

Mechanistic evaluation of the signaling events regulating curcumin-mediated chemosensitization of breast cancer cells to 5-fluorouracil

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5-Fluorouracil (5-FU) is the first rationally designed antimetabolite, which achieves its therapeutic efficacy through inhibition of the enzyme thymidylate synthase (TS), which is essential for the synthesis and repair of DNA. However, prolonged exposure to 5-FU induces TS overexpression, which leads to 5-FU resistance in cancer cells. Several studies have identified curcumin as a potent chemosensitizer against chemoresistance induced by various chemotherapeutic drugs. In this study, we report for the first time, with mechanism-based evidences, that curcumin can effectively chemosensitize breast cancer cells to 5-FU, thereby reducing the toxicity and drug resistance. We found that 10 μ M 5-FU and 10 μ M curcumin induces a synergistic cytotoxic effect in different breast cancer cells, independent of their receptor status, through the enhancement of apoptosis. Curcumin was found to sensitize the breast cancer cells to 5-FU through TS-dependent downregulation of nuclear factor- κ B (NF- κ B), and this observation was confirmed by silencing TS and inactivating NF- κ B, both of which reduced the chemosensitizing efficacy of curcumin. Silencing of TS suppressed 5-FU-induced NF- κ B activation, whereas inactivation of NF- κ B did not affect 5-FU-induced TS upregulation, confirming that TS is upstream of NF- κ B and regulates the activation of NF- κ B in 5-FU-induced signaling pathway. Although Akt/PI3kinase and mitogen-activated protein kinase pathways are activated by 5-FU and downregulated by curcumin, they do not have any role in regulating the synergism. As curcumin is a pharmacologically safe and cost-effective compound, its use in combination with 5-FU may improve the therapeutic index of 5-FU, if corroborated by *in vivo* studies and clinical trials.

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Eradication of breast cancer is currently a demanding arena owing to the challenges faced in developing an adequate drug for its treatment because of the heterogeneity in receptor status of breast cancer tissues. Drugs for breast cancer treatment are selected based on estrogen, progesterone and HER2/neu receptor status of the tissue. In the case of triple-negative breast cancer, which is devoid of ER/PR/HER2/neu receptors, the currently available chemotherapeutic regimens fail, demanding better treatment modalities that can act independent of the receptor status.

The major lacunae in conventional chemotherapy are their adverse side effects and the development of chemoresistance, which they induce by upregulating various survival signaling pathways and multidrug resistance genes. Any compound, which can downregulate these survival signals, can act as a chemosensitizer and can increase the efficacy of chemotherapy. Several phytochemicals have been reported to act as potent chemosensitizers in combination with conventional chemotherapeutic drugs^{1,2} and are preferred over synthetic chemicals because they have been proven to be pharmacologically safe.³ The antimetabolite agent 5-fluorouracil (5-FU) is widely used in the treatment of many

types of cancers including breast cancer.⁴ It inhibits cancer cell growth and initiates apoptosis by targeting thymidylate synthase (TS), an enzyme crucial for the *de novo* synthesis of DNA,⁵ and also by inducing DNA and RNA strand breaks by direct incorporation of fluorinated nucleotides.^{6,7} 5-FU chemotherapy is hampered by side effects like leukopenia, diarrhea, anorexia and vomiting.⁸

In vitro and *in vivo* studies have established a strong association between increased TS expression and development of 5-FU chemoresistance.^{9,10} Multiple clinical investigations have shown an improved response to 5-FU-based therapy in patients with low TS expression in the tumor tissues.¹¹ In addition to the well-established concept that TS is a target of fluoropyrimidine, it is also reported as an oncogene.¹²

5-FU also upregulates several survival signals including NF- κ B and Akt. NF- κ B pathway is a major downstream effector pathway leading to chemoresistance.¹³ Several molecules, such as COX-2, cyclin D1, Bcl-2, Bcl-xL, survivin, XIAP, and so on, have been identified to be responsible for NF- κ B-mediated chemoresistance. In addition, there exists crosstalk between NF- κ B and other survival pathways, such

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Abbreviations: 5-FU, 5-fluorouracil; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; TS, thymidylate synthase

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as PI3kinase/Akt, EGFR, and so on, further contributing to chemoresistance. Thus, downregulation of NF- κ B by chemopreventives is an effective mechanism to tackle drug resistance.¹⁴ The therapeutic efficacy of 5-FU is significantly increased when NF- κ B nuclear translocation and activation is prevented.^{15–17}

Akt, which has a major function in cell survival and proliferation, has an important role in 5-FU chemoresistance.¹⁸ Evidences indicate that Akt is frequently activated in breast cancer and makes breast cancer patients more prone to tumor relapse and metastasis.¹⁹

Besides these survival pathways, ERK1/2 (p42/44), p38 and JNK – the three major mitogen-activated protein kinases (MAPKs) – have been widely implicated in the cancer chemoresistance owing to their constitutive and drug-induced activation.²⁰ Inhibition of ERK has been shown to enhance the activity of 5-FU.¹⁸

Curcumin is an ideal chemopreventive agent owing to its diverse effects on multiple signaling pathways²¹ and pharmacological safety.²² It sensitizes cancer cells to chemotherapeutic agents by inhibiting antiapoptotic pathways.^{2,23} Several studies have shown that curcumin can sensitize cancer cells of various origin to 5-FU.^{24–26} Although 5-FU is commonly used in breast cancer treatment, no study has been conducted on the effect of 5-FU and curcumin in breast cancer.

This study was carried out to find out whether curcumin can chemosensitize breast cancer cells to 5-FU, inducing a synergistic cytotoxicity to them, and to elucidate the signaling pathways regulating the synergism.

Results

A synergistic combination of 5-FU and curcumin induces enhanced cytotoxicity and apoptosis in breast cancer cells, while the normal immortalized breast cells are unaffected. 5-FU and curcumin were screened for their cytotoxicity, both individually and in combination, towards various breast cancer cell lines and the normal immortalized breast epithelial cell line MCF 10A by MTT assay (Figure 1a). Among the various combinations studied, the combination of 10 μ M 5-FU and 10 μ M curcumin was found to induce a synergistic cytotoxic effect compared with that caused by either of the two compounds alone in breast cancer cells. The combination index (CI) value was <1 in all breast cancer cells for the combination, indicating synergism, whereas >1 in the normal immortalized breast epithelial cell line MCF 10A, indicating an antagonistic effect (Supplementary Table 1). As the synergism was almost similar among all the breast cancer cells studied, we selected MDA-MB-231, the triple-negative cell line for further studies, to ensure that the synergism is independent of the receptor status. Although 10 μ M 5-FU and 10 μ M curcumin induced 29% and 12% cytotoxicity, respectively, in MDA-MB-231 cells, a combination of these two induced 58% cytotoxicity, which is more than that induced by 25 μ M 5-FU. This enhanced cytotoxicity is due to the synergistic effect of 5-FU and curcumin, as an additive effect of both these compounds should have induced only 41% cytotoxicity (Figure 1a). The synergism was further confirmed in MDA-MB-231 cells by a

more reliable [³H]thymidine incorporation assay (Figure 1b). These results indicate that curcumin and 5-FU, when used in combination, induce more than double the effect of 5-FU alone, whereas curcumin itself does not induce a significant cytotoxicity at this concentration. However, this combination was non-toxic to MCF 10A, indicating that the combination is biologically safe (Figure 1c). FACS analysis indicated that while 5-FU alone induces an S-phase arrest, treatment of curcumin along with 5-FU pushed the cells to apoptotic death as indicated by the tremendous increase (from 6.8 to 43.1%) in the number of cells in the sub-G0 phase (Figure 1d).

The enhancement of 5-FU-induced apoptosis by curcumin, as evidenced by Annexin V positivity, is through caspase-mediated cleavage of PARP, leading to DNA fragmentation. The results from Annexin V/PI staining were also in concordance with that of the MTT assay (Figure 2a) and clearly indicate that curcumin enhances externalization of phosphatidylserine. The combination induced a significant cleavage of procaspase-8 to its active fragments (p43/41) and procaspase-9 to its active fragments (p35/37) (Figures 2b and c) compared with cells treated with either of the two compounds alone. The combination also induced the cleavage of procaspase-3 to its active fragments (p17/19) and enhanced the cleavage of procaspase-7 to its active fragment (p20) (Figures 2d and e). In addition, it induced enhanced cleavage of PARP, the downstream target of caspase-3 (Figure 2f). The synergistic effect was confirmed through the enhancement of apoptosis when the combination induced momentous increase in the internucleosomal cleavage of DNA, the biochemical hallmark of apoptosis, compared with that induced by 5-FU and curcumin alone (Figure 2g).

5-FU induces time-dependent upregulation of TS, which is downregulated by curcumin and silencing of TS abrogates the synergism. The main mechanism of action of 5-FU is the inhibition of TS by fluorodeoxyuridine monophosphate, the active metabolite of 5-FU.⁴ However, studies indicate that 5-FU treatment induces TS expression, which might bypass deoxythymidine monophosphate depletion, leading to chemoresistance.⁹ This effect is due to the inhibition of the negative-feedback mechanism, where TS inhibits its translation by binding to its own mRNA.²⁷ Several studies have demonstrated strong association between increased TS expression and development of resistance to 5-FU.^{4,28} Reports indicate that the expression of this enzyme is significantly upregulated in various tumors including breast cancer.^{9,10} This is the first report demonstrating the ability of curcumin to downregulate the expression of TS in breast cancer cells. We checked whether 5-FU upregulates the expression of TS at a concentration of 10 μ M inducing synergism. We also observed the downregulation of TS by 5-FU at 6 h, followed by a time-dependent upregulation up to 48 h (Figure 3a). Curcumin pre-treatment could significantly downregulate 5-FU-induced upregulation of TS (Figure 3b). This prompted us to check whether the downregulation of TS by curcumin in breast cancer cells has any regulatory role in the synergism.

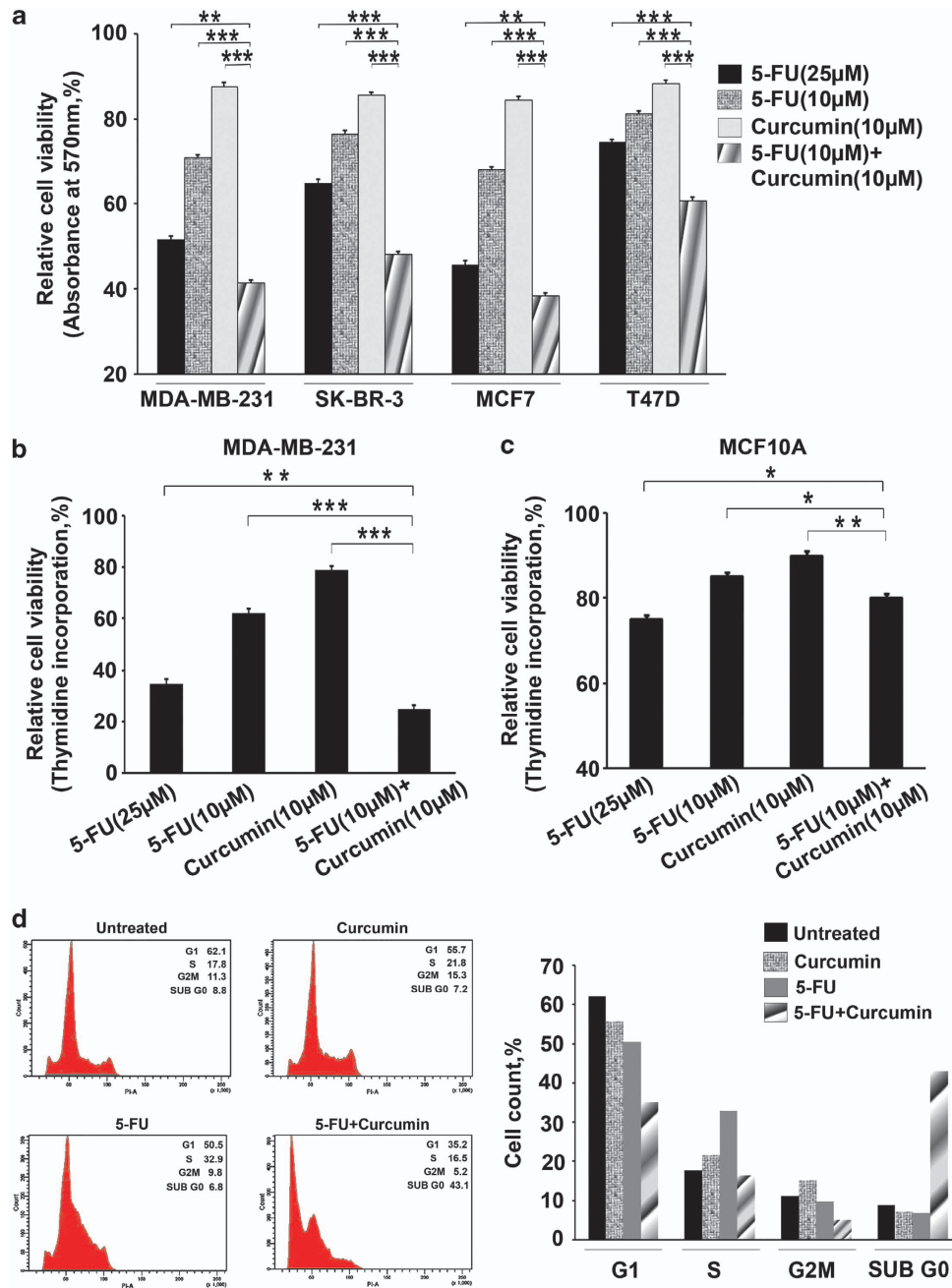


Figure 1 Curcumin sensitizes breast cancer cells to 5-FU-induced apoptosis, while normal breast cells are unaffected. (a) Effect of 5-FU and curcumin, alone or in combination, on various breast cancer cells. A total of 5000 cells in triplicates were exposed to the indicated concentrations of the drugs for 48 h and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Relative cell viability was determined as percentage absorbance over untreated control. Data represent three independent sets of experiments and results are shown as the mean \pm S.D. *** and ** represents P -values ≤ 0.0001 and ≤ 0.001 respectively. (b) Effect of 5-FU and curcumin, alone or in combination, on MDA-MB-231 cells. A total of 5000 cells in triplicates were exposed to the indicated concentrations of the drugs for 24 h and subjected to [3 H]thymidine incorporation assay. Relative cell viability was determined as percentage thymidine incorporation over untreated control. The data represent three independent experiments. *** and ** represents P -values ≤ 0.0001 and ≤ 0.001 respectively. (c) Effect of 5-FU and curcumin, alone or in combination, on normal immortalized breast epithelial cells using [3 H]thymidine incorporation assay as described above. ** and * represents P -values ≤ 0.001 , ≤ 0.05 respectively. (d) Effect of 5-FU and curcumin, alone or in combination, on cell cycle. Cells were harvested after 48 h of drug treatment, fixed in alcohol, stained with propidium iodide and assayed for DNA content by flow cytometry. Representative histograms on the right-hand panel indicate the percentages of cells in G1, S, G2/M and sub-G0 phases of the cell cycle. The percentage of cells with sub-G0 DNA content was taken as a measure of the apoptotic cell population. The data provided are representatives of three independent experiments

Hence, we silenced TS expression by transiently transfecting the MDA-MB-231 cells with TS siRNA, which completely silenced the TS expression at 200 p moles (Figure 3c) and the

viability of these cells treated with the compounds was compared with the data obtained for cells transfected with control siRNA. It was very interesting to note that the silencing

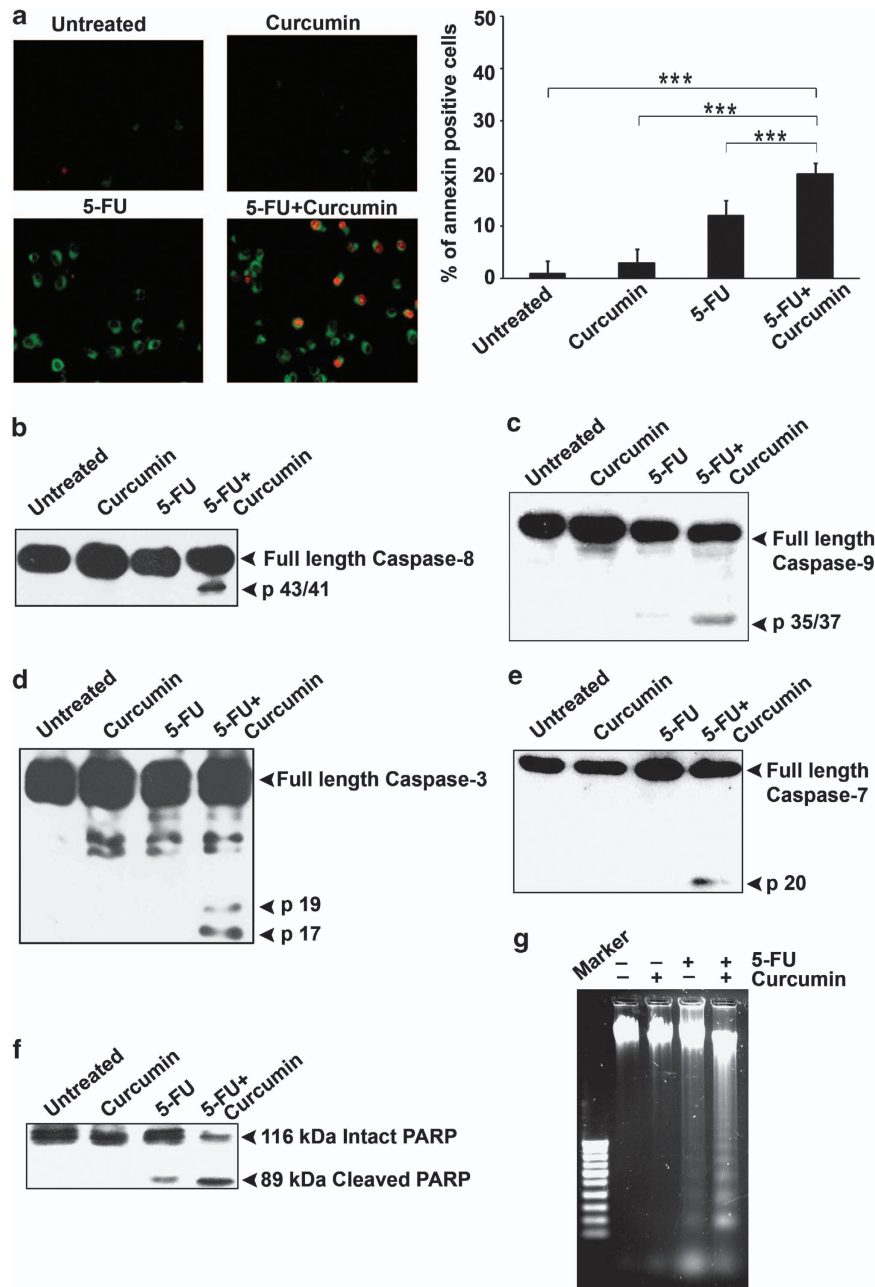


Figure 2 Curcumin potentiates 5-FU-induced membrane flip-flop, caspase activation, poly (ADP-ribose) polymerase (PARP) cleavage and DNA fragmentation. **(a)** MDA-MB-231 cells were treated with 5-FU and/or curcumin for 16 h and stained for Annexin V-propidium iodide (PI) positivity. Annexin V-positive cells in various fields were counted, and the average was taken. The green-stained cells are those that have taken only the Annexin V-FITC stain and indicate early stages of apoptosis, and red-stained cells are those that have taken up both Annexin-FITC and PI, which indicates nuclear membrane damage, and hence represents later stages of apoptosis. Representative histograms indicates percentage of annexin positive cells. *** represent P -value ≤ 0.0001 . **(b–e)** Western blots showing curcumin-mediated enhancement of 5-FU-induced caspase activation in MDA-MB-231 cells. Whole-cell extracts were prepared after treating MDA-MB-231 cells with 5-FU and/or curcumin for 48 h and probed using anticaspase antibodies. **(f)** Western blot showing curcumin-mediated enhancement of 5-FU-induced PARP cleavage in MDA-MB-231 cells. Whole cell extracts were prepared as described earlier and probed using anti-PARP antibody. **(g)** Agarose gel showing the effect of 5-FU and/or curcumin on internucleosomal DNA fragmentation in MDA-MB-231 cells. Cells were treated with 5-FU and/or curcumin for 48 h, DNA was isolated, run on an agarose gel and visualized. All experiments were repeated at least three times to confirm the reproducibility

of TS abrogated the synergism almost completely, although silencing of TS by itself could enhance the cytotoxicity of 5-FU (Figure 3d), confirming the pivotal involvement of TS in regulating the synergism. The loss of synergism was further confirmed when there was no enhancement in cleavage of PARP by the

combination when TS was inhibited, although the inhibition of TS expression through siRNA enhanced the cleavage of PARP induced by 5-FU itself as expected (Figure 3e), which confirms that downregulation of TS by curcumin has a decisive role in the synergistic effect of 5-FU and curcumin.

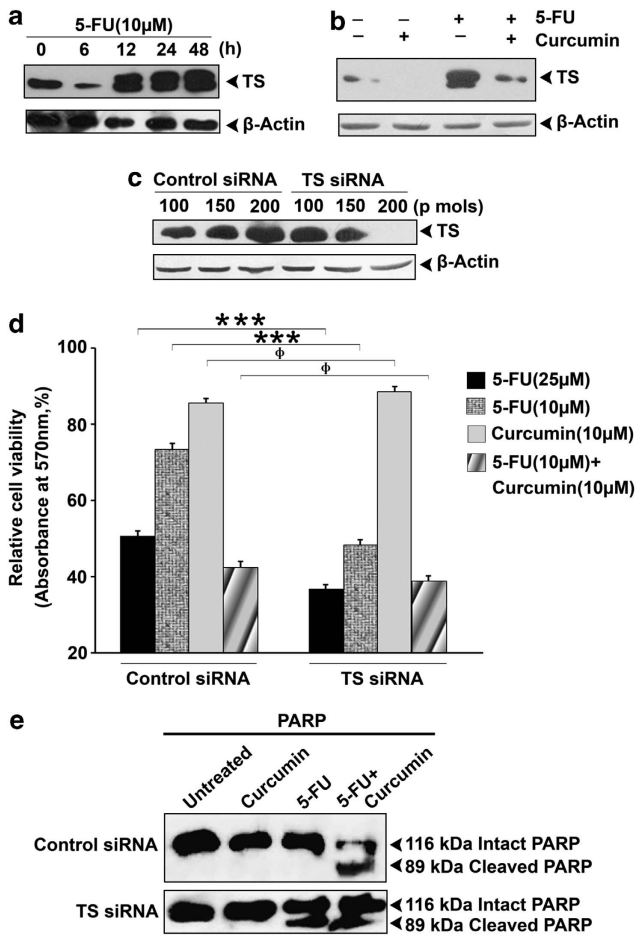


Figure 3 5-FU induces upregulation of TS, which is downregulated by curcumin, and inactivation of TS inhibits the synergism. (a) Kinetics of 5-FU-induced activation of TS in MDA-MB-231 cells at different time intervals (0–48 h). The whole cell lysate was immunoblotted against TS antibody and detected by enhanced chemiluminescent (ECL). β -Actin levels are shown as loading control. (b) Effect of curcumin on 5-FU-induced activation of TS. MDA-MB-231 cells were pre-treated with curcumin for 6 h and simultaneously exposed to 5-FU for 48 h and the whole cell lysate were immunoblotted against TS. (c) Small interfering RNA (siRNA)-mediated silencing of TS expression in MDA-MB-231 cells. Cells were transiently transfected with different concentrations of control and TS siRNA, and also checked for the expression of TS using western blotting. (d) Effect of 5-FU and curcumin, alone or in combination, on control and TS siRNA-transfected MDA-MB-231 cells. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described earlier in Figure 1a. Data represent three independent sets of experiments and results are shown as the mean \pm S.D. *** and ϕ represents *P*-values ≤ 0.0001 and > 0.05 respectively. (e) Effect of curcumin and/or 5-FU on cleavage of poly (ADP-ribose) polymerase (PARP) in control and TS siRNA-transfected MDA-MB-231 cells. Western blotting was carried out using anti-PARP antibody. All the data are representative of three independent experiments

Curcumin downregulates 5-FU-induced activation of NF- κ B, degradation of I κ B α and phosphorylation of IKK, and the inhibition of NF- κ B leads to abrogation of the synergism. Our next effort was to find out the signaling events induced by 5-FU, which may have a role in regulating the synergism. NF- κ B has a pivotal role in regulating the apoptotic program. We found a dose-dependent activation of NF- κ B up to 10 μ M, which decreases afterwards (Figure 4a),

and a time-dependent transient activation of NF- κ B up to 2 h by 10 μ M 5-FU (Figure 4b) in MDA-MB-231 cells, which was significantly downregulated by curcumin pre-treatment (Figure 4c). The specificity of NF- κ B bands was verified by supershift analysis (Figure 4d). The degradation of I κ B α (Figure 4e) corresponded to the activation pattern of NF- κ B, proving that NF- κ B activation by 5-FU is through the classical pathway. Curcumin inhibited the I κ B α degradation (Figure 4f) and IKK phosphorylation (Figure 4g) induced by 5-FU. To investigate the role of NF- κ B in regulating the synergism, we inhibited NF- κ B using the inhibitor SN50 as well as by transient transfection of I κ B α DM, in which both the phosphorylation sites are mutated so that I κ B α degradation is prevented. The synergism was drastically decreased in the presence of SN50 (Figure 4h) and on I κ B α DM transfection (Figure 4i), clearly indicating that NF- κ B has a significant role in regulating the synergism. Moreover, NF- κ B inhibition sensitized breast cancer cells to 5-FU-induced cytotoxicity, underscoring the importance of NF- κ B.

Akt and MAPK pathways do not regulate the synergism, although these are activated by 5-FU and downregulated by curcumin. We also checked the role of other survival signals, such as Akt and MAPKs, which have significant roles in regulating the apoptotic machinery. We observed that 10 μ M 5-FU induces phosphorylation of Akt up to 6 h (Figure 5a) and 10 μ M curcumin inhibits this phosphorylation (Figure 5b). 5-FU also phosphorylated the MAPKs up to 2 h (Figure 5c), which curcumin extensively downregulated (Figure 5d). However, 5-FU did not upregulate any of the unphosphorylated MAPK (data not shown). 5-FU also induced nuclear translocation of AP-1, the downstream target of MAPKs (Figure 5e), and curcumin downregulates the same (Figure 5f). The specificity of the AP-1 band was confirmed by incubating with anti-c-Jun antibody (Figure 5g). However, inhibition of Akt/PI3kinase or MAPK pathways by the corresponding inhibitors did not influence the synergism of 5-FU and curcumin (Figure 5h), indicating that these molecules do not have a significant role in regulating the synergism.

It is evident from the above studies that TS and NF- κ B have significant roles in regulating the synergism, while Akt/PI3kinase or MAPK pathways do not, although all these signaling pathways are upregulated by 5-FU and downregulated by curcumin.

NF- κ B is downstream and Akt and MAPKs are upstream of TS in 5-FU-induced signaling events. Our next effort was to locate the exact position of Akt, MAPKs, NF- κ B and TS in the 5-FU-induced signaling cascade. We checked whether there is any cross-talk between TS, NF- κ B, Akt and MAPK pathways in the 5-FU-mediated signaling events. We inhibited NF- κ B with SN50 (Figure 6a) and by I κ B α DM transfection (Figure 6b), and also checked whether 5-FU can still induce TS upregulation. 5-FU failed to induce I κ B α degradation and p65 phosphorylation (Figure 6c) in I κ B α DM-transfected cells, confirming the transfection efficiency. It was interesting to note that inhibition of NF- κ B by SN50 or by I κ B α DM did not prevent the upregulation of TS by 5-FU, indicating that NF- κ B is either downstream or independent of

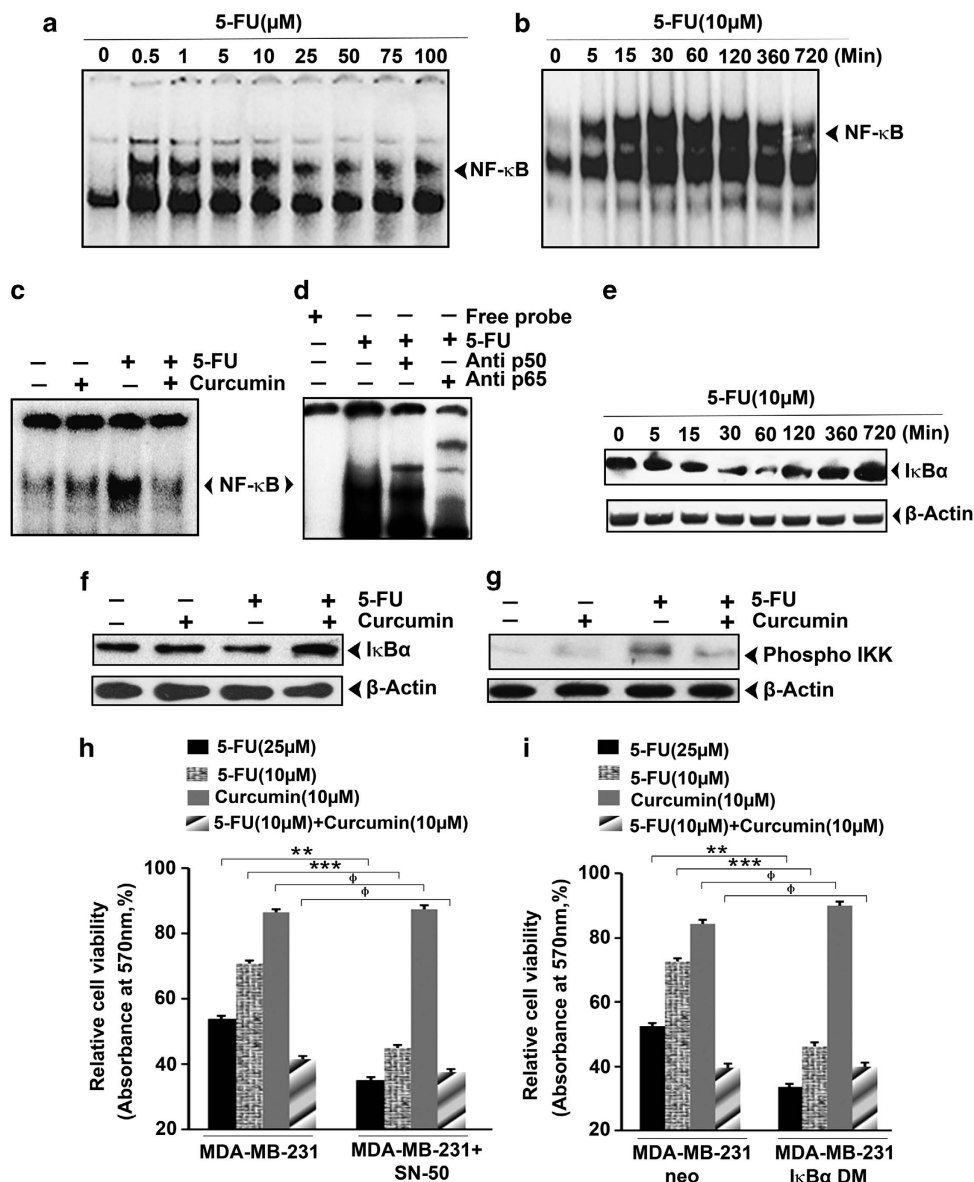


Figure 4 5-FU induces upregulation of NF- κ B, which is downregulated by curcumin, and inactivation of NF- κ B inhibits the synergism. (a) Dose dependence of 5-FU-mediated DNA binding activity of NF- κ B in MDA-MB-231 cells. Nuclear extracts prepared from MDA-MB-231 cells exposed to different concentrations of 5-FU (0–100 μ M) assayed for NF- κ B activation by electrophoretic mobility shift assay (EMSA). (b) Kinetics of 5-FU-induced activation of NF- κ B in MDA-MB-231 cells. Nuclear extracts were prepared after exposing the cells to 10 μ M 5-FU for different time intervals (0–12 h) and NF- κ B status was assessed by EMSA. (c) Individual and combined effects of 5-FU and curcumin on NF- κ B activation in MDA-MB-231 cells compared with untreated controls. NF- κ B activation was assayed by EMSA as described earlier. (d) Supershift analysis, using anti-p50 and p65 antibodies to indicate band specificity, is carried out as described in Materials and Methods. (e) Kinetics of I κ B α degradation corresponding to nuclear translocation of NF- κ B. Cytoplasmic extract collected after exposing the cells to 10 μ M 5-FU for different time periods were subjected to western blotting using antibody against I κ B α . (f) Inhibition of 5-FU-induced I κ B α degradation by curcumin. Cytosolic extract prepared from MDA-MB-231 cells after treating with 5-FU and curcumin, either alone or in combination, for 1 h was subjected to western blotting using anti-I κ B α antibody. (g) Effect of curcumin on 5-FU-induced phosphorylation of I κ B kinase (IKK). Cytosolic lysates from MDA-MB-231 cells after treatment with 5-FU and curcumin were subjected to western blotting using anti-phospho-IKK antibody. (h) Effect of 5-FU and curcumin, alone or in combination, on control and SN-50-pretreated MDA-MB-231 cells. Cell viability was checked using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described earlier. Data represent three independent sets of experiments and results are shown as the mean \pm S.D. ***, **, and ϕ represents P -values \leq 0.0001, \leq 0.001 and $>$ 0.05, respectively. (i) Effect of 5-FU and curcumin, alone or in combination, on empty vector (pcDNA3) or pcDNA3-I κ B- α DM plasmid-transfected MDA-MB-231 cells. Cell viability was checked using MTT assay as described earlier. Data represent three independent sets of experiments and results are shown as the mean \pm S.D. ***, **, and ϕ represents P -values \leq 0.0001, \leq 0.001 and $>$ 0.05 respectively

TS signaling pathway (Figures 6d and e). We noticed an upregulation of TS, both in the control as well as in 5FU-treated wells in I κ B α DM-transfected cells. Probably the cell is trying to upregulate TS through the MAPK pathway when

NF- κ B is inactivated. We observed a similar pattern of upregulation of the basal expressions of all the IAPs and cyclin D1 in I κ B α DM cells as well as Akt DN cells compared with control, in another study too.²⁹ Supporting this

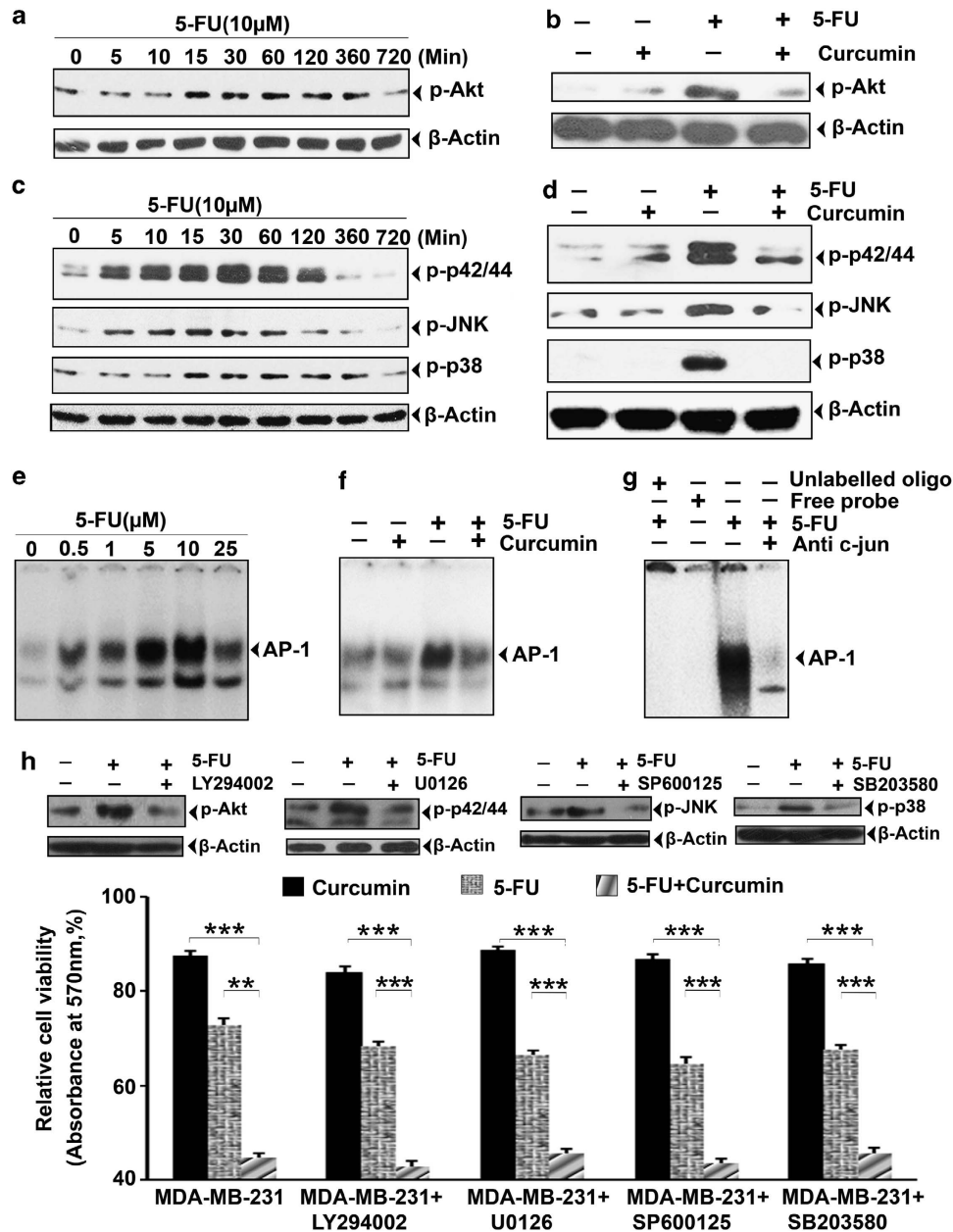


Figure 5 Even though 5-FU induces phosphorylation of Akt and MAPKs in MDA-MB-231 cells and curcumin inhibits this upregulation, the synergism of 5-FU and curcumin is independent of both these survival signals. (a) Kinetics of 5-FU-induced activation of Akt in MDA-MB-231 cells after treating them with 5-FU for different time intervals (0–12 h). The whole cell lysate was immunoblotted using antibody against phospho-Akt (ser473) antibody and detected by enhanced chemiluminescent (ECL). β -Actin levels are shown as loading control. (b) Curcumin-mediated downregulation of 5-FU-induced activation of Akt. Western blot analyses were performed with anti-phospho-Akt (ser473) on whole cell lysates after 30 min of drug exposure. (c) Activation status of various MAPKs in MDA-MB-231 cells after exposing to 10 μ M 5-FU for different time periods (0–12 h). The whole cell lysate was immunoblotted using phospho-specific antibodies against extracellular regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK) and p38. The expression level of β -actin is shown as loading control. (d) Downregulation of 5-FU-induced activation of various MAPKs in MDA-MB-231 cells by curcumin. Western blot analyses were performed using phospho-specific antibodies against the various MAPKs on cell lysates, after treating with indicated drugs for 30 min. (e) Dose-dependent activation of AP-1 by 5-FU in MDA-MB-231 cells. Nuclear extracts prepared from MDA-MB-231 cells after exposing them to different concentrations of 5-FU (0–25 μ M) were assayed for AP-1 activation by electrophoretic mobility shift assay (EMSA). (f) Inhibition of 5-FU-induced activation of AP-1 by curcumin in MDA-MB-231 cells. Nuclear extracts prepared after exposing MDA-MB-231 cells to 5-FU and curcumin, either alone or in combination for a period of 1 h, were assayed for AP-1 activation by EMSA. (g) Supershift analysis, using anti-c-jun antibody to indicate band specificity, is carried out as described in Materials and Methods. (h) Effect of 5-FU and curcumin, alone or in combination, in MDA-MB-231 cells treated with Akt and MAPKs inhibitors. A total of 5000 cells in triplicates were pre-treated with curcumin, LY294002 (1 μ M), U0126 (5 μ M), SP600125 (5 μ M) and SB203580 (1 μ M), followed by 5-FU treatment for 48 h and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data represent three independent sets of experiments and results are shown as the mean \pm S.D. ***, and ** represents *P*-values \leq 0.0001 and \leq 0.001 respectively. Inhibition status of Akt and various MAPKs were shown in inset

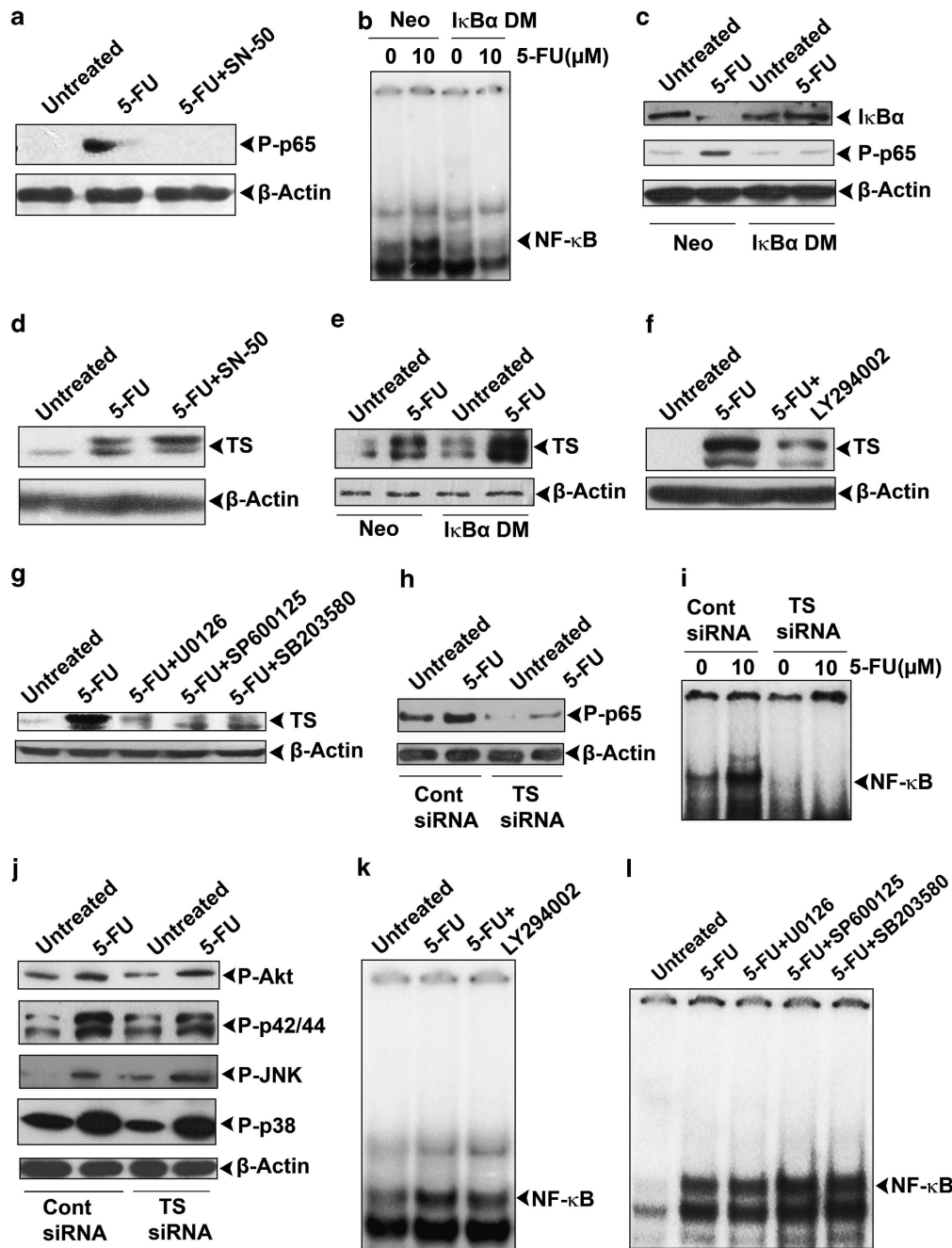


Figure 6 MAPK and Akt are upstream and NF- κ B is downstream of TS. (a) Inhibition of NF- κ B using the peptide inhibitor SN-50. Phospho-p65 status was used to check the inhibition of NF- κ B using SN-50. Western blot analysis was carried out using whole cell lysate from MDA-MB-231 cells pre-treated with SN-50 and subsequently to 5-FU. (b) 5-FU induced NF- κ B activation in MDA-MB-231-Neo cells, while it failed to induce the same in MDA-MB-231-I κ B- α DM cells. Nuclear extracts prepared after exposing Neo and I κ B- α DM cells to 10 μ M 5-FU were subjected to electrophoretic mobility shift assay (EMSA) to check activation of NF- κ B. (c) 5-FU failed to induce I κ B α degradation and p65 phosphorylation in NF- κ B-inhibited MDA-MB-231 cells. Western blot analysis was carried out after treating Neo and I κ B- α DM cells to 10 μ M 5-FU and expression of phospho-p65 and I κ B- α was checked. (d and e) Effect of NF- κ B inactivation on 5-FU-induced TS activation. NF- κ B expression was inhibited in MDA-MB-231 cells either by SN-50 or by transient transfection using I κ B- α DM plasmid. The cells were then treated with 5-FU for 1 h and subjected to western blotting using TS antibody. (f and g) Effect of inhibition of Akt and MAPKs on 5-FU-induced TS expression. MDA-MB-231 cells were pre-treated with LY294002 (10 μ M), U0126 (10 μ M), SP600125 (25 μ M) and SB203580 (20 μ M) for 30 min, and subsequently exposed to 5-FU for 48 h and western blotted against TS antibody. (h and i) Inhibition of 5-FU-induced NF- κ B activation and nuclear translocation in TS-silenced cells. MDA-MB-231 cells were transiently transfected with control and TS siRNA and then treated with 10 μ M 5-FU for 30 min and checked for phosphorylation of p65 by western blot and NF- κ B DNA-binding activity by EMSA. (j) Effect of 5-FU-induced phosphorylation status of Akt and MAPKs upon silencing of TS. MDA-MB-231 cells were transiently transfected with control and TS siRNA and then treated with 10 μ M 5-FU for 30 min and checked for phosphorylation of Akt and MAPKs by western blotting. (k) Effect of 5-FU induced NF- κ B DNA-binding activity upon silencing of Akt. MDA-MB-231 cells pre-treated with LY294002 (5 μ M) were treated with 5-FU for 1 h, nuclear extracts were prepared and EMSA was performed. (l) Effect of 5-FU-induced NF- κ B DNA-binding activity upon silencing of MAPKs. Nuclear extracts were prepared from MDA-MB-231 cells pre-treated with U0126 (10 μ M), SP600125 (50 μ M) or SB203580 (40 μ M), followed by 5-FU treatment for 1 h and EMSA was performed. The experiments were repeated at least three times to confirm reproducibility.

hypothesis, inhibition of Akt and MAPKs considerably inhibited 5-FU-induced TS upregulation (Figures 6f and g), although not completely, which obviously indicates that these molecules are upstream of TS in the 5-FU signaling cascade. However, further experiments are required to confirm this hypothesis. As the inhibitors of Akt or MAPKs could not completely inhibit 5-FU-induced upregulation of TS (Figures 6f and g), it clearly indicates that 5-FU can still operate through TS-dependent NF- κ B pathway and regulate the synergism.

To locate the position of TS and NF- κ B in 5-FU signaling, we transiently silenced TS expression using TS siRNA and checked the phosphorylation and nuclear translocation of NF- κ B and phosphorylation of Akt and MAPKs, upon exposure to 5-FU. Silencing of TS almost completely inhibited 5-FU-induced phosphorylation of p65 (Figure 6h) and inhibition of TS blocked NF- κ B nuclear translocation in TS-silenced cells (Figure 6i). These results clearly prove that NF- κ B is downstream of TS in 5-FU signaling. However, phosphorylation of Akt and MAPKs by 5-FU was unaffected when TS was silenced (Figure 6j), again confirming that they are upstream of TS in 5-FU-mediated signaling. Moreover, inhibition of Akt and MAPK pathways did not significantly inhibit 5-FU-induced activation of NF- κ B (Figures 6k and l), confirming that 5-FU directly induces TS-mediated activation of NF- κ B, independent of Akt and MAPK pathways. Hence, these experiments clearly prove that NF- κ B is downstream and Akt and MAPKs are upstream of TS in 5-FU-induced signaling events. It is also clear from our results that 5-FU-induced upregulation of TS and the simultaneous activation of NF- κ B have a pivotal role in 5-FU signaling and hence can be considered as the target for 5-FU chemotherapy, and as curcumin is a potent inhibitor of both TS and NF- κ B, it can be effectively used as a chemosensitizer against 5-FU-induced chemoresistance.

Curcumin sensitizes the breast cancer cell lines SK-BR-3 and MCF7 also, to 5-FU, through the downregulation of TS and NF- κ B. We checked whether the synergism exists in other breast cancer cells with different receptor status. Interestingly, 10 μ M 5-FU induced drastic upregulation of TS in both SK-BR-3 and MCF7 cells, which is almost completely downregulated by curcumin (Figure 7a). We also observed that NF- κ B is activated in both the cell lines by 5-FU (Figure 7b) and found that 10 μ M curcumin inhibits it almost completely (Figure 7c). We also silenced TS and shutdown NF- κ B and compared the synergism of 5-FU and curcumin in these cells with that of respective controls. As observed in MDA-MB-231, silencing of TS and inactivation of NF- κ B inhibited the synergism almost completely (Figure 7d), establishing that TS and NF- κ B play critical roles in regulating the chemotherapeutic effect of 5-FU in all breast cancer cells, independent of their receptor status.

Hence, our study indicates that curcumin can act as a chemosensitizer in 5-FU chemotherapy against breast cancer, independent of the receptor status. This is a very important finding, as the entire chemotherapeutic regimen currently available select the treatment mode considering the receptor status of the breast tissue, and frequent relapse of cancer is reported in patients with triple-negative receptor status. Therefore, our finding will be beneficial to the field of

breast cancer chemotherapy, if validated through *in vivo* studies.

Discussion

The development of drug resistance and dose-limiting cytotoxicity greatly impede the use of 5-FU, which will form a covalent ternary complex with 5, 10-methylenetetrahydrofolate and TS, resulting in the inhibition of DNA synthesis.³⁰ The expression of TS, which is an important therapeutic target of 5-FU, has been found to increase after prolonged exposure to 5-FU, leading to the maintenance of free enzyme in excess than that bound to 5-FU,^{10,31} which has been reported to be the reason for the chemoresistance of 5-FU.^{32,33} The overexpression of TS not only reflects drug resistance to fluoropyrimidine but also indicates the biological aggressiveness of cancer cells. The cytotoxicity of 5-FU was significantly increased when TS was downregulated as a result of the reduced amount of its protein target.³⁴ Several studies have been conducted based on the concept that development of a new therapeutic strategy that reduces TS expression would be clinically important. 5-FU, in combination with 3*n*-butyrate, was shown to have a synergistic effect in colorectal cancer xenografts through the enhanced reduction of TS and prevention of thymidine salvage in DNA synthesis.³⁵ Inhibitors of histone deacetylase and mammalian target of rapamycin have been shown to enhance the antitumor activity of 5-FU in various cancer cells, through downregulation of TS.^{36,37} Moreover, the antisense downregulation of TS and gefitinib-mediated downregulation of TS enhance the efficacy of 5-FU.^{38,39} Over and above these preclinical findings, several clinical studies showed consistent inverse relation between TS expression and 5-FU sensitivity.^{40,41} Consistent with these findings, we found a clear negative correlation between 5-FU cytotoxicity and TS expression in breast cancer cells, as evidenced by enhanced 5-FU cytotoxicity in response to the silencing of TS. Curcumin, a well-documented chemosensitizer, could clearly downregulate both basal and induced TS expression in breast cancer cells, thereby enhancing 5-FU-induced cytotoxicity in these cells, suggesting for the first time that TS downregulation by curcumin could be used as a major strategy in breast cancer treatment in combination with 5-FU.

Another major signaling pathway responsible for chemoresistance induced by various chemotherapeutics including 5-FU is NF- κ B. Several studies have shown that downregulation of NF- κ B by various means could enhance therapeutic efficacy of 5-FU.^{15,17} The ability of curcumin to downregulate constitutive and inducible NF- κ B, thereby enhancing the activity of various chemotherapeutics, has been well documented. The *in vitro* and *in vivo* studies from our laboratory have shown that the antitumor effects of paclitaxel could be enhanced by curcumin in cervical cancer cells through the downregulation of paclitaxel-induced activation of NF- κ B, Akt and Bcl-2.^{2,23,29} In this study, we observed that curcumin could drastically downregulate 5-FU-induced NF- κ B activation in all the breast cancer cells studied.

In addition to these pathways, Akt and MAPK pathways are also reported to be involved in 5-FU chemoresistance.^{15,18} However, our study indicates that these two pathways do not have any important role as far as the regulation of synergism is

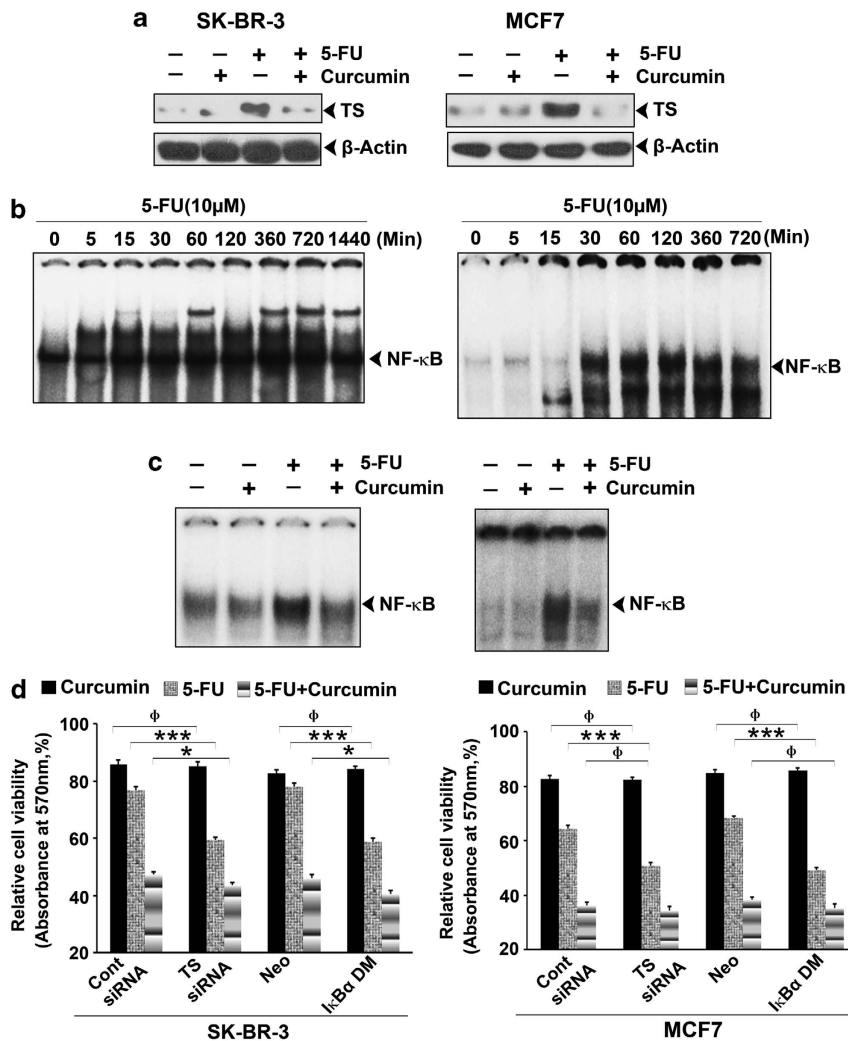


Figure 7 The synergism between 5-FU and curcumin is observed in various breast cancer cells of different receptor status. (a) Curcumin-mediated downregulation of 5-FU induced TS activation in SK-BR-3 and MCF7 cells. Cells were pre-treated with curcumin and subsequently exposed to 5-FU for 48 h and subjected to western blotting using TS antibody. (b) Kinetics of 5-FU-induced NF- κ B activation in SK-BR-3 and MCF7 cells. Nuclear lysates were prepared after exposing the cells to 10 μ M 5-FU for different time periods and electrophoretic mobility shift assay (EMSA) was performed. (c) Effect of curcumin in 5-FU-induced NF- κ B DNA-binding activity in SK-BR-3 and MCF7 cells. EMSA was performed using nuclear lysates prepared after exposing the cells to 5-FU and/curcumin for 30 min. (d) Effect of 5-FU and curcumin, alone or in combination, on TS- and NF- κ B-silenced SK-BR-3 and MCF7 cells. A total of 5000 cells in triplicates were exposed to the indicated concentrations of the drugs for 48 h and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data represent three independent sets of experiments and results are shown as the mean \pm S.D. ***, * and ϕ represents P -values ≤ 0.0001 , ≤ 0.05 and > 0.05 respectively

concerned, even though both these pathways are upregulated by 5-FU and downregulated by curcumin. Although reports have suggested that the activation of Akt and MAPK can activate NF- κ B,²⁹ in this study we found that inhibition of both these pathways did not have an effect on NF- κ B (Figures 6k and l). As the inhibitors of Akt or MAPKs could not completely inhibit 5-FU-induced upregulation of TS (Figures 6f and g), it clearly indicates that 5-FU can still operate through TS-dependent NF- κ B pathway and regulate the synergism.

Cross-talk between TS and NF- κ B pathways in 5-FU therapy has not been reported. However, high NF- κ B expression and nuclear activity has been observed in several TS inhibitor-resistant cell lines.⁴² It has also been shown that the downregulation of 5-FU-induced NF- κ B and TS could enhance the efficacy of 5-FU chemotherapy.⁴³ However, no

study has yet reported the interdependence of TS and NF- κ B. In this study, we found that silencing of TS considerably inhibits both constitutive and 5-FU-induced NF- κ B expression in the breast cancer cell line MDA-MB-231. We also observed that 5-FU upregulates TS and NF- κ B, and curcumin downregulates both of them. The inhibition of TS as well as NF- κ B considerably restrains the synergism of 5-FU and curcumin in all the breast cancer cells studied, independent of their receptor status. The signaling events regulating the synergism of 5-FU and curcumin are depicted in Figure 8.

Thus, this study opens a new strategy for enhancing the efficacy of 5-FU chemotherapy. The physiologically achievable concentration of 5-FU is 0.077–15.4 μ M.⁴⁴ As our study indicates that the combined use of curcumin with 10 μ M 5-FU can increase the cytotoxic effect of 5-FU to more than double,

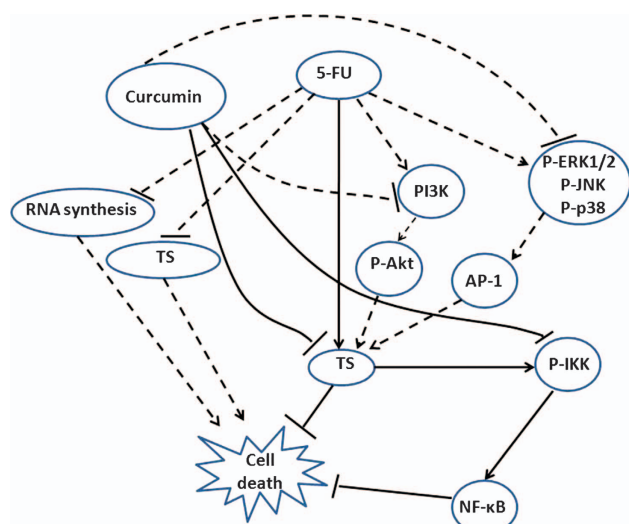


Figure 8 Proposed model for the synergistic effect of 5-FU and curcumin: 5-FU is a well-known inhibitor of TS but also leads to the upregulation of the same on prolonged exposure. It also activates NF- κ B, Akt and MAPKs in several cell systems. This study postulates that TS-dependent NF- κ B upregulation by 5-FU and its downregulation by curcumin have an important role in regulating the synergistic effect of 5-FU and curcumin. It also confirms that Akt and MAPK pathways do not have any regulatory role in the synergism, although they are activated by 5-FU and downregulated by curcumin. The bold lines illustrate signaling pathways regulating the synergism and dotted lines indicate pathways not involved in the synergism

it will significantly enhance the efficacy of 5-FU chemotherapy without inducing any side effects. Curcumin is a pharmacologically safe compound and could clearly downregulate NF- κ B, both directly and also via TS, thereby circumventing 5-FU resistance. The physiologically achievable concentration of curcumin when used orally is around 2 μ M.⁴⁵ However, recent studies including those from our lab indicate that liposomal as well as nanoformulations of curcumin are more water soluble^{46,47} and bioavailable,^{48,49} facilitating the intravenous administration of curcumin, rendering it amenable to systemic dosing. Moreover, several studies including that of ours indicate that bioavailable levels of curcumin are enough for producing synergistic cytotoxic effect with chemotherapeutics *in vivo*.^{1,2} A recent review from our lab has given a detailed account of various modifications of curcumin to improve its bioavailability and solubility in aqueous medium.³ Other flavonoids like apigenin⁵⁰ and tangeretin⁵¹ have also been shown to enhance the therapeutic effectiveness of 5-FU in breast cancer cells.

In conclusion, this study provides an important mechanism-based knowledge with potential utility in overcoming chemoresistance induced by 5-FU, thereby offering survival benefit to breast cancer patients. However, before proceeding for clinical trials, the proposed mechanism needs to be validated by *in vivo* studies, and our lab is currently carrying out experiments in this direction.

Materials and Methods

Cell lines. The breast cancer cell lines MCF7, MDA-MB-231, SK-BR-3 and T47D were purchased from National Center for Cell Sciences (Pune, India) and the normal immortalized breast epithelial cell line MCF10A (ATCC, Manassas, VA,

USA) was a gift from Dr. S Sreeja (Rajiv Gandhi Center for Biotechnology (RGCB), Thiruvananthapuram, India).

Chemicals. Dulbecco's modified Eagle's medium was obtained from Life Technologies (Grand Island, NY, USA), antibodies against caspases, phospho-ERK1/2, phospho-JNK, phospho-p38, phospho-Akt and phospho-p65 were obtained from Cell Signaling (Beverly, MA, USA) and those against c-Jun, p65, p50, TS and PARP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 5-FU was procured from Calbiochem (San Diego, CA, USA). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA).

Mode of treatment. In all combination treatments, curcumin (10 μ M) was added 6 h before 5-FU (10 μ M) treatment. The DMSO concentration in all experiments, including controls, was $\leq 0.2\%$.

MTT assay. Proliferative/cytotoxic effect of 5-FU and/or curcumin was determined by MTT assay as described earlier²³ and the relative cell viability percentage is expressed as (Abs₅₇₀ of treated wells/Abs₅₇₀ of untreated wells) $\times 100$.

Statistical analysis. The error bars represent \pm S.D. of the experiments. For the flow cytometry, data analysis was carried out using the BD FACS Diva software, version 5.0.2, Becton Dickinson and Company, Franklin Lakes, NJ, USA. The statistical analysis was carried out using Student's *t*-test. ***, **, * and ^o represents *P*-values ≤ 0.0001 , ≤ 0.001 , ≤ 0.05 and > 0.05 , respectively.

Determination of combinatorial effects. To assess whether 5-FU and curcumin act in a synergistic or additive manner with regard to cytotoxicity, the CI was determined, as proposed by Chou and Talalay.⁵² Combinations having CI value < 1 were taken as synergistic, those with CI value = 1 were taken as additive and those with CI values > 1 were taken as antagonistic. The most effective synergistic combination was selected for further studies.

[³H]thymidine incorporation assay. Inhibition of DNA synthesis induced by various drugs was assessed by [³H]thymidine incorporation assay as reported earlier.²³ Cells (5×10^3 per well) seeded in 96-well plates were treated with required concentrations of the drug. After 18 h incubation, [³H]thymidine was added (0.2 μ Ci per well) and incubated for 6 h. The cells were washed with PBS; proteins were precipitated with 5% trichloroacetic acid and solubilized in 0.2 N NaOH. The relative cell viability was calculated as percentage thymidine incorporation over untreated control.

Western blot analysis. Total protein isolated from cells after indicated treatments were subjected to western blotting as described earlier.²³ Briefly, 60 μ g of whole cell protein was resolved on a 10–15% polyacrylamide gel, transferred to a PVDF membrane, incubated with the corresponding antibody and detected by ECL (Millipore, Billerica, MA, USA).

Annexin staining. Phosphatidylserine externalization was observed by staining the cells with fluorescein isothiocyanate-conjugated Annexin V (Santa Cruz Biotechnology) according to the manufacturer's instructions and were photomicrographed.²⁹

Cell cycle analysis. The cell cycle analysis was conducted as reported earlier.² Briefly, the cells were treated with the compounds for 48 h, fixed in 70% ice-cold ethanol, treated with 100 mg/ml of RNase A and 50 mg/ml propidium iodide, and subjected to flow cytometry (BD Biosciences, San Jose, CA, USA).

Preparation of nuclear extracts and EMSA. EMSA was performed to evaluate DNA-binding activity of NF- κ B or AP-1 as described earlier.⁵³ In brief, 10 μ g of nuclear proteins was incubated for 30 min with ³²P-end-labeled double-stranded NF- κ B (5'-TTGTTACAAGGGACTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3', at 37 °C) or AP-1 (5'-CGCTTGATGACTCAGCCGGAA-3', at 30 °C) oligonucleotide and the DNA protein complex was resolved in 6.6% non-denaturing polyacrylamide gel, which was dried and visualized by Phosphor Imager (Personal Molecular Imager FX; Bio-Rad Laboratories, Hercules, CA, USA).

Transfection. Cells were transiently transfected with control siRNA, TS siRNA, l κ B α DM-pcDNA3 vector and empty vector using Lipofectamine 2000 reagent

according to the manufacturer's protocol (Invitrogen, Life Technologies, Grand Island, NY, USA).⁵³ Briefly, the diluted DNA sample as well as the transfection reagent were mixed in 1:1 ratio and added to 60–70% confluent cells and incubated for 5–7 h. The reaction was stopped later by replacing transfection media with the normal growth media.

Conflict of Interest

The authors declare no conflict of interest.

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