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Sensitization of Taxol-induced Apoptosis by Curcumin Involves Down-regulation of Nuclear Factor-κB and the Serine/Threonine Kinase Akt and Is Independent of Tubulin Polymerization*

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Taxol is the best anticancer agent that has ever been isolated from plants, but its major disadvantage is its dose-limiting toxicity. In this study, we report with mechanism-based evidence that curcumin, a nontoxic food additive commonly used by the Indian population, sensitizes tumor cells more efficiently to the therapeutic effect of Taxol. A combination of 5 nM Taxol with 5 μM curcumin augments anticancer effects more efficiently than Taxol alone as evidenced by increased cytotoxicity and reduced DNA synthesis in HeLa cells. Furthermore, our results reveal that this combination at the cellular level augments activation of caspases and cytochrome *c* release. This synergistic effect was not observed in normal cervical cells, 293 cells (in which Taxol down-regulates nuclear factor-κB (NF-κB)), or HeLa cells transfected with inhibitor κBα double mutant (IκBα DM), although the transfection itself sensitized the cells to Taxol-induced cytotoxicity. Evaluation of signaling pathways common to Taxol and curcumin reveals that this synergism was in part related to down-regulation of NF-κB and serine/threonine kinase Akt pathways by curcumin. An electrophoretic mobility shift assay revealed that activation of NF-κB induced by Taxol is down-regulated by curcumin. We also noted that curcumin-down-regulated Taxol induced phosphorylation of the serine/threonine kinase Akt, a survival signal which in many instances is regulated by NF-κB. Interestingly, tubulin polymerization and cyclin-dependent kinase Cdc2 activation induced by Taxol was not affected by curcumin. Altogether, our observations indicate that Taxol in combination with curcumin may provide a superior therapeutic index and advantage in the clinic for the treatment of refractory tumors.

Cervical cancer is the most frequently diagnosed cancer of females in developing countries and the second most frequent cancer affecting women worldwide (1). An estimated half-million new cases and almost as many deaths in 2000 have been reported (2). Current treatment modalities such as surgical ablation and/or external radiotherapy intervention remain largely palliative for cervical cancer patients because the disease recurs in a refractory form. Long term disease-free treatment consists of cytotoxic chemotherapeutic agents that kill cancer cells mainly by apoptosis. However, commonly used cytotoxic chemotherapy is largely associated with highly nonspecific cytotoxicity, narrow therapeutic indices, and undesirable side effects. Taxol (paclitaxel), isolated from *Taxus brevifolia*, is the drug of choice with significant antitumor activity toward cervical, breast, and ovarian cancer, among others (3–5). It interferes mechanistically with the dynamic instability of microtubules and thereby arrests the cell cycle at the G_2/M phase, finally leading to apoptotic cell death (6). However, the success of Taxol chemotherapy in cervical cancer patients is limited because of myelotoxicity and neurotoxicity (3, 7). Furthermore, tumors tend to acquire resistance to cytotoxic chemotherapeutic agents, including Taxol.

The molecular basis of resistance to Taxol is not well understood, although a few speculative mechanisms independent of microtubule stabilization have been suggested. In addition, Taxol has been implicated in regulating targeted cellular proteins that promote cell survival and block apoptosis (such as Bcl-2 and Bcl-XL) (8). Another suggestive mechanism of chemoresistance by cytotoxic drugs involves elevated levels of phosphorylated protein kinase B/Akt (9). Furthermore, mounting evidence supports the role of NF-κB[1] in promoting cell survival and up-regulating genes important for tumor proliferation and metastasis (10), thereby affording protection against programmed cell death (11). For instance, the proliferation of Hodgkin's disease is dependent on the constitutive activation of NF-κB (12). We hypothesize that blocking NF-κB activation may augment cancer chemotherapy. Thus, agents that induce apoptosis and stimulate NF-κB activity may be effective if given in combination with agents that could inhibit NF-κB. Evolving interest in recent years has focused on phytochemicals augmenting apoptosis as possible candidates for evaluation of their synergistic efficacy in combination with chemotherapeutic agents.

Curcumin, isolated from the rhizomes of the plant *Curcuma longa*, has been shown to possess potent anti-inflammatory and chemo-preventive properties. It possesses antiproliferative activities against tumor cells *in vitro* (13) and inhibits tumor promotion against skin, oral, intestinal, and colon carcinogenesis (14–16). Previous studies from our laboratory and elsewhere have shown that curcumin suppresses a number of key elements in cellular signal transduction pathways including NF-κB (17, 18), c-Jun/AP-1 activation, (19) and phosphorylation reactions catalyzed by protein kinases (20).

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[1] The abbreviations used are: NF-κB, nuclear factor κB; AFC, 7-amino-4-trifluoromethyl coumarin; EMSA, electrophoretic mobility shift assay; IKK, IκB kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase.

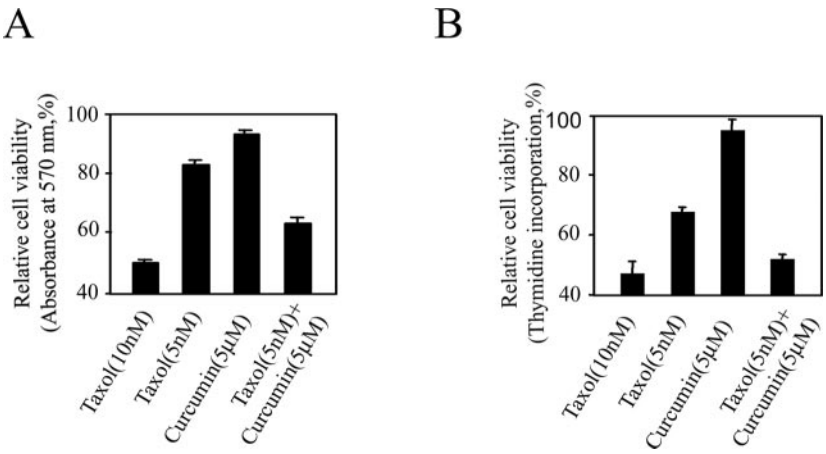

FIG. 1. **Taxol-induced growth inhibition and DNA synthesis are potentiated by curcumin.** *A*, HeLa cells were treated with Taxol and/or curcumin as indicated and incubated for 72 h, and cell viability was assessed by MTT assay as described under "Experimental Procedures." *B*, HeLa cells were treated with Taxol and/or curcumin as indicated for 24 h, and [^{3}H]thymidine incorporation was determined as described under "Experimental Procedures."

The present communication suggests a combination treatment synergism of great effectiveness by combining curcumin and Taxol. We assessed potential molecular mechanism supporting this synergistic effect with emphasis on determining whether the combination treatment enhanced inhibition of the antiapoptotic signal transducers Akt and NF-κB, leading to increased activation of caspase-mediated apoptosis. Moreover, combinatorial strategy dramatically lowered the inhibitory concentration of Taxol to induce a cytotoxic response, suggesting that curcumin may have potential for use in combination regimens with Taxol.

EXPERIMENTAL PROCEDURES

Cell Lines—The human cervical cancer cell lines HeLa, SiHa, CaSki, and ME-180 and the human kidney cell line 293 were obtained from the National Centre for Cell Science, Pune, India and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1× antibiotic-antimycotic.

Chemicals—Antibodies against caspase-8, -9, -3, and -7, poly(ADP-ribose) polymerase (PARP), Akt, phospho-Akt, and phospho-IKK were purchased from Cell Signaling (Beverly, MA), and those against cytochrome *c*, β-tubulin, IKK-β, p50, RelA, and Cdc2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Taxol and substrates for caspase-3 (acetyl-DEVD-AFC), caspase-8 (benzyloxycarbonyl-IETD-AFC), and caspase-9 (acetyl-LEHD-AFC) were procured from Calbiochem (San Diego, CA). All other chemicals were purchased from Sigma if not otherwise indicated.

Primary Culture—Normal cervical epithelial cells were isolated from a patient undergoing hysterectomy for fibroids who had a normal cervical smear before surgery. Ectocervical lips were collected aseptically in Hank's balanced salt solution containing antibiotics. After scraping off the keratinocyte layer, the ectocervical tissue was cut into fine pieces, treated with collagenase (0.2 mg/ml), dissolved in serum-free medium, and incubated overnight at 4 °C. The cells obtained were diluted in MCDB-131 medium containing 20% serum and growth factors, and the debris was allowed to settle. The supernatant was then centrifuged at 3,500 rpm for 7 min, and the pellet was seeded in complete medium.

Mode of Treatment—In all combination treatments curcumin was added 2 h before Taxol. For the MTT assay, [^{3}H]thymidine incorporation and annexin V-PI staining, 5×10^3 cells/well were seeded in 96-well plates. For the comet assay, Western immunoblot, spectrofluorimetric assay, transfection, and electrophoretic mobility shift assay (EMSA) 1×10^6 cells per 60-mm plate were seeded.

MTT Assay—Cytotoxic effect of Taxol and/or curcumin was determined by MTT assay as described earlier (21). Briefly, after a PBS wash, 100 μl of Dulbecco's modified Eagle's medium and 25 μl of MTT solution (5 mg/ml in phosphate-buffered saline) was added to cells (untreated and treated) cultured in 96-well plates. Cells were incubated for 2 h, and 0.1 ml of the extraction buffer (20% sodium dodecyl sulfate in 50% formamide) was added. After an overnight incubation at 37 °C, the optical densities at 570 nm were measured using a plate reader (Bio-Rad) with the extraction buffer as blank. The relative cell viabilities in percentage were calculated by comparing the viability of the treated cells with that of the control.

[^{3}H]Thymidine Incorporation—Inhibition of DNA synthesis induced by Taxol and/or curcumin was assessed by a [^{3}H] thymidine incorporation assay as described previously (18).

Comet Assay—DNA damage induced by Taxol and/or curcumin was studied by comet assay as described by Singh *et al.* (22).

Annexin V-Propidium Iodide Staining—The membrane flip-flop induced by Taxol and/or curcumin was assessed essentially as described previously (23) according to manufacturer's protocol (annexin V apoptosis detection kit, Santa Cruz Biotechnology).

Detection of Caspase Activation, PARP Cleavage, and Cdc2 Overexpression—Western blot analysis was done to detect Cdc2, caspase-8, -9, -3 and -7, and PARP as described previously (21) using enhanced chemiluminescence (Amersham Biosciences) or the alkaline phosphatase method.

Assay of Caspases—The enzymatic activities of caspase-3, -8, and -9 were assayed spectrofluorometrically (Perkin Elmer LS-50) as described earlier (23) with excitation and emission wavelengths of 400 and 505 nm, respectively.

Measurement of Cytochrome c Release—Mitochondria-free cytosol was isolated as described earlier (21) using a Dounce homogenizer and Western blotted against anti-cytochrome *c*.

EMSA—To detect NF-κB activation, EMSA was performed, and the specificity of the bands was confirmed by super shift (24) and visualized by a phosphorimaging device (Bio-Rad Personal FX).

Stable Transfection—Cells were transfected with pcDNA3-IκBα DM (double mutant) or the empty vector pcDNA3 using the calcium-phosphate transfection kit (Invitrogen) according to the manufacturer's protocol and grown in selection medium (600 μg/ml G418), and the clones that formed were picked up as described elsewhere (23).

Tubulin Polymerization Assay—Polymerized and non-polymerized tubulins were extracted as described elsewhere (25), and the respective levels were assessed by Western blotting against anti-β-tubulin.

RESULTS

Taxol-induced Growth Inhibition, DNA Synthesis, and Morphological Alterations Are Potentiated by Curcumin—Our objective in this study was to investigate whether there is any synergism between Taxol and curcumin and, if so, to find the most efficient combination of doses of the two. We screened four human-derived cervical cancer cell lines to evaluate their sensitivity to Taxol and curcumin by MTT assay, which is a very convenient method for assessing drug sensitivity. Our findings revealed 100 nM Taxol to be highly toxic to almost all of the investigated cell types, whereas curcumin up to 10 μM exhibited no significant effect on cell viability (data not shown). HeLa appeared to be the most sensitive among the panel of cell lines evaluated; we therefore continued our studies using HeLa cells. The latter were evaluated for their sensitivity toward various concentrations of Taxol and curcumin by MTT assay. Results obtained reveal that relative to control, 5 and 10 nM Taxol induced 17 and 50% cytotoxicity respectively, whereas a combination dose of 5 nM Taxol and 5 μM curcumin induced 35% cytotoxicity (Fig. 1*A*). Furthermore, we examined the effects of Taxol and curcumin individually and the effect of their combinatorial regimen on DNA synthesis. Whereas 10 and 5

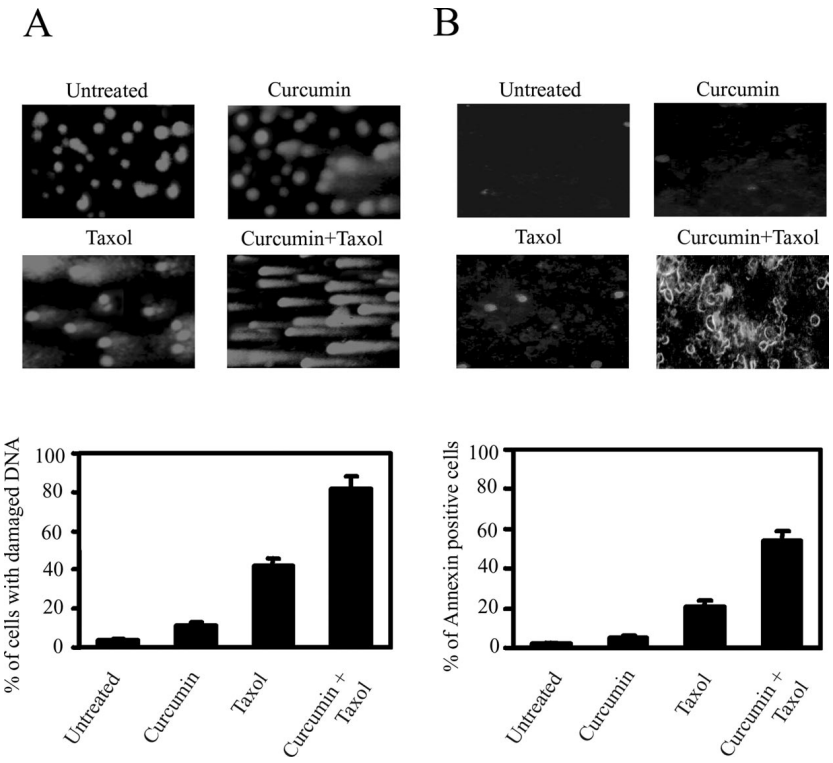

FIG. 2. **Curcumin potentiates Taxol-induced DNA fragmentation and membrane flip-flop.** *A*, HeLa cells were treated with Taxol (5 nM) and/or curcumin (5 μM) for 24 h, trypsinized, and pelleted, and a comet assay was done as described under "Experimental Procedures." Cells with damaged DNA in various fields were counted, and the average was taken. The experiment was repeated once more with similar results. *B*, HeLa cells were treated with Taxol (5 nM) and/or curcumin (5 μM) for 16 h and stained for annexin-V-PI positivity as described under "Experimental Procedures." Annexin V positive cells in various fields were counted, and the average was taken. The experiment was repeated three times with similar results

nM Taxol induced 47 and 68% thymidine incorporation respectively, a combination of 5 nM Taxol and 5 μM curcumin induced 52% of the same (Fig. 1*B*). These results indicate that curcumin and Taxol, when used in combination, induce almost double the amount of cell death compared with Taxol *per se*. Apoptotic bodies were observed to be comparatively more in wells treated with the combination as compared with individual treatments (data not shown).

Curcumin Potentiated Taxol-induced Cell Damage and DNA Fragmentation in HeLa Cells—Comet assay and annexin staining also extend support to the proposed combination hypothesis. Comparison of the tail length of the comets clearly reveals that 5 nM Taxol-induced DNA fragmentation (42%) is significantly enhanced by pre-treatment with 5 μM curcumin (82%; Fig. 2*A*). Bright green annexin fluorescence was imparted to membranes of the apoptotic cells revealing flip-flop of phosphatidylserine to the outer surface of the membrane, representative of the early stage event of apoptosis. In cells pre-treated with curcumin, 5 nM Taxol produced 47% cells with annexin positivity, whereas only 21% were annexin positive when Taxol was used alone. Untreated and curcumin-treated cells had a very minimum number of positively stained cells. Furthermore, cells treated with the combination of curcumin and Taxol also showed the red color of propidium iodide, signifying a higher extent of nuclear damage (Fig. 2*B*).

Curcumin Potentiates Taxol-induced Caspase Activation, PARP Cleavage, and Cytochrome c Release—To confirm apoptosis in response to combinatorial treatment with curcumin and Taxol, we evaluated caspase-8, -9, -7, and -3 by Western immunoblotting and spectrofluorimetry. Caspase-8 and -9 are considered to be the initiator caspases, and caspase-3 and -7 are considered the effectors of apoptosis. It was noted that the cleavage of procaspase-8 to its active fragments (p43/41 and p18) and procaspase-9 to its active fragments (p35/37) induced by 5 nM Taxol was significantly up-regulated by pre-treatment with 5 μM curcumin, which by itself alone did not induce any cleavage of caspase-8 or -9 (Fig. 3, *A* and *B*). Moreover, Taxol-induced cleavage of caspase-3 and -7 was also up-regulated by curcumin pre-treatment (Fig. 3, *C* and *D*). A spectrofluorimetric assay using artificial substrates corresponding to the upstream amino acid sequence of the respective caspase cleavage site and the fluorophor AFC confirmed these results (Fig. 3, *A–C*). Furthermore, the combinatorial regimen also induced more cleavage of the well characterized caspase-3 substrate, PARP, relative to individual treatments (Fig. 3*E*). At the cellular level, caspase-8 mediates cleavage of BID, subsequently leading to the release of cytochrome *c* from the mitochondria. The latter in the presence of Apaf-1 activates caspase-9, resulting in activation of downstream caspases-3 and 7. As shown in Fig. 3*F*, pre-treatment with curcumin augmented Taxol-induced release of cytochrome *c*. Taken together, the above results confirm that a combination of 5 nM Taxol and 5 μM curcumin makes HeLa cells more susceptible to apoptosis.

Taxol-induced NF-κB Activation and IκBα Degradation Are Inhibited by Curcumin—Taxol activates NF-κB in several cell lines (26–28). We also observed a clear dose-dependent activation of NF-κB in HeLa cells by Taxol up to 10 nM that diminishes at high concentrations (Fig. 4*A*). The result of the kinetics study shown in Fig. 4*B* reveals that NF-κB activation by Taxol starts within 15 min, reaches a plateau at 1 h, and then begins to recede. NF-κB is sequestered in the cytoplasm by inhibitory protein IκBα. To confirm whether the NF-κB activation induced by Taxol is through degradation of IκBα, immunoblotting was performed against anti-IκBα utilizing the cytoplasmic protein extracts derived from the kinetic study. As shown in Fig. 4*C*, IκBα degradation starts as early as 15 min after the start of Taxol treatment and is almost complete at 60 min, after which the resynthesis of IκBα begins. We noticed that curcumin pre-treatment clearly down-regulated NF-κB activation induced by Taxol (Fig. 4*D*). Both p50 and RelA antibodies shifted the active NF-κB complex (supershift), whereas incubation with excess unlabeled oligonucleotide containing the NF-κB binding site completely removed the active complex, confirming the specificity of the band (Fig. 4*D*). Nuclear extract of HeLa cells treated with tumor necrosis factor-α, a well known activator of NF-κB, was taken as the positive control. Interestingly, we found that curcumin effectively induces the resynthesis and/or blocks the degradation of IκBα in cells

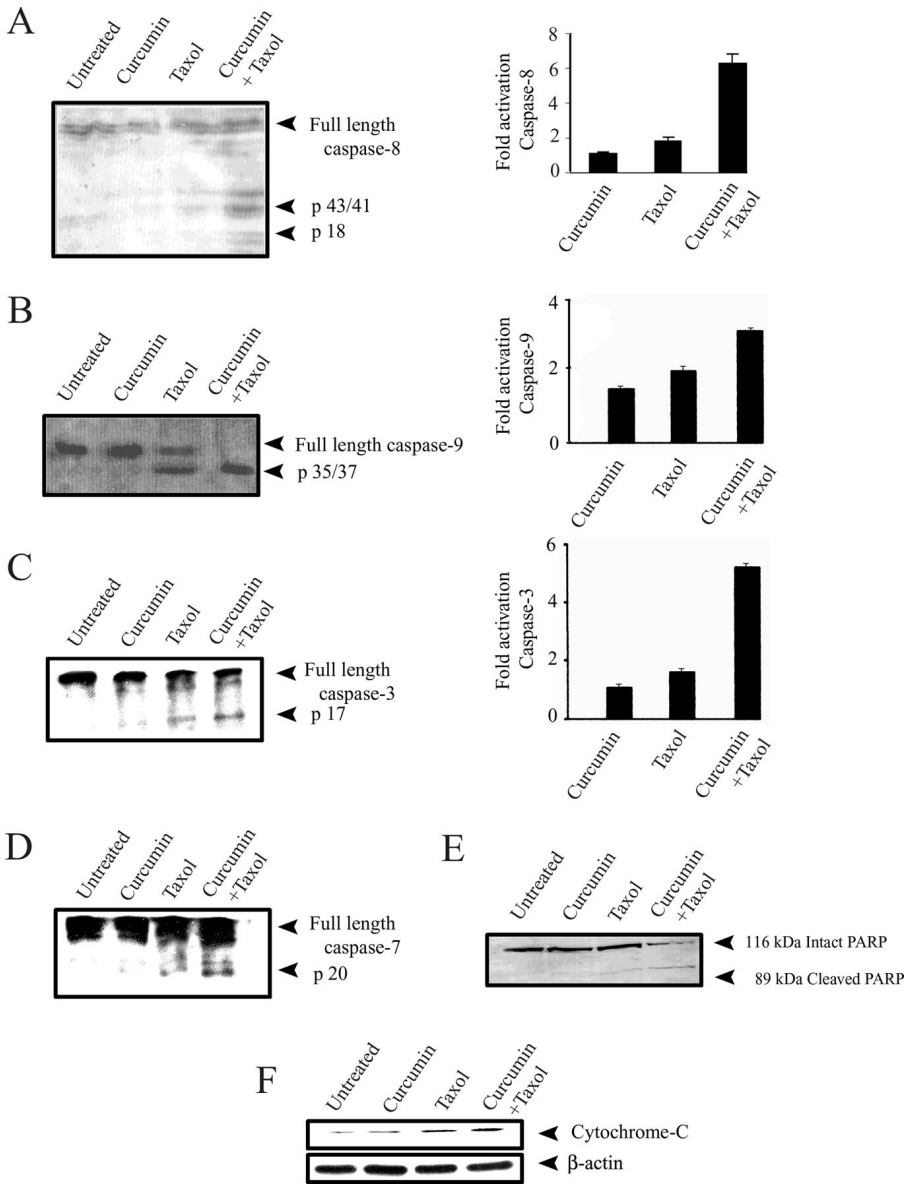

FIG. 3. **Taxol-induced caspase activation, PARP cleavage, and cytochrome *c* release are potentiated by curcumin.** *A–D*, HeLa cells were treated with Taxol (5 nM) and/or curcumin (5 μM) for 24 h. For Western blot, the whole cell lysate was resolved on a 12% gel, blotted against anti-caspase-8, -9, -3, and –7, respectively, and detected by enhanced chemiluminescence; for the spectrofluorimetric assay, total protein (50 μg) was incubated with caspase substrates (50 μM) in a total volume of 500 μl of reaction buffer, and the fluorophor released was quantitated spectrofluorimetrically as described under "Experimental Procedures." *E*, HeLa cells were treated with Taxol (5 nM) and/or curcumin (5 μM) for 24 h, and the whole cell lysate was resolved on 8% gel, blotted against anti-PARP, and detected by the alkaline phosphatase method. *F*, HeLa cells were treated with Taxol (5 nM) and/or curcumin (5 μM) for 24 h, and mitochondria-free cytosol was prepared as described under "Experimental Procedures," resolved on 15% gel, blotted against anti-cytochrome *c*, and detected by enhanced chemiluminescence.

treated with Taxol and curcumin (Fig. 4*E*), possibly by curcumin-mediated inhibition of IKK (Fig. 4*F*). These results confirm that lower concentrations of Taxol induce NF-κB in HeLa cells through the degradation of IκBα and that curcumin inhibits it by blocking degradation and/or inducing resynthesis of the inhibitory protein IκBα.

Curcumin Does Not Sensitize Taxol-induced Apoptosis in Normal Cervical Cells, Human 293 Cells, and HeLa-IκBα Cells—To study the effect of Taxol and curcumin on normal cervical cells, we evaluated their sensitivity toward Taxol (1–100 nM) and curcumin (1–10 μM) by MTT and [^{3}H]thymidine incorporation assays. Comparison of the cell viability revealed that Taxol, up to 100 nM, and curcumin, up to 50 μM, was non-toxic to normal cells. Apparently none of the combination doses of Taxol and curcumin examined induced any synergistic effect in normal cervical epithelial cells as assessed by MTT (data not shown) and thymidine incorporation assays (Fig. 5*A*). Thus far, Taxol (1–5000 nM) could not activate NF-κB in these cells whereas, on the contrary, it readily activated NF-κB in HeLa cells (Fig. 5*B*).

We also studied the combined effect of Taxol and curcumin in human 293 cells in which NF-κB is down-regulated by Taxol (29) and, predictably, did not find any synergistic effect for Taxol and curcumin in inhibiting DNA synthesis. Whereas 5 and 10 nM Taxol induced 29 and 55% cytotoxicity, respectively, in these cells, a combination treatment of 5 nM Taxol and 5 μM curcumin produced only 30% cytotoxicity, which is almost the same as that by 5 nM Taxol (Fig. 5*C*). Interestingly, Taxol (up to 1000 nM) could not activate NF-κB in human 293 cells either (Fig. 5*D*). These observations also suggest that the sensitization of Taxol-induced apoptosis by curcumin may be through the down-regulation of NF-κB.

To confirm the role of NF-κB in disrupting signaling through the survival pathway leading to cell death, we inactivated NF-κB by stably transfecting HeLa cells with pcDNA3-IκBα, a double mutant of IκBα, which lacks the serine residues that are essential for IκBα phosphorylation and NF-κB activation. We isolated the stable clones, confirmed IκBα overexpression by Western immunoblotting, and selected clone 3 with maximum expression (Fig. 5*E*) for further studies. HeLa cells transfected with the empty vector pcDNA3 (HeLa-Neo) were used as control. Curcumin pre-treatment (5 μM) brought down the viability of Taxol-treated (5 nM) HeLa-Neo cells from 83 to 63%, although it did not produce any effect in HeLa-IκBα-transfected cells. However, IκBα transfection itself sensitized the cells to Taxol-induced apoptosis (Fig. 5*F*). IκBα transfection highly

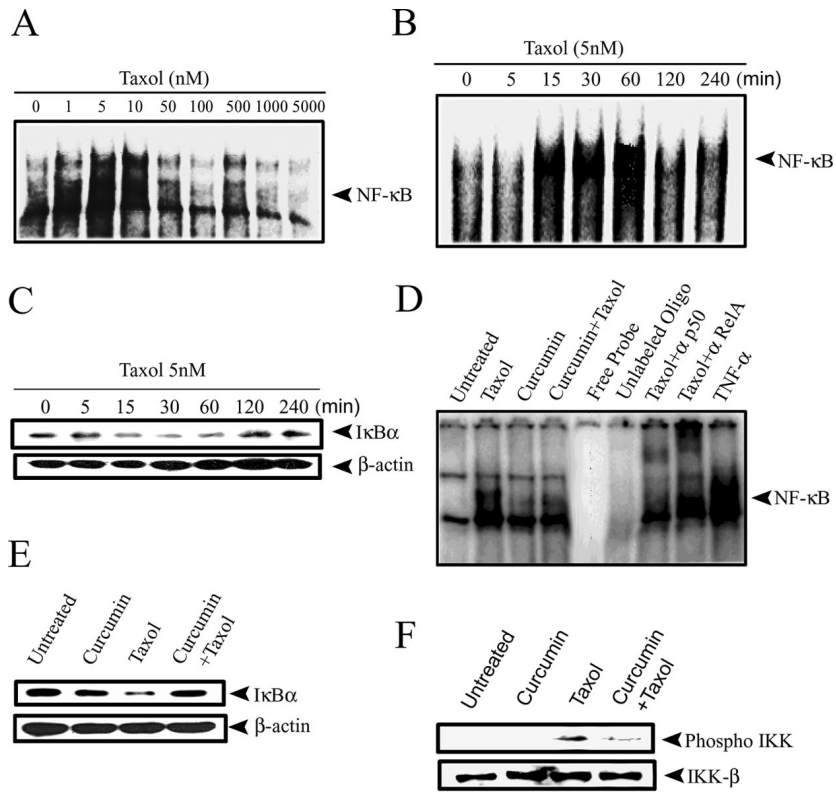

FIG. 4. **Curcumin inhibits Taxol-induced NF-κB activation and IκBα degradation.** *A*, HeLa cells were treated with Taxol (0–5000 nM), nuclear extracts were prepared, and EMSA was done as described under "Experimental Procedures." *B*, cells were treated with 5 nM Taxol for different time intervals (0–240 min), nuclear extracts were prepared, and EMSA was done as described under "Experimental Procedures." *C*, cytosol collected from the above experiment was blotted for IκBα. *D*, HeLa cells were treated with Taxol (5 nM) and/or curcumin (5 μM) for 1 h. Nuclear extracts were prepared, and EMSA was done as described under "Experimental Procedures." The nuclear extracts from Taxol-stimulated cells were also incubated with either Rel A or p50 antibody or unlabeled oligo. Cells treated with 0.1 nM tumor necrosis factor were kept as a positive control. The *arrowhead* indicates the positions of the active DNA binding complex of NF-κB. *E* and *F*, cytosols of control cells and those treated with Taxol and/or curcumin from the above experiment were collected and blotted against anti-IκBα and anti-phospho-IKK, respectively.

sensitized HeLa cells to Taxol-induced caspase activation and PARP cleavage, supporting our data from MTT assay. Moreover, no further enhancement of caspase activation was induced by curcumin pre-treatment (Fig. 5, *G* and *H*). These results clearly demonstrate that curcumin potentiates Taxol-induced apoptosis through the down-regulation of NF-κB.

Curcumin Pre-treatment Inhibits Taxol-induced Akt Activation in HeLa Cells, whereas Normal Cells Are Unaffected—The involvement of Akt, a survival signal that in many cases is regulated by NF-κB (30, 31), was also investigated. Dose-dependent phosphorylation was observed at lower concentrations of Taxol that gradually declined after 10 nM (Fig. 6*A*). Curcumin pretreatment almost completely abolished the phospho-Akt band in the combination, indicating a possible role for Akt in the synergistic effect of Taxol and curcumin (Fig. 6*B*). However, similarly as with NF-κB, Akt also was not activated by Taxol in normal cervical cells (Fig. 6*C*). These results indicate a possible role for Akt in chemosensitization of Taxol-induced apoptosis.

Sensitization of Taxol-induced Apoptosis by Curcumin Is Independent of Tubulin Polymerization and Cell Cycle Arrest—To examine whether curcumin is also influencing the ability of Taxol to stabilize microtubules by disrupting the dynamic equilibrium between soluble tubulin dimers and their polymerized form (6), we isolated the insoluble polymerized tubulin and the soluble non-polymerized tubulin from HeLa cells treated with Taxol and/or curcumin and immunoblotted against anti-β-tubulin. In the absence of Taxol, the majority of tubulin was found in the soluble non-polymerized form, whereas the amount of insoluble polymerized tubulin was increased upon Taxol treatment as reported earlier (32). Curcumin did not induce any change in the amount of soluble or insoluble tubulin when treated alone or with Taxol (Fig. 7*A*). The role of Cdc2, which plays a critical role in Taxol-induced cell cycle arrest, was also investigated in this context. We could not observe any significant effect for curcumin on the up-regulation of Cdc2 by Taxol either (Fig. 7*B*).

Taken together, these results indicate that curcumin potentiates Taxol-induced apoptosis through a pathway that is independent of cell cycle arrest and tubulin polymerization. Our study also suggests that this synergistic effect is at least partly, if not fully, regulated by NF-κB and Akt. A model for the regulation of Taxol-induced apoptosis by curcumin incorporating the contributions of the present study is given in Fig. 8.

DISCUSSION

The primary mechanism of the action of Taxol is attributed to its ability to bind to microtubules and prevent their assembly, causing cells to arrest in the G_2/M phase and thereby blocking cell cycle progression (6). Although this explains the underlying mechanism of Taxol-mediated growth arrest, its efficacy exceeds that of conventional microtubule-disrupting agents, suggesting that additional cellular effects may be operating via pathways independent of mitotic arrest (33, 34). Our results also support this notion. We observed a dose-dependent cytotoxic effect by Taxol in HeLa cells, which was potentiated by pre-treatment with 5 μM curcumin. We provide herein proof of the principle of curcumin pre-treatment augmenting membrane flip-flop, caspase activation, PARP cleavage, and cytochrome *c* release by Taxol. Throughout the present study we did not see a noticeable apoptosis induction by curcumin alone (5 μM), even though we noticed induction of all of the above-mentioned apoptotic parameters by curcumin at a higher concentration of 25 μM (21).

Tumor cells often evade apoptosis by overexpressing anti-apoptotic proteins such as Bcl-2, NF-κB, Akt, etc., which give them a survival advantage (35–37). Some conventional chemotherapeutic drugs in low concentrations cause up-regulation of survival signals, thereby necessitating increment of the effective dose of treatment. We have previously reported the protective effect of NF-κB against apoptosis (18). Taxol activates NF-κB in several cell systems, probably through the principal kinase IKK-β (26). On the contrary, curcumin promotes apoptosis reportedly by interfering in cell survival signaling pathways (17, 21, 38, 39). It inhibits the NF-κB pathway at a step before IκB phosphorylation (17) by inhibiting IKK activity,

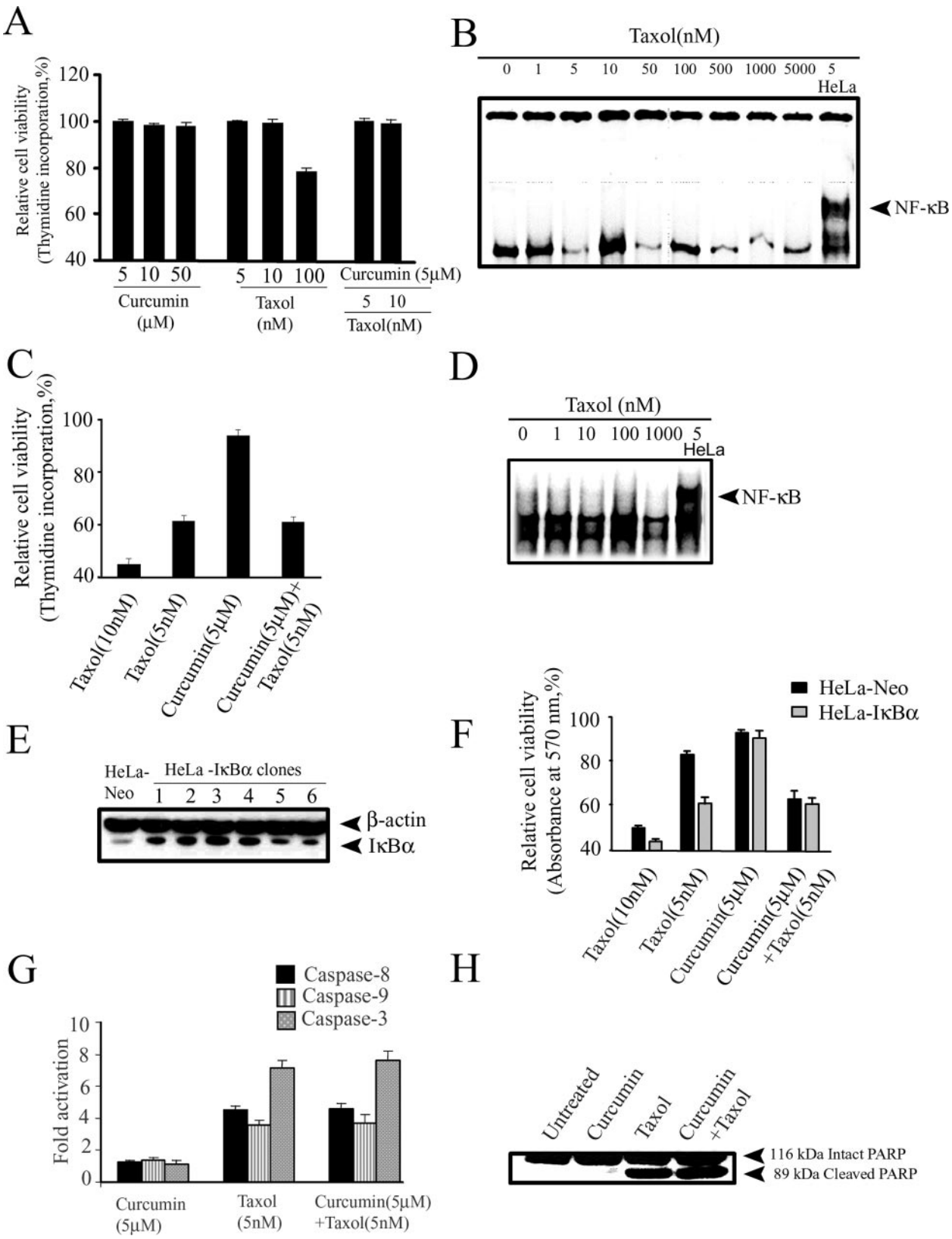

FIG. 5. **Curcumin does not sensitize Taxol-induced apoptosis in normal cervical cells, human 293 cells, or HeLa-IκBα cells.** *A*, normal cervical cells were treated with Taxol and/or curcumin as indicated for 24 h, and [^{3}H]thymidine incorporation was determined as described under "Experimental Procedures." *B*, normal cervical cells were treated with Taxol (0–5000 nM), nuclear extracts were prepared, and EMSA was done as described under "Experimental Procedures." The nuclear extracts of HeLa cells treated with 5 nM Taxol were loaded as a positive control. *C*, human 293 cells were treated with Taxol and/or curcumin as indicated for 24 h, and [^{3}H]thymidine incorporation was determined as described under "Experimental Procedures." *D*, human 293 cells were treated with Taxol (0–1000 nM), nuclear extracts were prepared, and EMSA was done as described under "Experimental Procedures." Nuclear extracts of HeLa cells treated with 5 nM Taxol were loaded as a positive control. *E*, HeLa cells were transfected with pcDNA3 vector or the pcDNA3-IκBα construct using a calcium phosphate transfection kit, and the G418-resistant clones were selected as described under "Experimental Procedures." Cell lysates from the vector-transfected HeLa-Neo cells and the different clones of HeLa-IκBα cells were immunoblotted for IκBα and β-actin. *F*, HeLa-Neo and HeLa-IκBα cells were treated with Taxol and/or curcumin

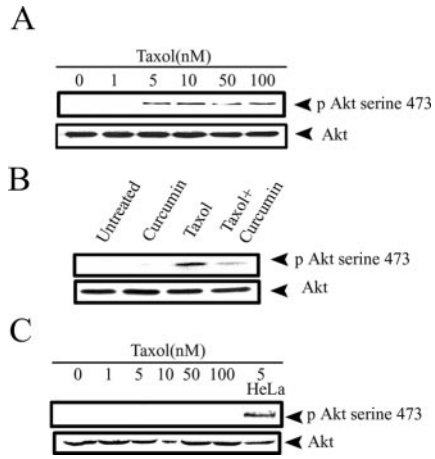

FIG. 6. **Taxol-induced Akt activation is down-regulated by curcumin in HeLa cells, whereas normal cells remain unaffected.** *A*, HeLa cells were treated with Taxol (0–100 nM) for 1 h, and the whole cell lysate was resolved on 10% gel and blotted against phospho-Akt serine 473. *B*, HeLa cells were treated with Taxol (5 nM) and/or curcumin (5 μM) for 1 h, and the whole cell lysate was resolved on a 10% gel and blotted against phospho-Akt serine 473. *C*, normal cervical cells were treated with Taxol (0–100 nM) for 1 h, and whole cell lysate was resolved on a 10% gel and blotted against phospho-Akt serine 473. Cell lysates of HeLa cells treated with 5 nM Taxol were loaded as a positive control.

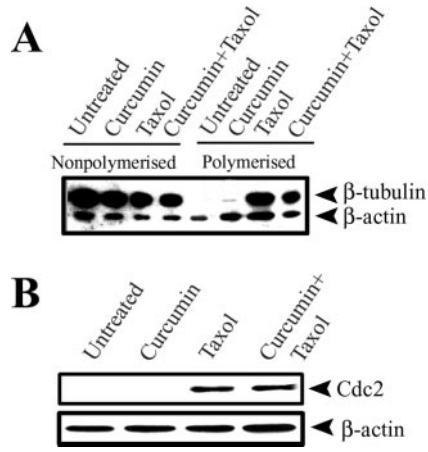

FIG. 7. **Sensitization of Taxol-induced apoptosis by curcumin is independent of tubulin polymerization and cell cycle arrest.** *A*, HeLa cells were treated with Taxol (5 nM) and/or curcumin (5 μM) for 24 h, and polymerized and non-polymerized tubulin were isolated as described under "Experimental Procedures" and resolved on a 10% gel and blotted against anti-β-tubulin and anti-β-actin. *B*, HeLa cells were treated with Taxol (5 nM) and/or curcumin (5 μM) for 1 h, and whole cell lysate was resolved on a 10% gel and blotted against anti-Cdc2.

probably via a NIK-IKK signaling complex (40). Several research groups, including ours, have reported that curcumin inhibits NF-κB activation induced by various agents (17, 18, 41). In the present study we observed that Taxol-induced NF-κB activation in HeLa cells is down-regulated by curcumin, which mechanistically may be contributing to the sensitization of HeLa cells to Taxol-induced apoptosis. Interestingly, Taxol *per se* could not activate NF-κB in normal cervical cells or in human 293 cells in which Taxol down-regulates NF-κB. This highlights the possible reason for the absence of a synergistic effect in these cells. The involvement of this mechanism was further confirmed when we could not find any synergistic effect in HeLa-IκBα cells even though they became sensitive to lower concentrations of Taxol, supporting our earlier studies (23). As we have used a non-toxic concentration of curcumin in this study, an additive effect in terms of cytotoxicity of both the compounds cannot be expected.

Studies published previously describe Taxol as an activator of Akt, a serine/threonine protein kinase and a downstream target of phosphoinositide 3-kinase (42, 43). We observed down-regulation of Taxol-induced Akt activation by curcumin in HeLa cells. Many workers have shown that Akt suppresses apoptosis by activating NF-κB (30, 31). According to a recent report, treatment with LY294002, a specific inhibitor of phosphoinositide 3-kinase, resulted in enhancement of Taxol-induced cytotoxicity followed by suppression of NF-κB transcriptional activity, indicating that NF-κB may be the crucial intermediary step connecting Akt to the intrinsic susceptibility of cancer cells to chemotherapeutic agents (44). Inhibition of Akt by curcumin and its derivatives is also known (38, 45, 46). But whether Taxol-induced up-regulation and curcumin-induced down-regulation of Akt is regulated only through NF-κB is neither clear from these studies nor from ours. However, several studies have shown that Taxol directly activates the survival pathways such as Bcl-2, Akt, Cox-2, mitogen-activated protein kinase, etc. (42, 47, 48) independently of NF-κB. Moreover, Taxol did not induce Akt activation in normal cells, which also may be a contributing factor for the absence of synergistic effect of Taxol and curcumin in these cells. Furthermore, control experiments indicate that curcumin alone did not lead to apoptosis but that it is the pretreatment that caused sensitization, leading to down-regulation of NF-κB and Akt, augmenting apoptosis.

Some retinoids have been reported to have synergistic cytotoxic effects with Taxol independent of tubulin polymerization (49). To see whether curcumin is inducing any cell cycle-specific effects and influencing the tubulin polymerization induced by Taxol in HeLa cells, we examined the level of polymerized and non-polymerized tubulin in cells exposed to curcumin and/or Taxol. Our results indicate that curcumin does not interfere with the tubulin-polymerizing action of Taxol at the investigated concentration (5 μM), although Holy (50) observed disruption of mitotic spindle structure and induction of micronucleation by curcumin at a higher concentration (25 μM). Up-regulation of the cell cycle protein Cdc2 by Taxol plays a critical role in Taxol-induced mitotic arrest (51). We did not observe any noticeable effect of curcumin on Taxol-induced Cdc2 synthesis, even though Jaiswal *et al.* (52) have reported a slight down-regulation of Cdc2 by curcumin at a higher concentration (20 μM). These results lead to the conclusion that the synergistic effect of Taxol and curcumin in inducing apoptosis in cervical cancer cells follows a pathway that is independent of tubulin polymerization and cell cycle arrest, at least at lower concentrations of curcumin.

Whether curcumin can regulate Taxol-induced activation of other antiapoptotic factors is not yet known, although curcumin inhibits some of these factors in several cell systems (38, 39, 53). Further studies are in progress in our laboratory to delineate the role of these proteins in the execution of the synergistic effect by Taxol and curcumin. In conclusion, this study unravels important mechanism-based knowledge with

as indicated and incubated for 24 h, and the cell viability was assessed by the MTT assay as described under "Experimental Procedures." *G*, HeLa-IκBα cells were treated as indicated for 24 h. Total protein (50 μg) was incubated with a caspase-8, -9, or -3 fluorimetric substrate (50 μM) in a total volume of 500 μl of reaction buffer, and the fluorophor released was quantitated spectrofluorimetrically as described under "Experimental Procedures." *H*, HeLa-IκBα cells were treated with Taxol (5 nM) and/or curcumin (5 μM) for 24 h, and the whole cell lysate was resolved on 8% gel and blotted for PARP.

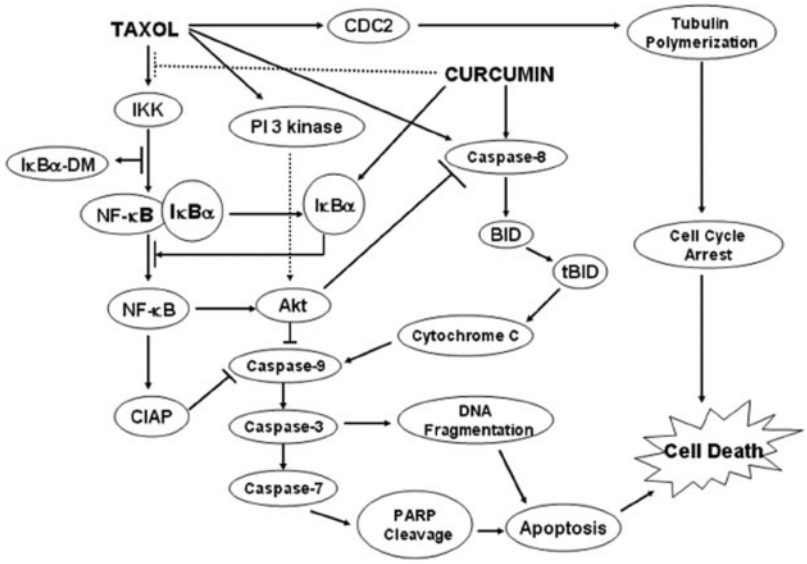

FIG. 8. **Proposed model for the synergistic effect of Taxol and curcumin.** Taxol is a well known inducer of tubulin polymerization that leads to cell cycle arrest and finally cell death. It also activates NFκB and Akt in several cell systems. Curcumin induces apoptosis through a caspase-dependent mitochondrial pathway. The present study postulates that curcumin enhances Taxol-induced apoptosis by down-regulating NFκB and Akt.

potential utility in treating refractory tumors conveying a survival benefit to cancer patients. The potential of such synergism has yet to be realized in the clinic.

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