3 Curcumin: A Potent Candidate to be Evaluated as a Chemosensitizer in Paclitaxel Chemotherapy Against Cervical Cancer

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Abstract

Rigorous efforts in searching for novel chemosensitizers and unraveling their molecular mechanism have identified curcumin as one of the promising candidates. Our earlier report has shown that cervical cancer cells can be sensitized by curcumin to paclitaxel-induced apoptosis through down-regulation of NF-κB and Akt. The present study is an attempt to decipher the signaling pathways regulating the synergism of paclitaxel and curcumin and to determine whether the synergism exists in vivo. The study has clearly proved that Akt and NF-κB function successively in the sequence of paclitaxel-induced signaling events where Akt is up-stream of NF-κB. Inactivation of NF-κB did not affect the activation of Akt and survivin, while that of Akt significantly inhibited NF-κB and completely inhibited up-regulation of survivin. Up-regulation of cyclin-D1, COX-2, XIAP, and c-IAP1 and phosphorylation of MAPKs were completely inhibited on inactivation of NF-κB assigning a key regulatory role to NF-κB in the synergism. While up-regulation of survivin by paclitaxel is regulated by Akt, independent of NF-κB, inactivation of neither Akt nor NF-κB produced any change in Bcl-2 level suggesting a distinct pathway for its action. Mouse cervical multistage squamous cell carcinoma model using 3-methylcholanthrene and a xenograft model of human cervical cancer in NOD-SCID mice using HeLa cells were used to evaluate the synergism

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in vivo. The results suggest that curcumin augments the antitumor action of paclitaxel by down-regulating the activation and down-stream signaling of antiapoptotic factors and survival signals such as NF-κB, Akt, and MAPKs.

Keywords
Paclitaxel • Curcumin • NF-κB • Akt • MAPKs • Synergism

3.1 Introduction

In parallel to the identification of chemotherapeutic agents, a great deal of effort is put in for the identification of phytochemicals known as chemosensitizers, which can enhance the efficacy of conventional chemotherapy. Paclitaxel, isolated from *Taxus brevifolia*, is one of the commonly prescribed chemotherapeutic drugs against a wide spectrum of epithelial cancers. However, administration of lower doses of this drug has been shown to activate various survival signals, leading to chemoresistance (Aggarwal et al. 2005; Mabuchi et al. 2004). Earlier studies, including ours, have shown that curcumin, the active principle of *Curcuma longa*, has synergistic effect with paclitaxel in inducing apoptosis in cancer cells and reducing tumorigenesis (Bava et al. 2005; Aggarwal et al. 2006), and this has been correlated with the down-regulation of various survival signals by curcumin (Aggarwal et al. 2006; Anto et al. 2000; Chen and Tan 1998).

Studies from our laboratory as well as that of others have clearly established that paclitaxel activates NF-κB and Akt in several cell systems, inhibition of which sensitizes cancer cells to paclitaxel (Bava et al. 2005; Mabuchi et al. 2004; Aggarwal et al. 2005). However, no study has yet explored whether these two molecules act independently or interdependently in the synergism of paclitaxel and curcumin. Moreover, the regulatory role of other survival signals such as mitogen-activated protein kinases (MAPKs), AP-1, Bcl-2, COX-2, cyclin D1, and IAPs have never been correlated to this synergism. Hence, this study investigates the major signaling molecules regulating the synergism.

Moreover, apart from a xenograft study using human breast cancer cells (Aggarwal et al. 2005), the combination of paclitaxel and curcumin has not been tested in vivo. Based on our earlier in vitro observations on the synergistic anticancer activity of curcumin and paclitaxel in cervical cancer cells (Bava et al. 2005), we carried out two preclinical studies: one a carcinogen-induced multistage tumor model that better reflects the architectural and cellular complexity of patient-derived tumor specimens and the other a human cervical cancer xenograft model in NOD-SCID mice. We have identified for the first time that activation of NF-κB is associated with 3-methylcholanthrene-induced tumorigenesis in *Swiss albino* mice and the suppression of NF-κB activation and other survival signals by curcumin leads to the augmentation of paclitaxel-induced apoptosis, thereby enhancing the therapeutic outcome of paclitaxel in vivo.

3.2 Materials and Methods

3.2.1 Reagents

All the cell culture reagents were purchased from Life Technologies Inc. Radiolabeled (γ-^{32}P) ATP was purchased from Bhabha Atomic Research Centre, India. The primers used in the PCR studies and the oligos for electrophoretic mobility shift assay (EMSA) were custom synthesized by Genosys, Sigma. Antibodies against β-actin and IκBα and all secondary antibodies were purchased from Sigma. Paclitaxel, U0126, SP600125, SB203580, and LY294002 were purchased from Calbiochem (San Diego, CA). Antibodies against phospho-ERK1/2, phospho-p38,

phospho-Akt, Akt, c-Jun, cyclin D1, PCNA, caspase-3, caspase-7, caspase-8, caspase-9, c-IAP1, phospho-p38, phospho-SAPK/JNK, and survivin- and rhodamine-conjugated secondary antibody were obtained from Cell Signaling Technology (Beverly, MA) and those against Bcl-2, MDR-1, MMP-2, MMP-9, VEGF, p50, RelA, Bcl-2, c-IAP, XIAP, survivin, COX-2, phospho-JNK, ERK-2, JNK-1, p38 DAPI, and Annexin V apoptosis detection kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-XIAP was procured from BD Biosciences (San Diego, CA). All other reagents and antibodies were obtained from Sigma.

3.2.2 Cell Culture

HeLa cells were purchased from the National Centre for Cell Sciences (NCCS), Pune, India. For immunoblotting, FACS, and electrophoretic mobility shift assay (EMSA), 1×10^6 cells were seeded/60 mm plate and for MTT assay 5,000 cells/well were seeded/96-well plate.

3.2.3 Western Blot Analysis

Cell/tissue samples were lysed in ice-cold RIPA buffer with protease inhibitors and Western blotting was performed as described (Bava et al. 2005).

3.2.4 MTT Assay

Proliferative/cytotoxic effect of paclitaxel and/or curcumin was determined by MTT assay as described earlier (Anto et al. 2002).

3.2.5 Drug Treatment

In all combination treatments, curcumin (5 μM) was added 2 h before adding paclitaxel (5 nM). Inhibitors of MAPKs and Akt were added 1 h before the addition of paclitaxel.

3.2.6 Stable Transfection

HeLa cells were stably transfected with IκBα DM-pcDNA3, Akt-DNpcDNA3, and Akt WT-pcDNA3 vector using the calcium phosphate transfection kit (Invitrogen) according to manufacturer's protocol. Stable clones were isolated using G418. Reverse Transcription Polymerase Chain Reaction (RT-PCR) – Total RNA isolated was reverse transcribed to cDNA using MMLV reverse transcriptase. The PCR products were resolved by electrophoresis.

3.2.7 Annexin V-Propidium Iodide Staining

The membrane flip-flop induced by paclitaxel and/or curcumin was assessed as described earlier (Anto et al. 2003) according to manufacturer's protocol.

3.2.8 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extract was isolated from cells after drug treatment and nuclear translocation of NF-κB and AP-1 was detected by EMSA and the specificity of the bands was confirmed by supershift and cold competition as described earlier (Anto et al. 2002; Amato et al. 1998). Nuclear extracts from tissue samples were prepared and EMSA was performed to evaluate DNA-binding activity of NF-κB or AP-1 as described elsewhere (Banerjee et al. 2002; Chaturvedi et al. 2000).

3.2.9 Immunocytochemical Analysis

For immunocytochemical localization of intracellular proteins, the cells were grown on glass cover slips and exposed to various concentrations of the drugs for the desired time. The cells were then washed with PBS, fixed with 4 % paraformaldehyde, permeabilized with 0.4 % Triton X-100 for 20 min at room temperature,

and blocked with 3 % normal goat serum in PBS for 1 h. Antibody (anti-p65) diluted 1:100 in PBS containing 3 % normal goat serum was added to cover the cells and incubated overnight at 4 °C. Unbound antibody was washed off with PBS, and the cells were incubated with 2 μg/ml rhodamine-conjugated secondary antibody for 1 h at room temperature. The unbound secondary antibody was washed off and the cells were covered with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. The cover slips with cells were mounted in glycerol, examined, and photographed under a fluorescence microscope.

3.2.10 Animal Experiments

All animal studies were done in accordance with Institute Animal Ethics Committee-approved protocols. Murphy's string method (Hussain and Rao 1991; Murphy 1961) was followed for tumor induction in the uterine cervices of 6-week-old, virgin female *Swiss albino* mice. Carcinogen (∽600 μg)-impregnated cotton threads or similar cotton threads without carcinogen were inserted into the canal of the uterine cervix of *Swiss albino* mice. Tumor development was checked by palpation thrice a week, starting from the 15th day of tumor induction. After 30 days, the 3-MC-treated animals were randomized into four groups ($n = 12$) to yield even distribution of tumor sizes and control animals into two groups ($n = 12$), and drug administration was started the same day as indicated in Fig. 3.1a. Curcumin was encapsulated into a uni-lamellar liposome formulation containing phosphatidyl choline and cholesterol (Kuttan et al. 1985) and injected intraperitoneally on alternate days at the dose of 25 mg/kg body weight. Paclitaxel was dissolved in Cremophor vehicle (Cremophor EL/ethanol 1:1, diluted 1:4 with PBS) and injected i.p. at 10 mg/kg doses twice weekly. The control animals were injected with empty liposomes and/or Cremophor vehicle. The drug treatment was continued until the animals were sacrificed on the 120th day and the cervical tumors or the normal cervices were excised. Tumor volume was calculated as (length × width^2)/2.

The xenograft studies to evaluate the efficacy of paclitaxel and curcumin, either alone or in combination against human cervical cancer cells in vivo, were carried out in female, 6-week-old, nulliparous non-obese diabetic severe combined immunodeficient (NOD-SCID) mice. HeLa cells (5×10^6) were injected subcutaneously in the flank of a NOD-SCID female mouse, and after 4 weeks, when the xenograft tumor reached a volume of ∽2.5 cm^3, the tumor was excised, cut into small pieces (∽0.5 mm × 0.5 mm), and these tumor fragments were subcutaneously transplanted into the left flank of 24 NOD-SCID mice using an 11-gauge trocar. Six animals each were randomly assigned to four study cohorts including a positive control group where the animals were left untreated, a paclitaxel-alone group, a curcumin-alone group, and the combination group where the animals received both paclitaxel and curcumin. Drug administration was started when the tumors reached the volume of ∽50 mm^3. The route and dose of drug administration were exactly the same as that followed in the carcinogen-induced cervical tumor model in *Swiss albino* mice and continued up to 6 weeks, after which the animals were sacrificed and the tissue samples were collected for further analyses.

3.2.11 Histology and Immunohistochemistry

For histopathologic examination, 5 μm sections cut from the formalin-fixed, paraffin-embedded tissues were deparaffinized, rehydrated, stained with hematoxylin and eosin, and mounted with DPX. Immunolocalization of specific proteins in the tissue sections was done using the Iso-IHC kit (Biogenex) following manufacturer's instructions. Photomicrographs were captured using a Nikon Eclipse E600 microscope.

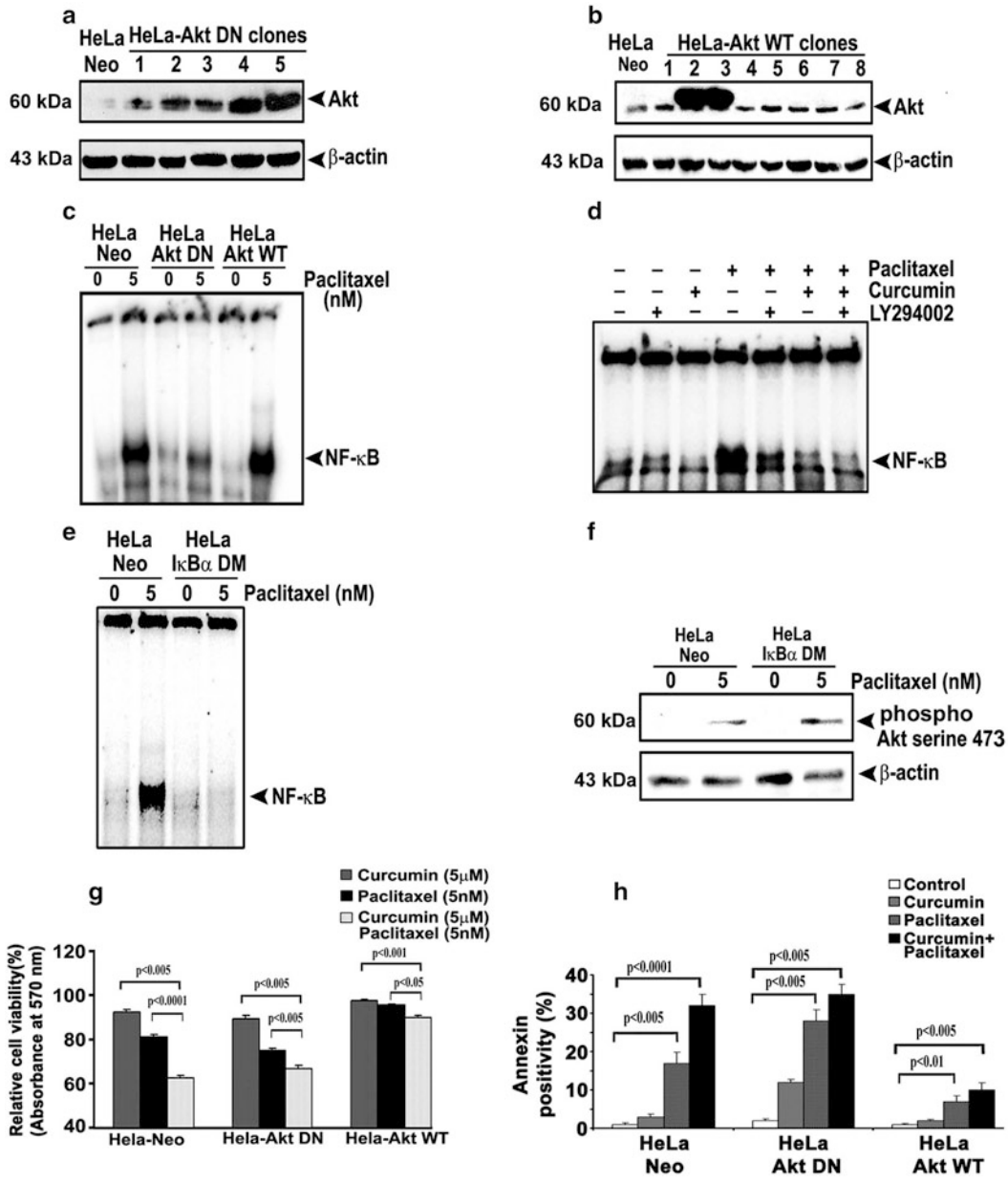


Fig. 3.1 *Dependence of NF-κB and Akt in paclitaxel-mediated signaling events and on the synergistic effect of paclitaxel and curcumin*. (**a**-**b**) HeLa cells were transfected with pcDNA3-vector or pcDNA3-AktDN or pcDNA3-Akt WT construct by calcium phosphate transfection method, G418-resistant clones were selected, and expression level of Akt was detected by Western blot. (**c**) HeLa-Neo, HeLa-AktDN, and HeLa-Akt WT cells were treated with paclitaxel for 1 h. Nuclear extracts were prepared and EMSA was done to detect NF-κB activation in response to paclitaxel. (**d**) HeLa cells pretreated with curcumin or LY294002 (5 μM) were treated with paclitaxel for 1 h, nuclear extracts were prepared, and EMSA was done to study NF-κB status in response to paclitaxel treated with the Akt inhibitor, LY294002, and/or curcumin. (**e**) HeLa-Neo and Hela-IkBα DM cells were treated with paclitaxel for 1 h and nuclear extracts were prepared to do EMSA using γ-32P labeled NF-κB oligonucleotide. (**f**) HeLa-Neo and Hela-IkBα DM cells were treated with paclitaxel for 1 h whole cell lysates were prepared for immunoblotting against p-Akt serine 473. (**g**) HeLa-Neo, HeLa-Akt DN, and HeLa-Akt WT cells were treated with paclitaxel and/or curcumin as indicated, incubated for 72 h and the cell viability was assessed by MTT assay (**h**) HeLa cells were treated with paclitaxel and/or curcumin for 16 h and stained for Annexin V-PI positivity. Annexin V-positive cells in five different fields were counted, and the average was taken

3.2.12 RNA Extraction and Real-Time Q-PCR

Total RNA was extracted from tissue samples using TRIZOL reagent (Invitrogen Corporation). Five microgram of RNA was used for cDNA synthesis using MMLV reverse transcriptase and random hexamers (Promega). Real-time quantitative PCR analysis was performed on an ABI 7500 real-time PCR system (Applied Biosystems) with 2 μL of cDNA in a total reaction volume of 20 μL using the SYBR Green PCR mix (Eurogentec, Belgium). Fold change in expression levels between normal and other treatment groups was calculated relative to the endogenous gene β-actin using the 2-ΔΔCt method (Livak and Schmittgen 2001).

3.2.13 Annexin Staining

To identify phosphatidylserine externalization, cells were stained with FITC-conjugated Annexin V (Santa Cruz Biotechnology) according to the manufacturer's instructions followed by flow cytometry.

3.2.14 Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL) Assay

TUNEL assay was performed to detect apoptosis in formalin-fixed, paraffin-embedded xenograft tumor tissue sections using DeadEnd™ Colorimetric TUNEL System (Promega) following manufacturer's instructions.

3.2.15 Statistical Analysis

Data are presented as mean ± SE of three independent experiments. Two-tailed Student's *t*-test was used for statistical analysis. *P* value <0.05 was considered statistically significant.

3.3 Results

3.3.1 NF-κB and Akt Act Sequentially in the Synergism of Paclitaxel and Curcumin, and Inactivation and Over-expression of Akt in HeLa Cells Lead to Partial Inhibition of the Synergistic Effect of Paclitaxel and Curcumin

Based on our earlier finding that NF-κB and Akt have a critical role in regulating the chemosensitization of HeLa cells by curcumin (Bava et al. 2005), we investigated whether these two important pathways work independently or concurrently in the synergism of paclitaxel and curcumin. To explore this, stable clones of DN Akt, WT Akt, and HeLa-Neo were developed.

Clones with maximum expression were selected for further studies (clone 5 in the case of DNAkt and clone 2 in the case of wild-type Akt) (Fig. 3.1a–b). Interestingly, compared to HeLa-Neo cells, paclitaxel-induced NF-κB DNA binding was much less in HeLa-Akt DN cells, while in HeLa-Akt WT cells it was much higher confirming that Akt mediates paclitaxel-induced NF-κB activation (Fig. 3.1c). It was noteworthy that inactivation of Akt either by transfection of DN Akt or by using the inhibitor LY294002 did not completely inhibit NF-κB DNA-binding ability by paclitaxel (Fig. 3.1d), indicating that paclitaxel-induced NF-κB activation is only partially regulated by Akt. Earlier studies from our laboratory have shown that paclitaxel activates NF-κB and Akt in HeLa cells, while curcumin down-regulates both of these (Bava et al. 2005). The extent of NF-κB inhibition by LY294002 was less than that of curcumin (Fig. 3.1d), suggesting that curcumin acts not only by inhibiting Akt but has other mediators through which it down-regulates NF-κB. Further, the pretreatment with LY294002 and then curcumin, synergistically reduced the

paclitaxel-induced NF-κB activation (Fig. 3.1d). To examine whether NF-κB has any role in regulating the activation of Akt, we used HeLa-IkBα DM cells (Bava et al. 2005) and confirmed the activity of IkBα DM cells by EMSA (Fig. 3.1e). However, in these cells paclitaxel did not produce any difference in the phosphorylation status of Akt (Fig. 3.1f), thereby proving that paclitaxel-induced phosphorylation of Akt is independent of NF-κB.

While pretreatment with curcumin brought down the viability of paclitaxel-treated HeLa-Neo cells from 80 to 62 %, the synergistic effect was significantly reduced ($p < 0.0001$), though not completely inhibited in HeLa-Akt DN cells, even though the inactivation of Akt itself sensitized the cells to paclitaxel and curcumin (Fig. 3.1g). Moreover, the over-expression of WT Akt abolished the effect of paclitaxel and curcumin either alone or in combination (Fig. 3.1g). Over-expression of WT Akt increased the resistance to paclitaxel producing negligible synergistic effect with curcumin (Fig. 3.1g). It was remarkable to note that neither inhibition nor over-expression of Akt completely inhibited the synergistic effect of paclitaxel and curcumin, suggesting that Akt has only a partial role in regulating the synergism.

The apoptotic status of the cells was evaluated by Annexin V/PI staining. The results were in concordance with that of the MTT assay. In HeLa-Neo cells, synergism of paclitaxel and curcumin was evident (Fig. 3.1h). But when Akt was shut down, the synergistic effect was significantly reduced ($p < 0.005$) even though the inactivation of Akt itself sensitized the cells to paclitaxel- and curcumin-induced externalization of phosphatidylserine (Fig. 3.1h) highlighting the role of Akt in paclitaxel-induced signaling. Supporting this observation, Akt over-expression significantly inhibited paclitaxel-induced apoptosis ($p < 0.01$), which was not appreciably affected by curcumin (Fig. 3.1h).

3.3.2 COX-2 and Cyclin D1 Play Regulatory Roles in the Synergistic Effect of Paclitaxel and Curcumin in an NF-κB-Dependent Manner, and Among the IAPs that Play Regulatory Roles in the Synergistic Effect of Paclitaxel and Curcumin, Survivin Alone is Independent of NF-κB

As reported earlier (Aggarwal et al. 2005), we observed that paclitaxel induced the expression of COX-2, which is an immediate early gene regulated by NF-κB. In our study, we observed a down-regulation of paclitaxel-induced COX-2 expression by curcumin in HeLa cells both in the protein level (data not shown) as well as in the RNA level (Fig. 3.2a). We observed a complete inhibition of COX-2 expression in HeLa-IκBα DM cells (Fig. 3.2a) and a partial inhibition of the same in HeLa-Akt DN cells (Fig. 3.2a), whereas in the HeLa-Akt WT cells, COX-2 expression was found to be higher than that of HeLa-Neo cells (Fig. 3.2a). Collectively, these data indicate that paclitaxel-induced COX-2 expression is completely regulated by NF-κB and partly by Akt. It is also evident that paclitaxel can induce NF-κB independent of Akt confirming the hypothesis that NF-κB is the key regulator of paclitaxel-induced signaling events.

As several studies have reported that NF-κB is the key regulator of cyclin D1, we investigated whether cyclin D1 has any role in the synergism of paclitaxel and curcumin. We observed a significant down-regulation of paclitaxel-induced cyclin D1 expression by curcumin (Fig. 3.2b), and as expected, inactivation of NF-κB by transfection of IκBα DM completely inhibited the up-regulation of cyclin D1 expression by paclitaxel (Fig. 3.2b). However, the basal expression of cyclin D1 was significantly higher in IκBα DM cells compared to control.

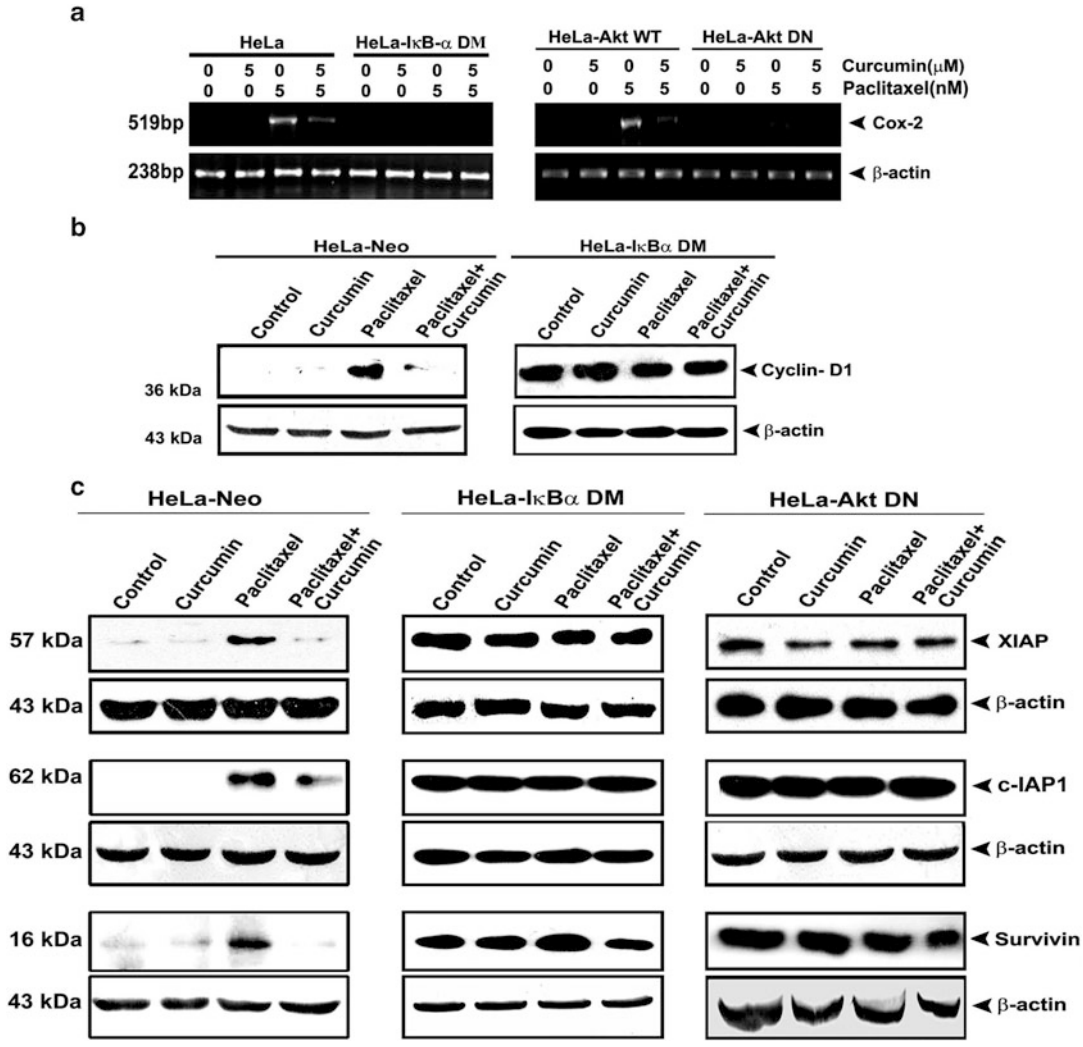


Fig. 3.2 *Regulatory role of NF-κB and Akt in paclitaxel-induced expression of COX-2, cyclin D1, and IAPs*. (**a**) HeLa, HeLa-IkBα DM, HeLa-Akt DN, and HeLa-Akt WT cells were treated with paclitaxel and/or curcumin for 24 h and total RNA was isolated. The RT-PCR products were then resolved by agarose gel electrophoresis. β-actin was used as the internal control. (**b**) HeLa and HeLa-IkBα DM cells were treated with paclitaxel and/or curcumin for 24 h and whole cell lysate was immunoblotted against anti-cyclin D1. β-actin was used as the internal control. (**c**) HeLa-Neo, HeLa-IkBα DM, and HeLa-Akt DN cells were treated with paclitaxel and/or curcumin for 24 h and the whole cell lysate was immunoblotted using antibodies against XIAP, c-IAP1, and survivin and detected by ECL. β-actin was used as the loading control

IAPs are a family of proteins that function as ubiquitin ligases and promote the degradation of caspases and are considered to be regulated by NF-κB. We observed that paclitaxel induced the up-regulation of XIAP, c-IAP1, and survivin in HeLa cells which are significantly down-regulated by curcumin (Fig. 3.2c). Another interesting observation was that the up-regulation of XIAP and c-IAP1 by paclitaxel was completely inhibited when NF-κB was shut down, while that of survivin remained unaffected (Fig. 3.2c). Though survivin is generally considered as an NF-κB-dependent gene, recent reports have clearly demonstrated regulation of survivin by PI3K/Akt pathway (Zhao et al. 2010). This made us to check the status of all the IAPs in

response to paclitaxel in HeLa-Akt DN cells. Supporting the observation of Zhao et al. (2010), we observed a complete inhibition in the up-regulation of survivin as well as that of XIAP and c-IAP1 (Fig. 3.2c) indicating that paclitaxel-induced up-regulation of survivin is regulated by Akt in an NF-κB-independent manner. However, as in the case of cyclin D1, the basal expressions of all the IAPs were significantly higher in IκBα DM cells as well as Akt DN cells compared to control.

3.3.3 MAPKs and AP-1 Contribute to the Synergism of Curcumin and Paclitaxel in an NF-κB-Dependent Manner, While Bcl-2 Regulates the Synergism, Independent of NF-κB and Akt

We also investigated the involvement of MAPKs, another set of survival signals induced by lower concentrations of paclitaxel. Our study revealed a transient phosphorylation of ERK1/2, JNK, and p38 that started within 5 min of the paclitaxel treatment, peaked at 15 min, and then receded by 30 min (Fig. 3.3a). Interestingly, curcumin significantly reduced this phosphorylation of MAPKs, signifying a possible role for MAPKs in regulating the synergism (Fig. 3.3b). Further, we also evaluated the role of NF-κB in paclitaxel-induced phosphorylation of MAPKs. Interestingly, we found that inhibition of NF-κB pathway by IκBα-DM transfection completely inhibited the phosphorylation of all the three MAPKs by paclitaxel (Fig. 3.3c) clearly indicating that NF-κB is regulating the phosphorylation of MAPK pathway by paclitaxel.

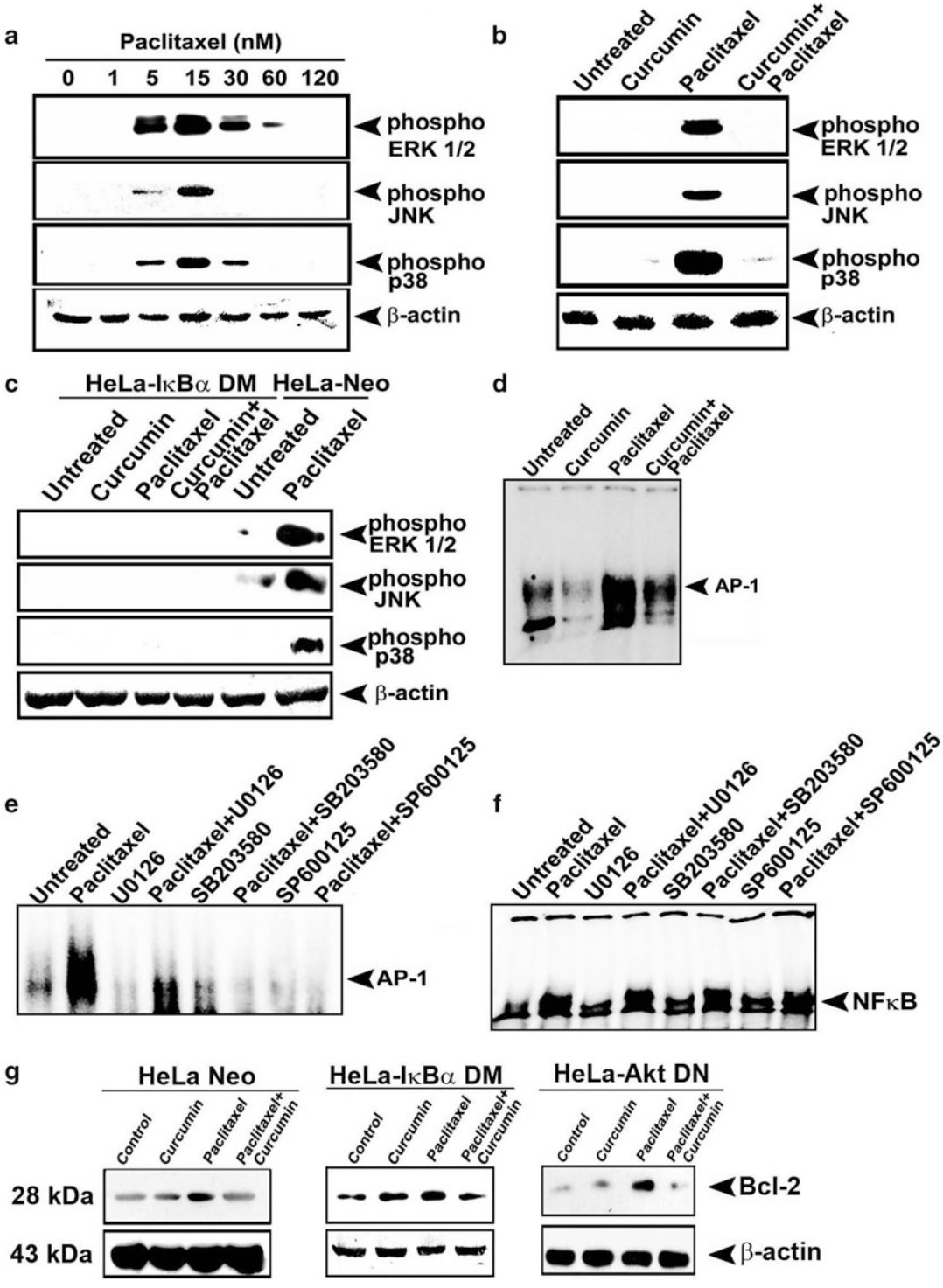
AP-1 transcription factors are complexes of DNA-binding proteins made up of homodimers of Jun family members or heterodimers of Jun and Fos family members. We observed a dose-dependent increase in the DNA-binding activity of AP-1 in response to paclitaxel in HeLa cells (data not shown). It was also found that pretreatment with curcumin significantly down-regulated the AP-1 activation induced by paclitaxel (Fig. 3.3d). We also examined whether ERK1/2, JNK, or p38 phosphorylation in response to paclitaxel contributes to the activation of AP-1 by treating HeLa cells with paclitaxel and/or U0126 (10 μM), SP600125 (50 μM), and SB203580 (40 μM) which are inhibitors of ERK1/2, JNK, and p38, respectively. As expected the inhibitors of all the MAPKs especially that of p38 and JNK significantly inhibited the AP-1 DNA-binding activity induced by the paclitaxel, suggesting the involvement of ERK1/2, JNK, and p38 in the activation of AP-1 induced by paclitaxel (Fig. 3.3e). Hence, the study demonstrates that the partial down-regulation of the AP-1 DNA binding by curcumin may be due to the inhibition of these MAPKs which acts up-stream of AP-1.

We also explored whether ERK1/2, p38, and JNK can regulate the NF-κB signaling events induced by paclitaxel. It was noteworthy that none of the inhibitors produced a significant downregulation to paclitaxel-induced NF-κB activation (Fig. 3.3f), strongly correlating with our previous observation that NF-κB is up-stream of MAPK pathway in the regulation of paclitaxel signaling.

Deregulated expression of the antiapoptotic protein Bcl-2 plays an important role in cell death as well as in the nonsensitiveness of cancer cells to chemotherapy. Several studies have demonstrated that NF-κB and Akt have regulatory roles in endogenous Bcl-2 protein expression. We observed a dose-dependent up-regulation of Bcl-2 protein by paclitaxel in HeLa cells (data not shown), which was significantly abrogated by curcumin (Fig. 3.3g). However, we observed up-regulation of Bcl-2 protein in HeLa-IκBα DM as well as HeLa-Akt DN cells, indicating that paclitaxel is inducing Bcl-2 in HeLa cells independent of NF-κB as well as Akt (Fig. 3.3g).

3.3.4 Curcumin Potentiates the Antitumor Effects of Paclitaxel in 3-MC-Induced Cervical Tumors in Mice

The schematic representation of various groups employed in the study is depicted in Fig. 3.4A.


a
Paclitaxel (nM)
0 1 5 15 30 60 120
phospho ERK 1/2
phospho JNK
phospho p38
β-actin
b
Untreated
Curcumin
Paclitaxel
Curcumin+ Paclitaxel
phospho ERK 1/2
phospho JNK
phospho p38
β-actin
c
HeLa-IκBα DM
HeLa-Neo
Untreated
Curcumin
Paclitaxel
Curcumin+ Paclitaxel
Untreated
Paclitaxel
phospho ERK 1/2
phospho JNK
phospho p38
β-actin
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e
Untreated
Paclitaxel
U0126
Paclitaxel+U0126
SB203580
Paclitaxel+SB203580
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Paclitaxel+U0126
SB203580
Paclitaxel+SB203580
SP600125
Paclitaxel+SP600125
NFκB
g
HeLa Neo
HeLa-IκBα DM
HeLa-Akt DN
Control
Curcumin
Paclitaxel
Paclitaxel+ Curcumin
28 kDa
43 kDa
Bcl-2
β-actin

Following tumor induction with 3-MC, palpable tumors began to appear within 20–25 days. Tumors developed in the group I and curcumin-alone-treated group III animals continued to grow during the span of the experiment and reached the maximum volume up to 5,500 mm^3, whereas >60 % of the tumors in the paclitaxel-alone group and >85 % of the combination-treated tumors did not grow beyond a volume of ∽3,200 mm^3. The final tumor incidence percentage (Fig. 3.4B) and the mean tumor volume (Fig. 3.4C) among different groups were determined after sacrificing the animals on day 120. In group I, 66 % of the animals treated with 3-MC (positive control) developed tumors with a mean tumor volume of 1,613 mm^3. In group II and group III, where the animals received either paclitaxel or curcumin, respectively, the tumor incidence percentages were 44 and 60, and the mean tumor volume among these groups were 965 and 1,294 mm^3, respectively. Mice of group IV, which received both paclitaxel and curcumin, showed a tumor incidence of only 24 %, and the mean tumor volume was reduced to 656 mm^3. None of the animals from group V and group VI (negative controls) show any signs of tumor development during the span of the study. Moreover, 3-MC treatment or administration of paclitaxel and/or curcumin had no adverse effects on the tumor-bearing animals or normal controls (group VI) as assessed by body weight observation and liver function tests (data not shown). Microscopic analysis of the formalin-fixed paraffin-embedded tissue sections showed different grades of squamous cell carcinoma, and this observation was verified by a histopathologist. Representative photomicrographs of H&E-stained tissue sections showing 3-MC-induced multistage squamous cell carcinogenesis are given in Fig. 3.4D.

3.3.5 Curcumin Inhibits Constitutively Active and Paclitaxel-Induced NF-κB in 3-MC-Induced Cervical Tumors and Down-regulates the Transcription of NF-κB Target Genes

Since most of the carcinogens and infectious agents linked with cancer have been shown to activate the NF-κB pathway, we investigated whether NF-κB is associated with 3-MC-induced cervical tumorigenesis. While constitutive activation of NF-κB and higher levels of nuclear DNA-binding activity were observed in 3-MC-induced cervical tumors and 3-MC+ paclitaxel-treated animals, administration of curcumin abolished the constitutively activated as well as paclitaxel-induced NF-κB. Incubation of DNA-protein complexes with p65 antibody resulted in the supershift of active band (Fig. 3.5A). To further confirm the activation of NF-κB by 3-MC and paclitaxel, we examined the NF-κB DNA-binding activity in 10 tissue samples each from the six

Fig. 3.3 *Involvement of MAPK Pathway and Bcl-2 in regulating the synergism of paclitaxel and curcumin*. (**a**) Kinetics of paclitaxel-induced phosphorylation of MAPKs-ERK1/2, JNK, and p38 in HeLa cells were studied by treating them with paclitaxel for different time intervals (0–120 min). The whole cell lysate was immunoblotted against phospho-ERK1/2, phospho-JNK, and phospho-p38 antibodies and detected by ECL. β-actin was used as the loading control. (**b**) The effect of curcumin on phosphorylation of MAPKs was detected by pretreating HeLa cells with curcumin and then with paclitaxel for 15 min and immunoblotting the whole cell lysate against phospho-ERK1/2, phospho-JNK, and phospho-p38 antibodies. (**c**) HeLa-IkBα DM cells were treated with paclitaxel and/or curcumin for 15 min and the whole cell lysate was immunoblotted against phospho-ERK1/2, phospho-JNK, and phospho-p38 antibodies. HeLa-Neo cells treated with and without paclitaxel were used as controls. (**d**) HeLa cells were pretreated with curcumin and then with paclitaxel (5nM) for 30 min, nuclear extracts were prepared, and EMSA was done to study the effect of curcumin on activation of AP-1. The arrowhead shown indicates the position of the active DNA-binding complex of AP-1. (**e–f**) HeLa cells pretreated with U0126 (10 μM), SP600125 (50 μM), or SB203580 (40 μM) were treated with paclitaxel for 30 min. Nuclear extracts were prepared and EMSA was done using radiolabeled AP-1 primer to detect the AP-1 status and radiolabeled NF-κB primer to detect the NF-κB status. (**g**) HeLa-Neo, HeLa-IkBα DM, and HeLa-Akt DN cells were treated with paclitaxel and/or curcumin for 24 h and the whole cell lysate was immunoblotted against Bcl-2 antibody

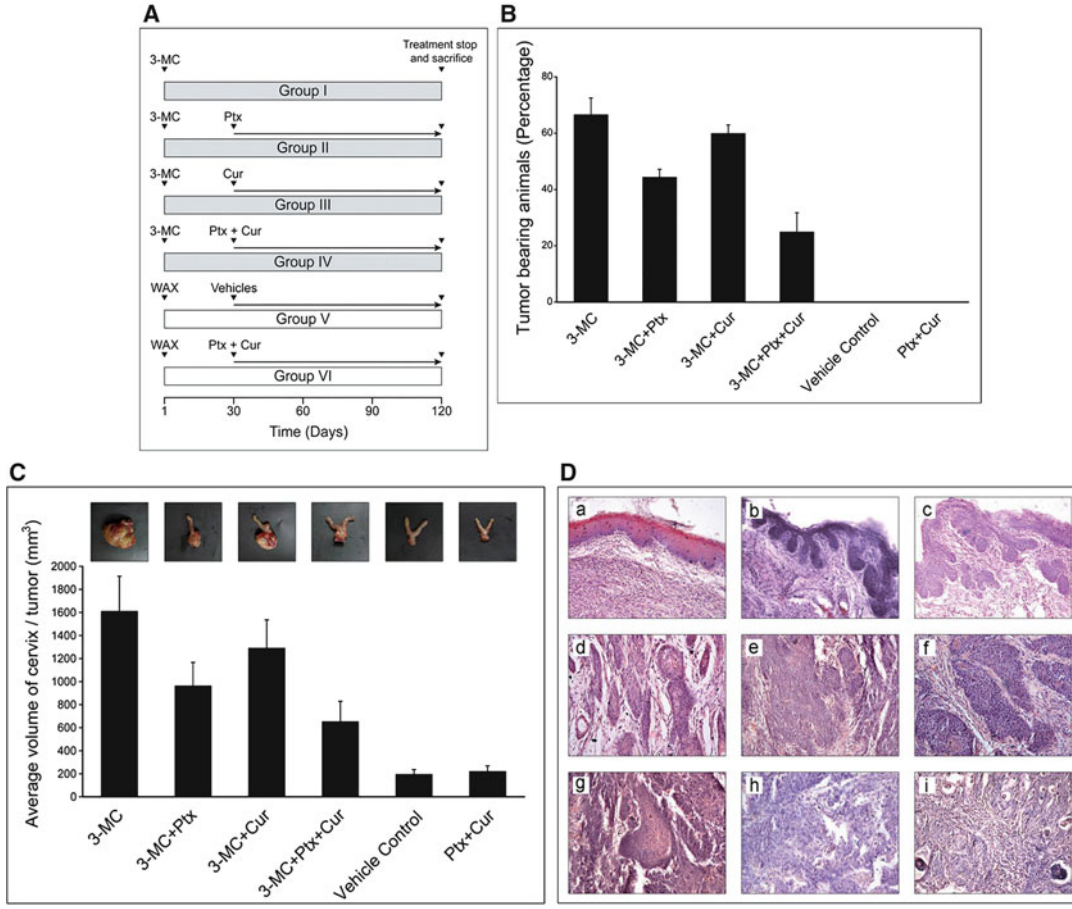


Fig. 3.4 *Combination of paclitaxel and curcumin reduces tumor incidence and tumor volume in cervical tumor model*. (**A**) Schematic representation of experimental protocols for evaluating the anticancer properties of paclitaxel and curcumin against 3-MC-induced carcinogenesis in *Swiss albino* mice. (**B**) Effect of paclitaxel and curcumin, alone or in combination, on 3-MC-induced cervical tumors. The tumors were distinguished from normal cervices based on size as well as histopathology, and the percentage of tumor-bearing animals per group is shown. Data represents three sets of experiments carried out independently. (**C**) The mean tumor volume and representative photographs of cervix samples from different experimental groups are shown. (**D**) Histological changes induced by 3-MC treatment in the cervical epithelium of *Swiss albino* mice. Formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin. The representative photomicrographs show progressive stages of squamous cell carcinoma. The panels depict (**a**) normal epithelium, (**b**–**c**) squamous hyperplasia, (**d**–**f**) dysplastic epithelium, and (**g**–**i**) SCC with characteristic keratin pearl formation

experimental groups. But in a few (<10 %) of the tumor-bearing mice, curcumin was unable to inhibit the NF-κB activity, probably because of the need for a higher dose or any genetic variability among animals (Fig. 3.5B). Immunohistochemical analysis of tissue samples harvested from 3-MC-alone and 3-MC+ paclitaxel-treated animals revealed increased expression and prominent nuclear localization of the p65 subunit of NF-κB in comparison with normal cervical epithelium (Fig. 3.5C). Consistent with the results obtained from EMSA, curcumin could down-regulate the expression and nuclear localization of p65 in tissue samples derived from curcumin and curcumin + paclitaxel-treated mice. Having established the association between NF-κB activation and 3-MC-induced tumor progression, we analyzed the functional status of NF-κB activation by

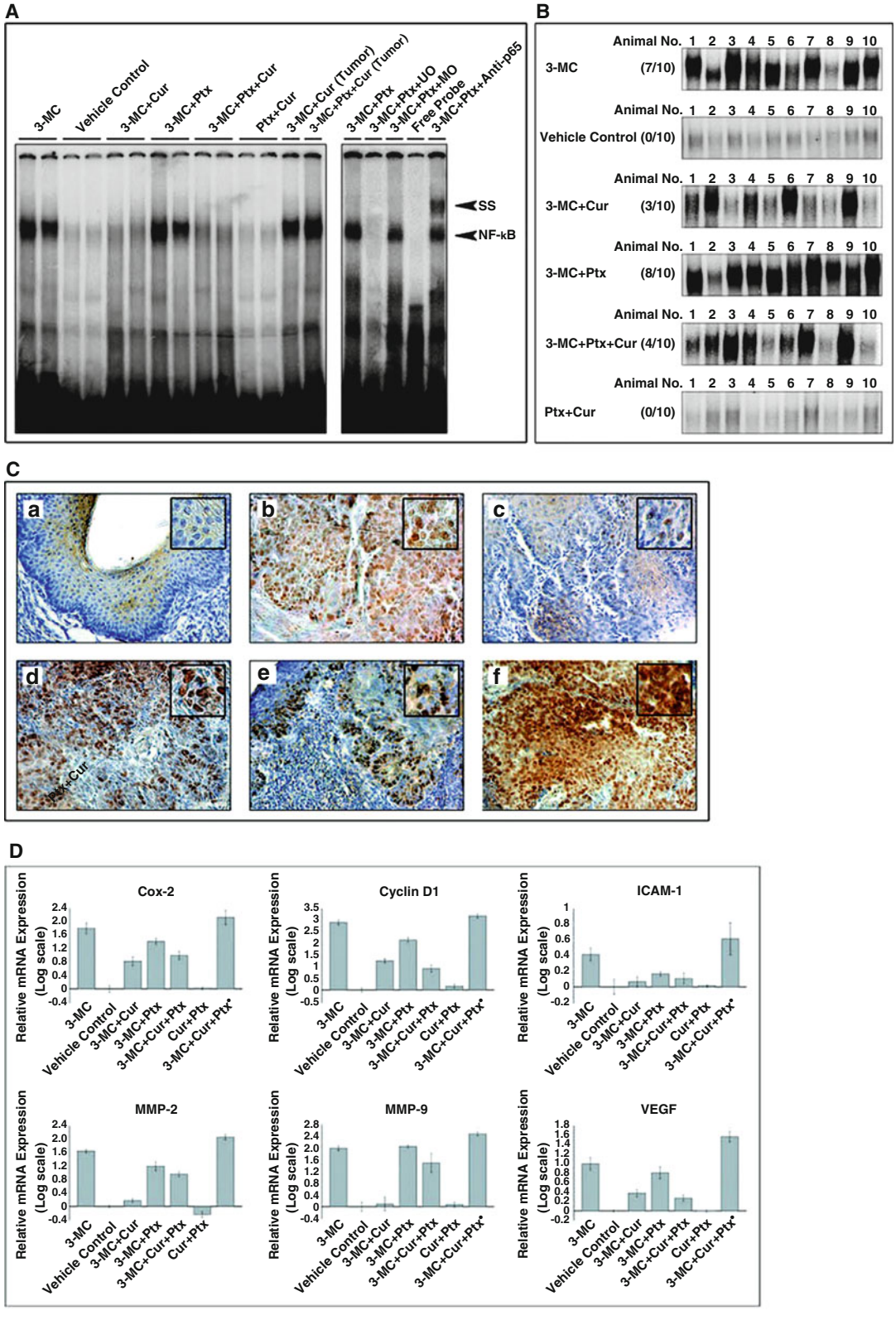
assessing the mRNA expression levels of NF-κB target genes such as COX-2, cyclin D1, ICAM-1, VEGF, MMP-2, and MMP-9 by real-time quantitative RT-PCR. Tissue samples from groups I and II showed high mRNA expression levels of these genes, whereas low levels were detected in the tumor samples collected from animals treated with curcumin either alone or in combination with paclitaxel (Fig. 3.5D). Curcumin in combination with paclitaxel could not down-regulate the transactivation of these target genes in a few of the highly aggressive cervical tumors (indicated with "*").

3.3.6 Curcumin Represses NF-κB-Dependent Gene Products, Potentiates the Antiproliferative Effect of Paclitaxel, and Enhances Paclitaxel-Induced Caspase Activation

Western blot analyses revealed that paclitaxel either further induced or did not down-regulate 3-MC-induced activity of various NF-κB-regulated gene products COX-2, cyclin D1, MMP-2, MMP-9, and VEGF that are directly involved in tumor cell proliferation, angiogenesis, and metastasis (Fig. 3.6A). Immunohistochemical analysis for the cell proliferation marker PCNA in the tissue samples revealed that curcumin significantly enhanced paclitaxel-induced inhibition of proliferation as evidenced by reduced PCNA immunoreactivity compared with the positive control group (Fig. 3.6B). Diverse antiapoptotic proteins such as Bcl-2, c-IAP1, survivin, and XIAP which are reported to be transactivated by NF-κB were also found to be up-regulated in most of the tumor samples collected from 3-MC-alone and 3-MC+paclitaxel groups (Fig. 3.6C). The expression levels of these molecules were significantly down-regulated by curcumin. We also observed the cleavage and activation of procaspases 8, 9, 3, and 7 in paclitaxel-treated cervical tumors of mice, which was significantly up-regulated when curcumin was co-administered (Fig. 3.6D).

3.3.7 Curcumin-Mediated Augmentation of Antitumor Effects of Paclitaxel in 3-MC-Induced Cervical Tumors Involves the Down-regulation of MDR1, Akt, MAPK, and AP-1 Pathways

3-MC-induced tumors as well as those treated with paclitaxel exhibited a strong over-expression of MDR1, which was significantly down-regulated by curcumin (Fig. 3.7a). Immunoblotting with a phospho-specific antibody against Akt at Ser473 points toward the hyperactivation of Akt by phosphorylation in 3-MC-induced tumors compared to normal controls. More interestingly, the animals that were administered with curcumin either alone or in combination with paclitaxel showed relatively low levels of phospho-Akt in comparison with the carcinogen-alone- or paclitaxel-treated groups. But, over-expression and hyperactivation of Akt was observed in well-developed tumors from curcumin-treated animals (Fig. 3.7b). We also found hyperphosphorylation at Ser136 of Bad, a well-known direct target of phosphorylation and inactivation by Akt, indicating a possible antiapoptotic effect of activated Akt. The expression and activation status of the three important MAP kinases – extracellular signal-regulated protein kinase (ERK), c-Jun-NH2 kinase (JNK), and p38 – were analyzed by Western blotting, and extensively high phosphorylation of ERK-1/2 and JNK was observed in most of the 3-MC-induced tumor samples when compared to normal controls. Paclitaxel treatment alone did not alter the phosphorylation levels of ERK-1/2 and JNK in the tumor tissues, whereas administration of curcumin, alone or in combination with paclitaxel, inhibited the phosphorylation of these molecules significantly. On the other hand, the phosphorylation and expression status of p38 was not found to significantly vary among the experimental groups, indicating a lesser role of p38 in mediating the carcinogenic action of 3-MC as well as in the chemotherapeutic action of paclitaxel and curcumin (Fig. 3.7c) though we observed a


A
3-MC
Vehicle Control
3-MC+Cur
3-MC+Ptx
3-MC+Ptx+Cur
Ptx+Cur
3-MC+Cur (Tumor)
3-MC+Ptx+Cur (Tumor)
3-MC+Ptx
3-MC+Ptx+UO
3-MC+Ptx+MO
Free Probe
3-MC+Ptx+Anti-p65
SS
NF-kB
B
Animal No. 1 2 3 4 5 6 7 8 9 10
3-MC (7/10)
Vehicle Control (0/10)
3-MC+Cur (3/10)
3-MC+Ptx (8/10)
3-MC+Ptx+Cur (4/10)
Ptx+Cur (0/10)
C
a
b
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f
Ptx+Cur
D
Relative mRNA Expression (Log scale)
Cox-2
Cyclin D1
ICAM-1
MMP-2
MMP-9
VEGF
3-MC
Vehicle Control
3-MC+Cur
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downregulation of paclitaxel-induced activation of p38 by curcumin in the human cervical cancer cell line, HeLa (Fig. 3.3b). The expression of phospho-c-Jun, a proto-oncogene which is the direct target of both JNK and ERK-1/2, and the central component of all activator protein-1 (AP-1) complexes, was found elevated in those samples with high levels of phospho-JNK and phospho-ERK1/2 (Fig. 3.7c). Moreover, the significantly higher levels of nuclear DNA-binding activity of AP-1 detected in 3-MC-induced tumor samples were suppressed by curcumin alone or in combination with paclitaxel (Fig. 3.7d).

3.3.8 Combined Treatment of Paclitaxel and Curcumin Decreases the Tumor Volume, Down-regulates NF-κB, and Enhances Apoptosis in Human Cervical Cancer Xenografts Compared to Individual Treatments, in NOD-SCID Mice

The mean tumor volume at the time of necropsy in the untreated control group was 2.66 cm^3, and administration of curcumin alone did not show any significant effect on the xenograft tumor growth, and the mean tumor volume was 2.47 cm^3. Paclitaxel treatment alone suppressed the growth of HeLa xenograft tumors and reduced the final mean tumor volume to 1.83 cm^3, whereas curcumin considerably enhanced the antitumor activity of paclitaxel, and the remarkable synergistic antitumor efficacy of these compounds resulted in a mean tumor volume of 1.32 cm^3 at the time of necropsy (Fig. 3.8a, b). Nuclear extracts prepared using tumor samples were used for electrophoretic mobility shift assay, and higher NF-κB activation levels were observed in the untreated tumors while paclitaxel treatment resulted in a further up-regulation of NF-κB activation levels. On the contrary, curcumin treatment either alone or in combination with paclitaxel significantly down-regulated the nuclear DNA-binding activity of NF-κB, in vivo (Fig. 3.8c). These results confirmed that suppression of paclitaxel-induced NF-κB activation by curcumin is an important molecular mechanism by which this compound sensitizes HeLa xenograft tumors in NOD-SCID mice to paclitaxel therapy. The in vivo apoptotic effects of curcumin and/or paclitaxel in ectopically implanted human cervical cancer xenografts in NOD-SCID mice were assessed by TUNEL staining. As shown in Fig. 3.8d, tumor sections from control and curcumin-alone-treated groups did not show any considerable positivity for apoptotic cells, while paclitaxel-treated samples showed substantial amount of TUNEL staining. More interestingly, in tumor samples collected from animals treated with both the agents, curcumin could significantly enhance the apoptotic effects of paclitaxel in comparison with the individual treatment.

3.4 Discussion

Tumor cells often escape apoptosis by overexpressing antiapoptotic proteins which give them survival advantage (Gagnon et al. 2008). Some conventional chemotherapeutic drugs in

Fig. 3.5 *Curcumin reduces the activation of NF-κB and its downstream targets induced by paclitaxel and 3-MC.* (**A**) Individual and combined effects of paclitaxel and curcumin on NF-κB activation in 3-MC-induced cervical tumors and normal controls are shown. Equal concentrations of nuclear extracts prepared from tissue samples were analyzed by EMSA. Supershift analysis using anti-p65 antibody is also done as described in "Materials and Methods." (**B**) EMSA results showing NF-κB activation status in ten different animals from each of the six experimental groups. (**C**) Immunohistochemical localization of the p65 subunit of NF-κB in the tissue sections correlates with EMSA results. The different panels indicate (**a**) the cytoplasmic localization of p65 in normal cervical epithelium, increased nuclear localization of p65 in (**b**) 3-MC-alone-treated (**c**) or 3-MC+paclitaxel-treated groups, inhibition of nuclear translocation of p65 in (**d**) 3-MC+curcumin or in (**e**) 3-MC+paclitaxel+curcumin, and (**f**) a highly aggressive tumor from paclitaxel+curcumin group shows strong nuclear positivity for p65. (**D**) Effect of paclitaxel and/or curcumin on transactivation of NF-κB-dependent genes in 3MC-induced cervical tumors and control tissues. Relative mRNA expression levels of target genes were determined by quantitative RT-PCR

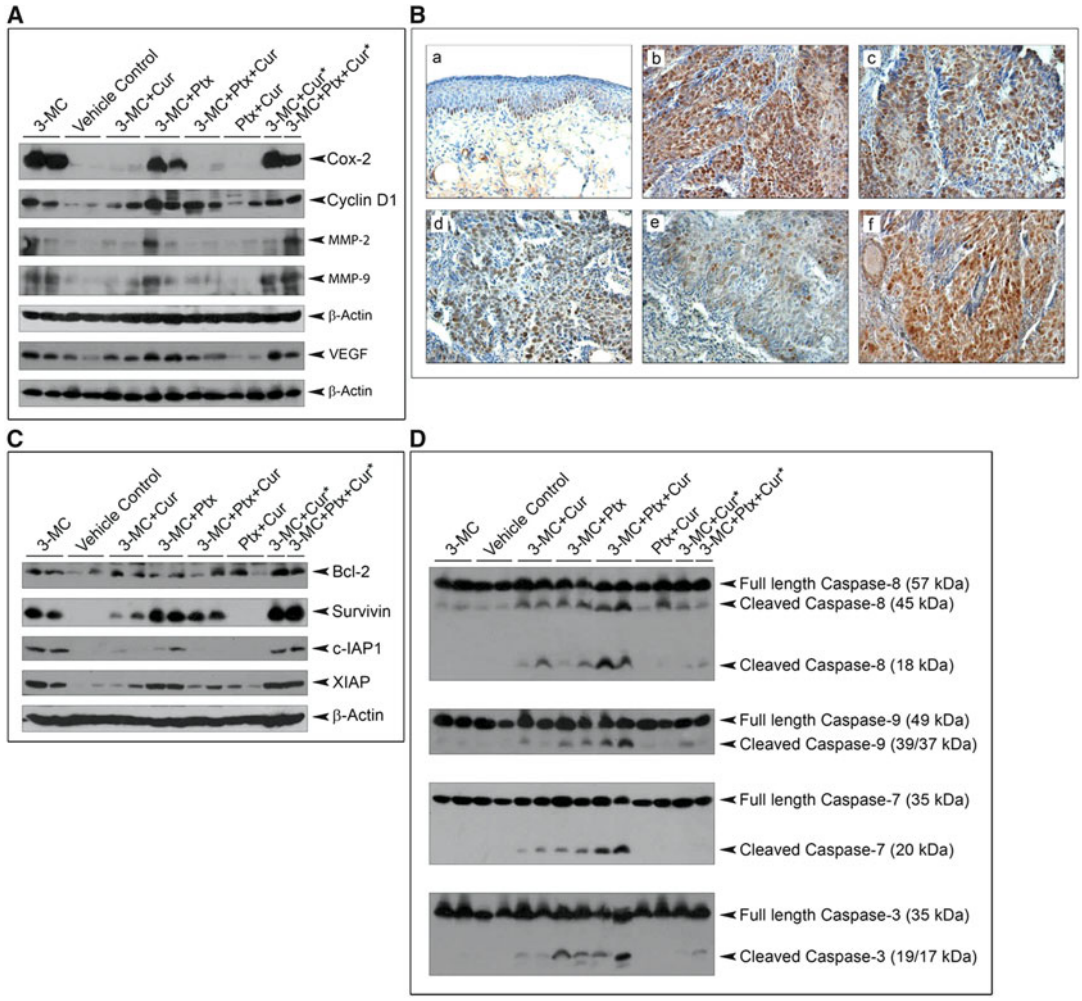

Fig. 3.6 *Curcumin inhibits NF-κB-dependent genes and the proliferative effect of paclitaxel and augments paclitaxel-induced caspase activation*. (**A**) Western blot analysis for different NF-κB-regulated gene products in 3-MC-induced tumors or control tissue samples harvested from mice of different treatment groups. At least six samples per group were analyzed, and representative data of two randomly selected animals from each group is shown. (**B**) Immunohistochemical analysis on tissue sections using PCNA antibody. Representative staining patterns in mouse cervical epithelium of (**a**) normal control, (**b**) 3-MC-treated, (**c**) 3-MC+Cur-treated, (**d**) 3-MC+Ptx-treated, (**e**) 3-MC+Cur+Ptx-treated, and (**f**) a very aggressive tumor from 3-MC+Cur+Ptx-treated group are shown. (**C**) Effect of curcumin and/or paclitaxel on the expression levels of various NF-κB-regulated antiapoptotic proteins in 3-MC-induced cervical tumors was detected by Western blotting. (**D**) Western blots showing curcumin-mediated enhancement of paclitaxel-induced caspase activation in the tumor tissue samples

low concentrations cause up-regulation of survival signals, thereby necessitating increment of the effective dose of treatment. Several studies including ours have shown that paclitaxel activates NF-κB and Akt, which have critical roles in regulating cell survival, proliferation, invasion, and metastasis (Huang and Fan 2002; Bava et al. 2005). On the contrary, curcumin, the natural polyphenolic compound, promotes apoptosis by interfering in various cell survival signaling pathways including NF-κB and Akt (Anto et al. 2002; Aggarwal et al. 2006). Our earlier findings (Bava et al. 2005) have clearly shown that paclitaxel-induced activation of NF-κB and

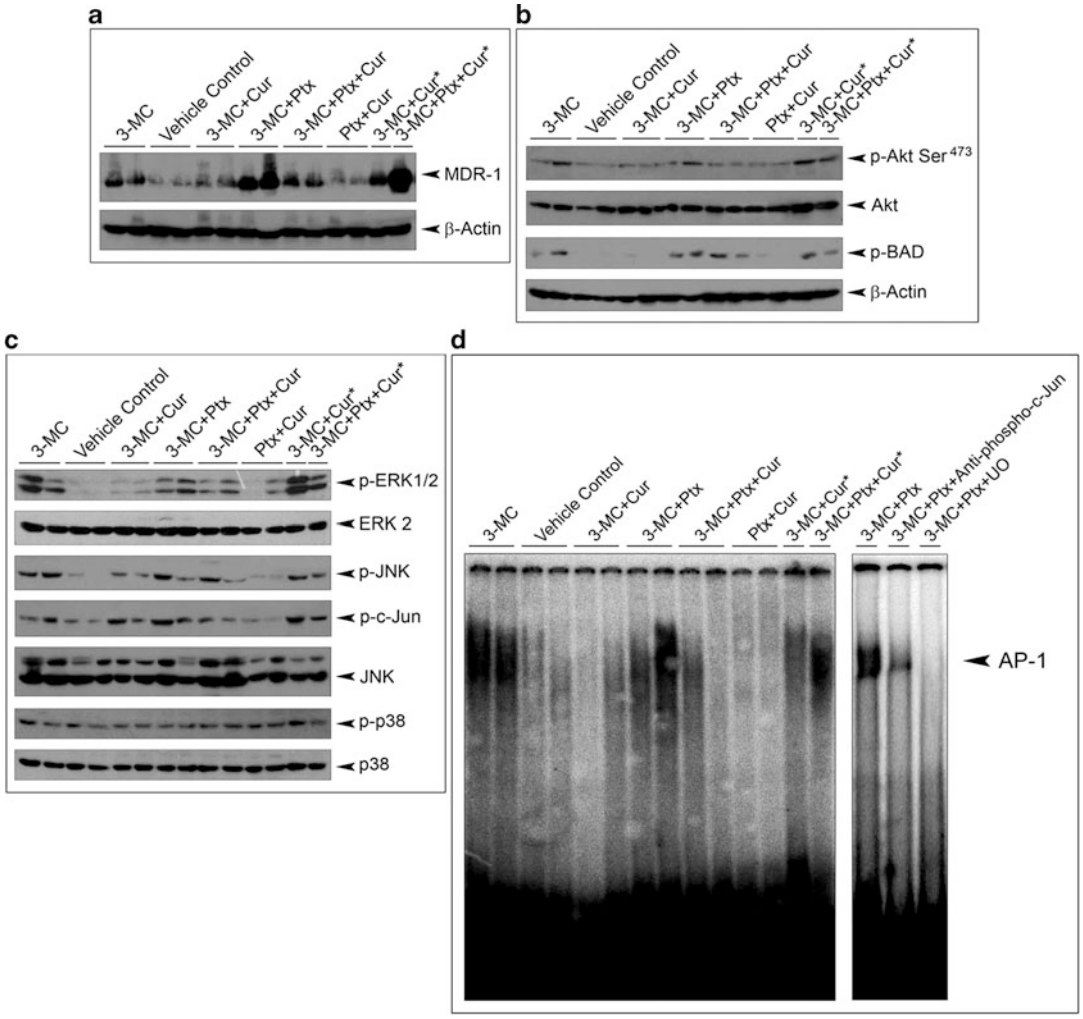

Fig. 3.7 *Curcumin enhances paclitaxel-induced chemotherapy by downregulating Akt, MAPK, and AP-1 pathways in 3-MC-induced cervical tumors.* (**a**) Expression of MDR-1 protein in tissue samples collected from different experimental groups. Western blot analysis was carried out with anti-MDR-1 and β-actin levels are shown as loading control. (**b**) Down-regulation of Akt activation in 3-MC-induced cervical tumors by curcumin. Western blot analyses were performed with anti-phospho-Akt, anti-Akt, and anti-phospho-Bad on tissue lysates. (**c**) Inhibition of MAPK phosphorylation by curcumin in 3-MC-induced tumors. Activation status of various MAPKs in tissue samples was detected by Western blotting using phosphorylation-specific antibodies against ERK1/2, JNK, phospho-c-jun, and p38. The expression levels of total MAPKs are shown as loading control. (**d**) 3-MC-induced cervical carcinogenesis in mice is associated with constitutive activation of AP-1. Curcumin inhibits the constitutive activation of AP-1 when given alone or along with paclitaxel. Nuclear extracts prepared from tumor or control tissue samples were assayed for AP-1 activation by EMSA. The *arrowhead* denotes active AP-1-DNA complex

Akt in HeLa cells involves phosphorylation of IKK, and its down-regulation by curcumin which contributes to the sensitization of HeLa cells to the paclitaxel-induced apoptosis involves inhibition of the same. Here we have tried to dissect out the pathways contributing to the synergism of paclitaxel and curcumin.

In the current study, we observed a significant down-regulation of paclitaxel-induced NF-κB activation upon Akt inactivation. While

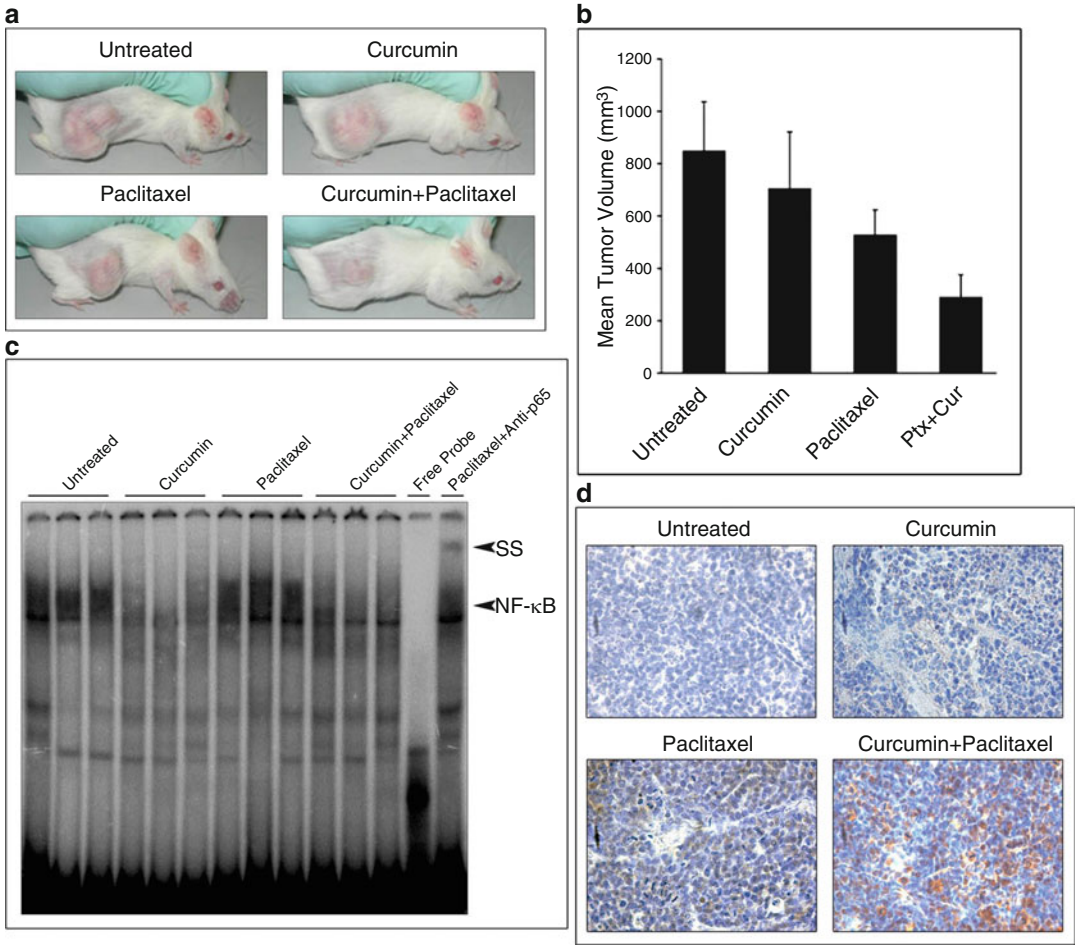

Fig. 3.8 *Curcumin enhances the antitumor activity of paclitaxel against human cervical cancer xenografts in NOD-SCID mice*. (**a**) Representative gross images of mice bearing subcutaneous tumors with or without drug treatment after 6 weeks. (**b**) The mean tumor volumes in different treatment groups after 6 weeks of drug treatment. Individual tumor volume was calculated by the standard formula a × b2/2, where a is the longest diameter and b is the shortest diameter. Data is represented as mean tumor volume + standard error ($P < 0.05$). (**c**) Effect of curcumin treatment, alone or in combination with paclitaxel, on NF-κB activation in HeLa xenograft tumors in SCID mice. Equal concentrations of nuclear extracts prepared from tumor samples or normal controls were analyzed by EMSA as described under "Materials and Methods." Tumor samples from five animals per group were analyzed individually and representative data from three randomly selected tumors from each group is shown. (**d**) Representative photomicrographs showing TUNEL staining pattern in formalin-fixed, paraffin-embedded xenograft tumor sections from different treatment groups. Dark brown staining is indicative of nuclei with fragmented DNA. Cell nuclei were counterstained with hematoxylin. *NOTE*: All error bars indicate standard deviation between three independent experiments

over-expression of Akt resulted in an increase in paclitaxel-mediated NF-κB activation, no change in Akt activation was observed when NF-κB was shut down, clearly indicating that Akt is up-stream of NF-κB in the paclitaxel-mediated signaling events in HeLa cells. Furthermore, analysis of paclitaxel-induced COX-2 expression in the HeLa-Akt WT cells and the HeLa-Akt DN cells shows that the expression of COX-2, an NF-κB-dependent gene, is also regulated by Akt. While inactivation of Akt reduced COX-2 expression, over-expression of Akt resulted

in the up-regulation of COX-2. Supporting this observation, a reduction in the synergistic effect of paclitaxel and curcumin was noted in the cell viability and apoptotic studies using the Akt DN-transfected HeLa cells. Over-expression of Akt made the cells resistant to paclitaxel as well as to the synergistic effect. Up-regulation of cyclin D1, another NF-κB-dependent gene, was also completely inhibited when NF-κB was shut down, implicating the key regulatory role of NF-κB in paclitaxel signaling.

Interestingly a marked difference was noted in the synergism in the case of HeLa-Akt DN and HeLa-IκBα DM cells. While a complete inhibition in synergism was observed in the case of HeLa-IκBα DM cells, only partial inhibition was noted in HeLa-Akt DN cells suggesting that inhibition of Akt by curcumin is not the only pathway by which curcumin inhibits NF-κB.

While expression of XIAP and c-IAP1 was dependent on NF-κB, that of survivin was independent though curcumin effectively down-regulated paclitaxel-induced expression of all these. However, we observed a complete inhibition of survivin up-regulation in HeLa-Akt DN cells confirming that Akt is regulating survivin signaling independent of NF-κB as observed by other investigators (Zhao et al. 2010; Zhu et al. 2008). Our study also indicates that paclitaxel-mediated up-regulation of Bcl-2 is independent of NF-κB and Akt even though contradictory reports exist (Catz and Johnson 2003).

Paclitaxel-induced activation of MAPK signaling pathway is already reported (Bacus et al. 2001). Among these three kinases, ERK1/2 module has most often been implicated in cell proliferation (Cobb 1999) while the biological effects of JNK and p38 activation are highly conflicting and, depending on the cellular context and stimuli, they exert both protective as well as proapoptotic functions (Lee et al. 1998; Seidman et al. 2001). Even though JNK signaling is attributed to apoptotic responses, it has been shown that the JNK cooperates with other signaling pathways like NF-κB to mediate cell survival. Sustained JNK activation with the absence of a survival pathway may lead to apoptosis (Lamb et al. 2003), while transient JNK activation mediates survival (Liu et al. 1996). Likewise, there are reports indicating the involvement of p38 in paclitaxel-induced activation (Oh et al. 2006; Seidman et al. 2001) as well as inhibition (Yu et al. 2001) of apoptosis. In the present study we observed a transient activation of all the three MAPKs, ERK1/2, JNK, and P38, by paclitaxel and significant down-regulation of the same by curcumin, suggesting that curcumin could be a useful adjunct in therapeutic regimen using paclitaxel. The study also revealed that phosphorylation of MAPKs is completely inhibited when NF-κB is shut down indicating that NF-κB is up-stream of MAPKs in paclitaxel signaling.

AP-1 is a well-known down-stream effector of MAPK pathway. As reported (Amato et al. 1998), we also observed that paclitaxel induces DNA binding of AP-1 and curcumin significantly down-regulated it. Since curcumin inhibited paclitaxel-induced activation of all the three MAPKs that contribute to the increased expression and activation of AP-1, inhibition of AP-1 by curcumin was predictable. Curcumin may also directly inhibit the binding of AP-1 to the DNA response element (Hahm et al. 2002).

Both ERK and JNK signaling pathways have been implicated in NF-κB activation through phosphorylation and subsequent degradation of the inhibitory subunit IκBα (Chen et al. 1999). p38-dependent activation of NF-κB via IKK pathway has also been reported (Chio et al. 2004). Thus, both the transcription factors AP-1 and NF-κB are the targets of MAP kinase signaling cascades (Lee et al. 2002). In our study also, pretreatment with specific inhibitors of MAPKs significantly inhibited nuclear translocation of AP-1, while that of NF-κB was unaffected further confirming that NF-κB is up-stream of MAPKs in paclitaxel signaling. Various studies indicate that NF-κB and AP-1 may modulate the activity of each other. NF-κB transactivation has been found to be elevated by agents that can also activate AP-1 during tumor progression (Li et al. 1997). Besides, NF-κB inhibitors have been found to attenuate AP-1 activity (Li et al. 1998). Further, the response to AP-1 is strikingly enhanced

when NF-κB subunits are present and vice versa (Fujioka et al. 2004; Stein et al. 1993).

NF-κB signaling pathway has been considered as a highly attractive target for the development of chemotherapeutic drugs, and several compounds which inhibit this pathway are currently under preclinical or clinical trials. While several of them are general inhibitors of NF-κB activation, some inhibit target-specific steps and others target multiple steps in the NF-κB pathway (Gupta et al. 2010). However, none of them has been approved for human use because most of them lack specificity and thus interfere with the physiological role of NF-κB in maintaining cellular homeostasis. Hence, the most important challenge is to develop NF-κB inhibitors based on their ability to specifically inhibit pathways leading to carcinogenesis, so that the risk of undesired side effects can be avoided. Paclitaxel is a very effective chemotherapeutic drug which acts mainly through induction of tubulin polymerization and cell cycle arrest. However, studies have shown that paclitaxel induces apoptotic cell death via a pathway independent of mitotic arrest (Huang et al. 2000; Lieu et al. 1997). Since lower concentration of paclitaxel leads to the concomitant activation of several survival signals, it fails to induce apoptosis at these concentrations necessitating a higher concentration of the drug for the purpose which in turn becomes the main reason for its toxicity. Hence, compounds like curcumin, which can counteract these survival signals, can be of therapeutic benefit when used in combination with paclitaxel.

Taken together, our results indicate that NF-κB is the central player in the synergism of paclitaxel and curcumin. Though not the only regulator, Akt is a major regulator of NF-κB, which through the phosphorylation of MAPKs regulates a set of survival signals. The study also reveals that NF-κB is the regulator of COX-2, cyclin D1, XIAP, and c-IAP1. While survivin is regulated by Akt, independent of NF-κB, Bcl-2 is operating through a pathway independent of both. However, further studies are required to rule out the role of direct down-stream targets of Akt in the synergistic effect of paclitaxel and curcumin.

Our next attempt was to evaluate the efficacy of the synergism using in vivo tumor models. For this, we used a chemical carcinogen-induced preclinical tumor model and the xenograft cervical cancer model and have shown how a suboptimal dose of the nontoxic dietary phytochemical curcumin circumvents the mechanisms of paclitaxel resistance and sensitizes tumor cells to paclitaxel treatment. The inconsistent findings on curcumin bioavailability in preclinical and clinical studies continue to be a major concern (Anand et al. 2007), and recent evidences suggest that liposomal encapsulation enhances its bioavailability and makes this compound amenable to systemic dosing (Li et al. 2007; Narayanan et al. 2009). In this study we encapsulated curcumin into unilamellar liposomes and injected intraperitoneally in order to improve its bioavailability and to eliminate dosing variability.

The initial gross observations on the percentage of tumor-bearing animals as well as mean tumor volume among different experimental groups provided an early hint about the synergistic anticancer activity of paclitaxel and curcumin against 3-MC-induced carcinogenesis. More interestingly, paclitaxel-curcumin combination therapy afforded significantly better tumor response than standard single-drug treatment. Consistent with previous reports (Chhabra et al. 1995), histopathological analysis of 3-methylcholanthrene-induced cervical tumors derived from our study revealed different stages of squamous cell carcinoma, and such progressive multistage carcinogenesis models better reflect the molecular and cellular complexity of human epithelial tumors.

Consistent with the role of NF-κB in oncogenesis and survival, cumulative experimental and epidemiologic evidence demonstrates the prevalence of aberrant constitutive activation of NF-κB in human tumors of diverse tissue origin and in various cancer cell lines (Aggarwal 2004; Karin et al. 2002). Reports also indicate that inhibition of NF-κB ameliorates the pathogenesis and overcomes therapeutic resistance (Ahn et al. 2007). Moreover, paclitaxel induces NF-κB activation in several cell types, and adjuvants

that inhibit NF-κB function can enhance its therapeutic efficiency (Bava et al. 2005; Inoue et al. 2008). To our knowledge this is the first report to reveal the strong association of constitutive NF-κB activity with 3-MC-induced tumorigenesis in mice, and we found that curcumin suppresses the nuclear translocation and DNA-binding activity of NF-κB very effectively in the tumor tissues when given alone or in combination with paclitaxel. The results of the xenograft experiments using HeLa cells also corroborate the central findings of the 3-MC-induced multistage carcinogenesis study, and together they suggest that the administration of curcumin along with paclitaxel can potentially enhance the therapeutic outcome of paclitaxel in vivo.

The transcriptional activation and overexpression of NF-κB target gene products such as the stress response protein COX-2, cell cycle regulatory protein cyclin D1, cell adhesion molecule ICAM-1, proteolytic enzymes such as matrix metalloproteinases 2 and 9, and the angiogenic factor VEGF in human cancers is well documented (Aggarwal et al. 2005). Our data substantiates the ability of curcumin to circumvent the detrimental effects of oncogenic NF-κB, such as induction of proliferation, cell invasion, metastasis, and angiogenesis. The expression patterns of several NF-κB-dependent antiapoptotic genes such as Bcl-2, survivin, XIAP, and c-IAP in the tissue samples showed excellent correlation with the NF-κB activity, and consequently cells with elevated NF-κB activity will be more resistant to paclitaxel-induced cell death. Since these proteins play central roles in the suppression of apoptosis, their inhibition by curcumin can enhance the apoptotic activity of paclitaxel when given together. This notion is confirmed by increased caspase activation observed in the tumor samples treated with the combinatorial regimen.

In many human malignancies, over-expression of the multidrug resistance (MDR-1) gene product P-glycoprotein is a major obstacle to effective chemotherapy, and it functions as an energy-dependent efflux pump for which paclitaxel is a substrate (Bradley and Ling 1994). In addition, MDR-1 gene expression is regulated by NF-κB in different cell types (Bentires-Alj et al. 2003; Zhou and Kuo 1997), and several groups have shown that curcumin down-regulates MDR-1 expression in different cell types either through inhibiting the NF-κB pathway or by direct interaction (Choi et al. 2008; Ganta and Amiji 2009). Our data suggest that the inhibition of MDR-1 expression by curcumin in 3-MC-induced tumor samples can enhance the intracellular accumulation of paclitaxel and contribute at least in part to the synergistic effect of these agents.

Constitutive and drug-induced activation of the serine-threonine kinase Akt and the three major MAPKs – ERK1/2, p38, and JNK – have been widely implicated in cancer cell proliferation, survival, and resistance to apoptotic stimuli (Chang and Karin 2001; Hokeness et al. 2005). Paclitaxel has been reported to activate Akt (Hokeness et al. 2005) and MAPK pathways (Kuo et al. 2006; Yagi et al. 2009) and thereby compromises its apoptotic potential, whereas inhibition of these pathways by curcumin increases susceptibility of cancer cells to chemotherapeutic agents (Aggarwal et al. 2006; Bava et al. 2005; Gagnon et al. 2008). Although activated JNK contributes to some apoptotic responses, the JNK-dependent apoptotic signaling pathways can be blocked by activation of NF-κB and Akt pathways (Xia et al. 1995). Since curcumin remarkably inhibited the activation of MAPKs in 3-MC-induced tumors, it could attenuate the DNA-binding activity of the down-stream transcription factor AP-1. Furthermore, activated Akt can contribute to the induction of NF-κB activity (Kane et al. 1999). Nevertheless, down-regulation of Akt activation, ERK1/2 and JNK pathways, and DNA-binding activity of AP-1 by curcumin are possible mechanisms by which the 3-MC-induced tumors are sensitized to paclitaxel therapy.

Collectively, we have shown that curcumin, the pharmacologically safe chemosensitizer, can significantly augment the anticancer potential of paclitaxel by targeting multiple signaling events using well-defined preclinical models, and these in vivo evidences on the existence of therapeutic synergism between these agents underscore the essential need for validating this combination through clinical trials.

References

Aggarwal BB (2004) Nuclear factor-kappaB: the enemy within. Cancer Cell 6(3):203–208

Aggarwal BB, Shishodia S, Takada Y, Banerjee S, Newman RA, Bueso-Ramos CE, Price JE (2005) Curcumin suppresses the paclitaxel-induced nuclear factor-kappaB pathway in breast cancer cells and inhibits lung metastasis of human breast cancer in nude mice. Clin Cancer Res 11:7490–7498

Aggarwal S, Ichikawa H, Takada Y, Sandur SK, Shishodia S, Aggarwal BB (2006) Curcumin (diferuloylmethane) down-regulates expression of cell proliferation and antiapoptotic and metastatic gene products through suppression of IkappaBalpha kinase and Akt activation. Mol Pharmacol 69:195–206

Ahn KS, Sethi G, Aggarwal BB (2007) Nuclear factor-kappa B: from clone to clinic. Curr Mol Med 7(7): 619–637

Amato SF, Swart JM, Berg M, Wanebo HJ, Mehta SR, Chiles TC (1998) Transient stimulation of the c-Jun-NH2-Terminal Kinase/Activator Protein 1 pathway and inhibition of extracellular signal-regulated kinase are early effects in paclitaxel-mediated apoptosis in human B lymphoblasts. Cancer Res 58:241–247

Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB (2007) Bioavailability of curcumin: problems and promises. Mol Pharm 4(6):807–818

Anto RJ, Maliekal TT, Karunagaran D (2000) L-929 cells harboring ectopically expressed RelA resist curcumin-induced apoptosis. J Biol Chem 275:15601–15604

Anto RJ, Mukhopadhyay A, Denning K, Aggarwal BB (2002) Curcumin (diferuloylmethane) induces apoptosis through activation of caspase-8, BID cleavage and cytochrome c release: its suppression by ectopic expression of Bcl-2 and Bcl-xl. Carcinogenesis 23(1):143–150

Anto RJ, Venkatraman M, Karunagaran D (2003) Inhibition of NF-κB sensitizes A431Cells to EGF-induced apoptosis whereas its activation by ectopic expression of RelA confers resistance. J Biol Chem 278: 25490–25498

Bacus SS, Gudkov AV, Lowe M, Lyass L, Yung Y, Komarov AP (2001) Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53. Oncogene 20:147–155

Banerjee S, Bueso-Ramos C, Aggarwal BB (2002) Suppression of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-kappaB, cyclooxygenase 2, and matrix metalloprotease 9. Cancer Res 62(17):4945–4954

Bava SV, Puliappadamba VT, Deepti A, Nair A, Karunagaran D, Anto RJ (2005) Sensitization of taxol-induced apoptosis by curcumin involves down-regulation of nuclear factor-kappaB and the serine/threonine kinase Akt and is independent of tubulin polymerization. J Biol Chem 280(8):6301–6308

Bentires-Alj M, Barbu V, Fillet M, Chariot A, Relic B, Jacobs N, Gielen J, Merville MP, Bours V (2003) NF-kappaB transcription factor induces drug resistance through MDR1 expression in cancer cells. Oncogene 22(1):90–97

Bradley G, Ling V (1994) P-glycoprotein, multidrug resistance and tumor progression. Cancer Metastasis Rev 13(2):223–233

Catz SD, Johnson JL (2003) BCL-2 in prostate cancer: a mini review. Apoptosis 8:29–37

Chang L, Karin M (2001) Mammalian MAP kinase signalling cascades. Nature 410(6824):37–40

Chaturvedi MM, Mukhopadhyay A, Aggarwal BB (2000) Assay for redox-sensitive transcription factor. Methods Enzymol 319:585–602

Chen YR, Tan TH (1998) Inhibition of the c-Jun N-terminal kinase (JNK) signalling pathway by curcumin. Oncogene 17:173–178

Chen F, Demers LM, Vallyathan V, Ding M, Lu Y, Castranova V, Shi X (1999) Vanadate induction of NF-kappaB involves IkappaB kinase beta and SAPK/ERK kinase 1 in macrophages. J Biol Chem 274:20307–20312

Chhabra SK, Kaur S, Rao AR (1995) Modulatory influence of the oral contraceptive pill, Ovral, on 3-methylcholanthrene-induced carcinogenesis in the uterus of mouse. Oncology 52(1):32–34

Chio CC, Chang YH, Hsu YW, Chi KH, Lin WW (2004) PKA-dependent activation of PKC, p38 MAPK and IKK in macrophage: implication in the induction of inducible nitric oxide synthase and interleukin-6 by dibutyryl cAMP. Cell Signal 16:565–575

Choi BH, Kim CG, Lim Y, Shin SY, Lee YH (2008) Curcumin down-regulates the multidrug-resistance mdr1b gene by inhibiting the PI3K/Akt/NF kappa B pathway. Cancer Lett 259(1):111–118

Cobb MH (1999) MAP kinase pathways. Prog Biophys Mol Bio 71:479–500

Fujioka S, Niu J, Schmidt C, Sclabas GM, Peng B, Uwagawa T (2004) NF-{kappa}Band AP-1 connection: mechanism of NF-{kappa}B-dependent regulation of AP-1 activity. Mol Cell Biol 24:7806–7819

Gagnon V, Themsche CV, Turner S, Leblanc V, Asselin E (2008) Akt and XIAP regulate the sensitivity of human uterine cancer cells to cisplatin, doxorubicin and taxol. Apoptosis 13:259–271

Ganta S, Amiji M (2009) Coadministration of paclitaxel and curcumin in nanoemulsion formulations to overcome multidrug resistance in tumor cells. Mol Pharm 6(3):928–939

Gupta SC, Sundaram C, Reuter S, Aggarwal BB (2010) Inhibiting NF-kappaB activation by small molecules as a therapeutic strategy. Biochim Biophys Acta 1799:775–787. doi:10.1016/j.bbagrm.2010.05.004

Hahm ER, Cheon G, Lee J, Kim B, Park C, Yang CH (2002) New and known symmetrical curcumin derivatives inhibit the formation of Fos-Jun-DNA complex. Cancer Lett 184:89–96

Hokeness K, Qiu LH, Vezeridis M, Yan BF, Mehta S, Wan YS (2005) IFN-gamma enhances paclitaxel-induced apoptosis that is modulated by activation of caspases 8 and 3 with a concomitant down regulation of the AKT survival pathway in cultured human keratinocytes. Oncol Rep 13(5):965–969

Huang Y, Fan W (2002) IkappaB kinase activation is involved in regulation of paclitaxel-induced apoptosis in human tumor cell lines. Mol Pharmacol 61:105–113

Huang Y, Johnson KR, Norris JS, Fan W (2000) Nuclear factor-kappaB/IkappaB signaling pathway may contribute to the mediation of paclitaxel-induced apoptosis in solid tumor cells. Cancer Res 60:4426–4432

Hussain SP, Rao AR (1991) Chemopreventive action of mace (Myristica fragrans, Houtt) on methylcholanthrene-induced carcinogenesis in the uterine cervix in mice. Cancer Lett 56(3):231–234

Inoue M, Matsumoto S, Saito H, Tsujitani S, Ikeguchi M (2008) Intraperitoneal administration of a small interfering RNA targeting nuclear factor-kappa B with paclitaxel successfully prolongs the survival of xenograft model mice with peritoneal metastasis of gastric cancer. Int J Cancer 123(11):2696–2701

Kane LP, Shapiro VS, Stokoe D, Weiss A (1999) Induction of NF-kappaB by the Akt/PKB kinase. Curr Biol 9(11):601–604

Karin M, Cao Y, Greten FR, Li ZW (2002) NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer 2(4):301–310

Kuo HC, Lee HJ, Hu CC, Shun HI, Tseng TH (2006) Enhancement of esculetin on Taxol-induced apoptosis in human hepatoma HepG2 cells. Toxicol Appl Pharmacol 210(1–2):55–62

Kuttan R, Bhanumathy P, Nirmala K, George MC (1985) Potential anticancer activity of turmeric (Curcuma longa). Cancer Lett 29(2):197–202

Lamb JA, Ventura JJ, Hess P, Flavell RA, Davis RJ (2003) JunD mediates survival signaling by the JNK signal transduction pathway. Mol Cell 11:1479–1489

Lee LF, Li G, Templeton DJ, Ting JP (1998) Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH2-terminal kinase (JNK/SAPK). J Biol Chem 273:28253–28260

Lee SW, Han SI, Kim HH, Lee ZH (2002) TAK1-dependent activation of AP-1 and c-Jun N-terminal kinase by receptor activator of NF-kappaB. J Biochem Mol Biol 35:371–376

Li JJ, Westergaard C, Ghosh P, Colburn NH (1997) Inhibitors of both nuclear factor kappaB and activator protein-1 activation block the neoplastic transformation response. Cancer Res 57:3569–3576

Li JJ, Rhim JS, Schlegel R, Vousden KH, Colburn NH (1998) Expression of dominant negative Jun inhibits elevated AP-1 and NF-kappaB transactivation and suppresses anchorage independent growth of HPV immortalized human keratinocytes. Oncogene 16:2711–2721

Li L, Ahmed B, Mehta K, Kurzrock R (2007) Liposomal curcumin with and without oxaliplatin: effects on cell growth, apoptosis, and angiogenesis in colorectal cancer. Mol Cancer Therap 6(4):1276–1282

Lieu CH, Chang YN, Lai YK (1997) Dual cytotoxic mechanisms of submicromolar taxol on human leukemia HL-60 cells. Biochem Pharmacol 53:1587–1596

Liu ZG, Hsu H, Goeddel DV, Karin M (1996) Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell 87:565–576

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25(4):402–408

Mabuchi S, Ohmichi M, Nishio Y, Hayasaka T, Kimura A, Ohta T, Kawagoe J, Yada-Hashimoto N, Takahashi K, Seino-Noda H, Sakata M, Motoyama T, Kurachi H, Testa JR, Tasaka K, Murata Y (2004) Inhibition of inhibitor of nuclear factor-kappaB phosphorylation increases the efficacy of paclitaxel in *in vitro* and *in vivo* ovarian cancer models. Clin Cancer Res 10: 7645–7654

Murphy ED (1961) Carcinogenesis of the uterine cervix in mice: effect of diethylstilbestrol after limited application of 3-methylcholanthrene. J Natl Cancer Inst 27:611–653

Narayanan NK, Nargi D, Randolph C, Narayanan BA (2009) Liposome encapsulation of curcumin and resveratrol in combination reduces prostate cancer incidence in PTEN knockout mice. Int J Cancer 125(1):1–8

Oh SY, Song JH, Gil JE, Kim JH, Yeom YI, Moon EY (2006) ERK activation bythymosin-beta-4 (TB4) over expression induces paclitaxel-resistance. Exp Cell Res 312:1651–1657

Seidman R, Gitelman I, Sagi O, Horwitz SB, Wolfson M (2001) The role of ERK ½ and p38 MAP-kinase pathways in Taxol-induced apoptosis in human ovarian carcinoma cells. Exp Cell Res 268:84–92

Stein B, Baldwin AS Jr, Ballard DW, Greene WC, Angel P, Herrlich P (1993) Cross coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function. EMBO J 12:3879–3891

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270(5240):1326–1331

Yagi H, Yotsumoto F, Sonoda K, Kuroki M, Mekada E, Miyamoto S (2009) Synergistic anti-tumor effect of paclitaxel with CRM197, an inhibitor of HB-EGF, in ovarian cancer. Int J Cancer 124(6):1429–1439

Yu C, Wang S, Dent P, Grant S (2001) Sequence-dependent potentiation of paclitaxel mediated apoptosis in human leukemia cells by inhibitors of the mitogen-activated protein kinase kinase/mitogen-activated protein kinase pathway. Mol Pharmacol 60:143–154

Zhao P, Meng Q, Liu LZ, You YP, Liu N, Jiang BH (2010) Regulation of survivin byPI3K/Akt/p70S6K1 pathway. Biochem Biophys Res Commun 395: 219–224

Zhou G, Kuo MT (1997) NF-kappaB-mediated induction of mdr1b expression by insulin in rat hepatoma cells. J Biol Chem 272(24):15174–15183

Zhu N, Gu L, Li F, Zhou M (2008) Inhibition of the Akt/survivin pathway synergizes the antileukemia effect of nutlin-3 in acute lymphoblastic leukemia cells. Mol Cancer Ther 7:1101–1109