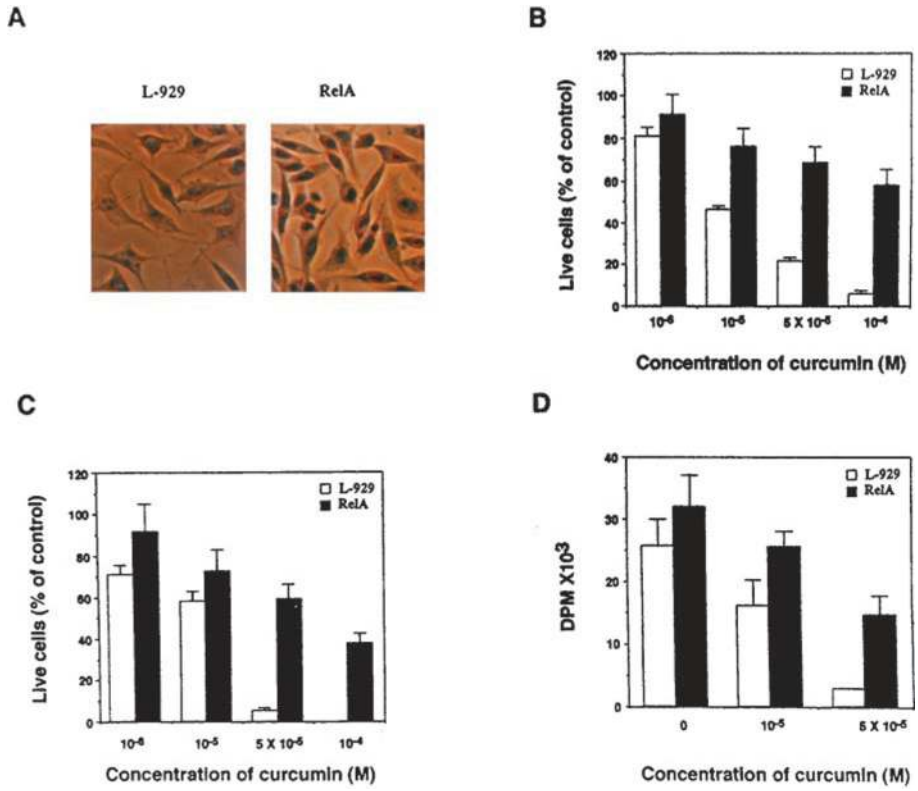

FIG. 1. **Changes in RelA expression and curcumin-induced cytotoxicity in parental and *relA*-transfected L-929 cells.** *A*, L-929 cells were transfected with human RelA by electroporation, and the stable pool of clones was selected with G418 as described under "Materials and Methods." Using a specific polyclonal p65/RelA antibody (Santa Cruz Biotechnology), the level of RelA expression was detected by immunocytochemical staining of parental and *relA*-transfected L-929 cells as per the details given under "Materials and Methods." *B*, cells were seeded into a 50-ml tissue culture flask, allowed to adhere, and then treated with different concentrations of curcumin for 24 h. The flasks were then washed twice with PBS (to remove the dead cells), and the live cells were collected by trypsinization and counted using a hemocytometer. The results are expressed as percent of control. *C*, cells were seeded into 50-ml tissue culture flasks and then treated with different concentrations of curcumin for 5 days. Live cells were counted as described above and expressed as percent of control. *D*, cells were seeded into 96-well plates, allowed to adhere, and then treated with or without the indicated concentrations of curcumin for 24 h. The medium was removed, and thymidine incorporation was measured as described under "Materials and Methods." The results are expressed as disintegrations/min (dpm). *Error bars* indicate S.D. of triplicate samples. All the above experiments were repeated at least two times with similar results.

tional 4 h, MTT was removed, Me_2SO was added, and cell viability was determined by measuring the absorbance at a test wavelength of 570 nm and a reference wavelength of 630 nm.

Immunocytochemical Analysis—For immunocytochemical analysis, the cells were cultured on coverslips, washed with PBS, and fixed with 3% formaldehyde for 30 min. Rabbit polyclonal antibody to RelA/p65 (sc-109, Santa Cruz Biotechnology) was used, and immunoreactivity was visualized using the vectastain ABC kit (Vector Laboratories Inc.) following the manufacturer's protocol.

Determination of Nuclear Condensation—cells were cultured for 24 h and treated with or without curcumin. The cells were trypsinized, fixed with 10% formaldehyde for 10 min, washed with PBS, and stained with ethidium bromide (50 μg/ml PBS) for 10 min. After washing, the cells were observed under a Nikon fluorescent microscope using a green filter ($\times$ 40 magnification).

Western Blotting—Western blotting analysis was performed as described previously (22) using a goat polyclonal antibody to the FLAG epitope (Santa Cruz Biotechnology, sc-807G).

DNA Fragmentation Analysis—This was carried out essentially as described previously (23).

Stable and Transient Transfections—Expression vector for RelA (pMT2T) has been described previously (24). For transfection, 1.0×10^6 cells were trypsinized, washed with PBS, and suspended in 0.8 ml of serum-free medium in a 0.4-cm cuvette and electroporated with 10 μg of DNA (empty or RelA expression vector) using the Gene Pulser II Electroporation System (Bio-Rad) under the conditions of 300 V and 950 microfarads with a time constant below 20. Cells were co-transfected with 2 μg of pcDNA3 vector (Invitrogen) containing the neomycin-resistant gene. Cells were then plated in 60-mm Petri dishes in Dulbecco's modified Eagle's medium, 10% newborn calf serum. After 36 h, G418 (0.5 mg/ml) was added and continued for a period of 10 days. The resistant clones were pooled and used. In transient transfection experiments, the super-repressor form of IκB-α fused downstream to a FLAG epitope in an expression vector (pCMV4) was used for electroporation (25).

[^{3}H]Thymidine Incorporation Assay—Cells were cultured and treated with or without curcumin. The medium was removed, and 0.2 μCi of [^{3}H]thymidine/well was added in serum-free medium and incubated for 6 h. The wells were washed with PBS, and proteins were precipitated with 5% trichloroacetic acid. Supernatant was removed and after washing with ethanol, the cells were solubilized with 0.2 N NaOH, and the radioactivity was counted using a liquid scintillation counter.

Electrophoretic Mobility Shift Assay—The assay was performed as described (22, 26).

RESULTS AND DISCUSSION

Earlier, we reported that curcumin and several natural or synthetic curcuminoids are cytotoxic to L-929 (murine fibrosarcoma) cells (1, 27). Now, we have examined the possible involvement of NF-κB in curcumin-induced apoptosis of L-929 cells, since its expression/activation is an important mechanism for apoptosis resistance/survival. To this end, L-929 cells were transfected with a human cDNA encoding the p65/RelA subunit of NF-κB, and the transfected pool of cells were selected for their resistance (achieved by co-transfection with a neomycin resistance gene) to G418. Immunocytochemical examination of the parental and *relA*-transfected cells using a specific polyclonal antibody to RelA revealed more intensive staining in the *relA*-transfected cells (Fig. 1*A*), confirming the expression of RelA. The staining pattern of cells transfected only with the pMT2T vector was similar to the untransfected parental cells (data not shown). The parental (L-929) and *relA*-transfected cells (RelA) treated with different concentrations of curcumin for 24 h at 37 °C clearly show that the transfected cells effectively resist curcumin-induced cytotoxicity

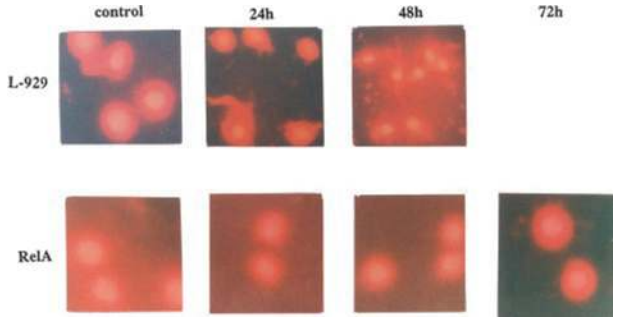

FIG. 2. **Nuclear condensation is induced by curcumin in L-929 cells but not in *relA*-transfected cells.** L-929 and *relA*-transfected cells were cultured in 60-mm Petri dishes and treated with or without (*control*) 10^{-6} M curcumin for 24, 48, and/or 72 h as shown. The cells were trypsinized, fixed, and stained with ethidium bromide, and nuclear condensation was examined as described under "Materials and Methods." The experiment was repeated again to confirm our results.

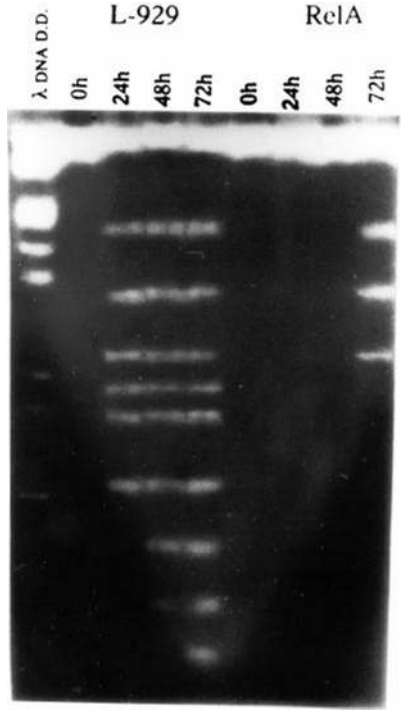

FIG. 3. **DNA fragmentation is induced by curcumin in L-929 cells but not in *relA*-transfected cells.** Cells were treated with or without 10^{-6} M curcumin. Cells were scraped out and DNA was extracted as described under "Materials and Methods." DNA fragments were resolved by 1.5% of agarose gel electrophoresis and visualized by ethidium bromide staining. Similar results were obtained in two independent experiments.

(more than 90% of cells survived with 10^{-6} M curcumin, and 50–58% were still viable at 10^{-4} M curcumin treatment), while the untransfected cells progressively underwent cell death (20% of cells died with 10^{-6} M curcumin, and more than 95% died with 10^{-4} M curcumin treatment) with the increase in concentration of curcumin (Fig. 1*B*). The dose-dependent cell death of the parental L-929 cells induced by curcumin and the resistance of *relA*-transfected cells to curcumin prompted us to check the time dependence of this phenomenon, and the results after a 5-day period of incubation with different concentrations of curcumin show that about 40% of the *relA*-transfected cells were still viable at 10^{-4} M curcumin, whereas none of the parental cells survived at this concentration (Fig. 1*C*). DNA synthesis as measured by the incorporation of labeled thymidine also confirmed that it is inhibited by curcumin in L-929 cells, whereas RelA can impart resistance to this inhibition of DNA synthesis by curcumin (Fig. 1*D*).

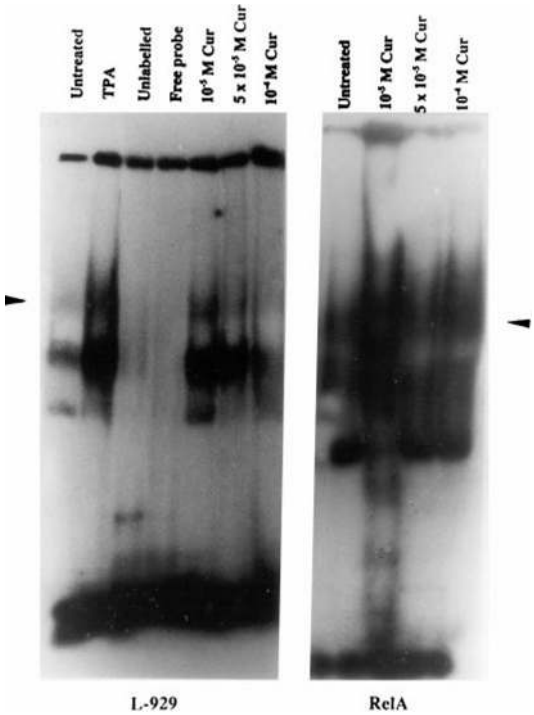

FIG. 4. **relA-transfected cells show constitutive NF-κB DNA binding activity unaffected by curcumin.** L-929 cells were treated with TPA (50 ng/ml) for 2 h at 37 °C to induce NF-κB. Untreated cells were taken as control. For experiment in L-929 cells, varying concentrations of curcumin was added together with TPA. Since the *relA*-transfected cells showed constitutive DNA binding activity, they were directly treated with curcumin without the addition of TPA. The *arrow* indicates the active heterodimeric NF-κB complex (p50-p65). Electrophoretic mobility shift assay was performed as described under "Materials and Methods." The specific nature of the binding proteins was ascertained by adding excess unlabeled oligonucleotide into the binding reaction. As a control the nuclear extract was omitted in one of the reactions (*Free probe*). The results were confirmed in another independent experiment.

These results show that NF-κB/RelA expression can effectively resist the antiproliferative and cytotoxic effects induced by curcumin in L-929 cells. Consistent with our results, proliferation rate of a human pancreatic cancer cell line (MDAPanc-28) showing constitutive activation of NF-κB remained unaffected upon treatment with 50 μM of curcumin (16).

To confirm whether the cytotoxic effects induced by curcumin in L-929 cells involve apoptotic changes and ascertain the role of NF-κB, L-929, and *relA*-transfected cells were examined for typical apoptotic patterns (nuclear condensation and fragmentation) with or without curcumin. Upon treatment with 10^{-6} M curcumin, nuclear condensation (examined by staining the cells with ethidium bromide) was visible in L-929 cells at 24 h and increased at 48 h, but the *relA*-transfected cells did not show nuclear condensation even up to 72 h treatment with curcumin (Fig. 2). Since one of the most distinct biochemical hallmarks of apoptosis is the cleavage of DNA into multiple internucleosomal fragments of 180–200 base pairs (28, 29), nuclear DNA isolated from the curcumin-treated cells was analyzed by agarose gel electrophoresis. The results show that in L-929 cells DNA fragmentation gradually increased with time (24–72 h) upon treatment with 10^{-6} M curcumin, whereas the *relA*-transfected cells did not show typical apoptotic DNA fragmentation patterns even up to 72-h treatment with curcumin, although some high molecular weight fragments appeared at 72 h (Fig. 3). In addition, relatively large numbers of apoptotic bodies were observed in curcumin-treated L-929 cells as compared with the *relA*-transfected cells during morphological ex-

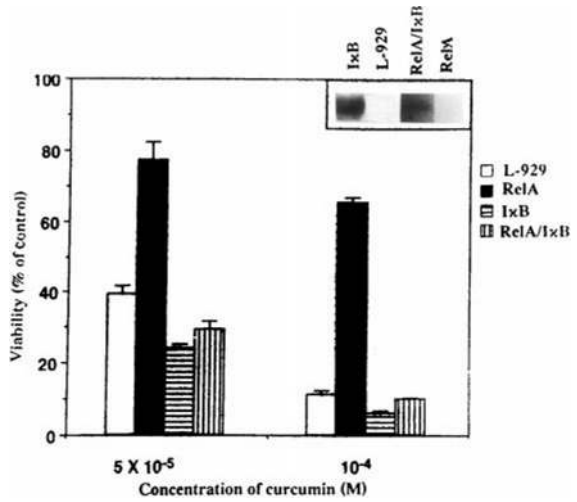

FIG. 5. **IκB reverses the resistance of *relA*-transfected cells to curcumin-induced cytotoxicity.** L-929 or *relA*-transfected cells were subjected to transient transfection with IκB-α super-repressor as described under "Materials and Methods." The untransfected and transfected cells were then treated with different concentrations of curcumin for 24 h. Then the medium was removed, MTT was added, and the cell viability was determined as described under "Materials and Methods," and the results are expressed as percent of control. The *inset* shows the results of Western blot analysis using a polyclonal antibody (Santa Cruz Biotechnology) to the FLAG epitope of the super-repressor form of IκB-α in L-929 or *relA*-transfected cells transfected with or without the super-repressor form of IκB-α. Triplicate samples were used to calculate the standard deviation (*bars*). The results were confirmed by repeating the experiment.

amination (data not shown). Taken together, these data suggest the involvement of apoptosis in curcumin-induced cytotoxicity in L-929 cells and also emphasize the anti-apoptotic potential of NF-κB expression. To our knowledge, the present work is the first attempt to unravel the anti-apoptotic role of NF-κB in curcumin-induced apoptosis of cancer cells. The present molecular approach of ectopic expression of RelA was considered as it is probably difficult to examine and interpret the effects of curcumin on cells with varying levels of NF-κB due to variations in their genetic background. For instance, in many human breast cancer cells, curcumin neither induced apoptosis nor any significant changes in the expression of apoptosis-related genes, including p53, Bcl2, cyclin B, and transglutaminase (10), although a multidrug-resistant human breast cancer cell line underwent apoptosis upon curcumin treatment and showed changes in p53 mRNA (30). Interestingly, human breast cancer cells are known to exhibit widely varying NF-κB expression levels, which negatively correlate with their estrogen receptor status (31).

Since curcumin is well known to block the activation of NF-κB induced by several agents, it became relevant to assess NF-κB DNA binding activity in L-929 cells. We used TPA to induce NF-κB, and treatment with curcumin inhibited TPA-induced activation of NF-κB in L-929 cells. The inactivation was complete at higher concentrations of curcumin (Fig. 4). Interestingly, the *relA*-transfected cells showed constitutive NF-κB DNA binding activity, and even 10^{-4} M curcumin could not inhibit this activity (Fig. 4), consistent with the capacity of these cells to resist curcumin-induced apoptosis (Figs. 1–3). If indeed, the constitutive NF-κB activity is responsible for the observed resistance to curcumin, blocking the activation is expected to reverse it. Super-repressor IκB-α is a mutant form of IκB-α (1–36 amino acids deleted) that has been shown to block the activation of NF-κB by other workers (12, 25). Hence, we transiently transfected the super-repressor form of IκB-α into *relA*-transfected cells and measured the viability by MTT assay, and the results show that the *relA*-transfected cells, which were resistant to curcumin (Fig. 1), also became responsive upon IκB transfection (Fig. 5). Apparently, even the basal level of NF-κ B in L-929 cells has some potential to block the action of curcumin as introducing IκB into these cells marginally increased curcumin-induced cytotoxicity (Fig. 5). These experiments demonstrate that NF-κB activation blocks curcumin-induced apoptosis, and NF-κB activation by the degradation of IκB-α is the major pathway for its activation in L-929 cells. Further work is needed to know whether NF-κB expression regulates apoptosis induced by other NF-κB inhibitors such as gliotoxin, pyrrolidine dithiocarbamate, and *N*-tosyl-L-phenylalanine chloromethyl ketone (32). Our results highlight a critical anti-apoptotic role for NF-κB in curcumin-induced apoptosis. The actual mechanism of curcumin-induced apoptosis is not clear but our results provide a plausible mechanism for cells resisting curcumin. Such a mechanism may also regulate the apoptotic susceptibility of curcumin-treated cells to other anti-cancer agents. Further work is also needed to understand the relative importance of NF-κB and other anti-apoptotic factors such as Bcl2 in the regulation of curcumin-induced apoptosis. Our findings have implications for developing curcumin-based anti-cancer therapies.

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