Citation: Cell Death and Disease (2013) 4, e505; doi:10.1038/cddis.2013.26

www.nature.com/cddis

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

BS Vinod[1,2], J Antony[1,2], HH Nair[1], VT Puliyappadamba[1], M Saikia[1], S Shyam Narayanan[1], A Bevin[1] and R John Anto*[,1]

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 μM 5-FU and 10 μM 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-κB (NF-κB), and this observation was confirmed by silencing TS and inactivating NF-κB, both of which reduced the chemosensitizing efficacy of curcumin. Silencing of TS suppressed 5-FU-induced NF-κB activation, whereas inactivation of NF-κB did not affect 5-FU-induced TS upregulation, confirming that TS is upstream of NF-κB and regulates the activation of NF-κB 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.

Cell Death and Disease (2013) **4,** e505; doi:10.1038/cddis.2013.26; published online 21 February 2013
Subject Category: Cancer

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-κB and Akt. NF-κB 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-κB-mediated chemoresistance. In addition, there exists crosstalk between NF-κB and other survival pathways, such

[1]Cancer Research Program, Division of Cancer Research, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala 695014, India
*Corresponding author: R John Anto, Cancer Research Program, Division of Cancer Research, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, 695014, India. Tel: +91 471 2529473; Fax: +91 471 2348096; E-mail: rjanto@rgcb.res.in
[2]These authors contributed equally to this work.
Keywords: 5-FU; curcumin; thymidylate synthase; NF-κB; chemosensitization; synergistic effect
Abbreviations: 5-FU, 5-fluorouracil; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; TS, thymidylate synthase
Received 06.12.12; revised 03.1.13; accepted 11.1.13; Edited by G Raschellá

as PI3kinase/Akt, EGFR, and so on, further contributing to chemoresistance. Thus, downregulation of NF-κB by chemopreventives is an effective mechanism to tackle drug resistance.[14] The therapeutic efficacy of 5-FU is significantly increased when NF-κB 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 pathways[21] 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 μM 5-FU and 10 μM 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 μM 5-FU and 10 μM 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 μM 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 [^{3}H]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 μM 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.

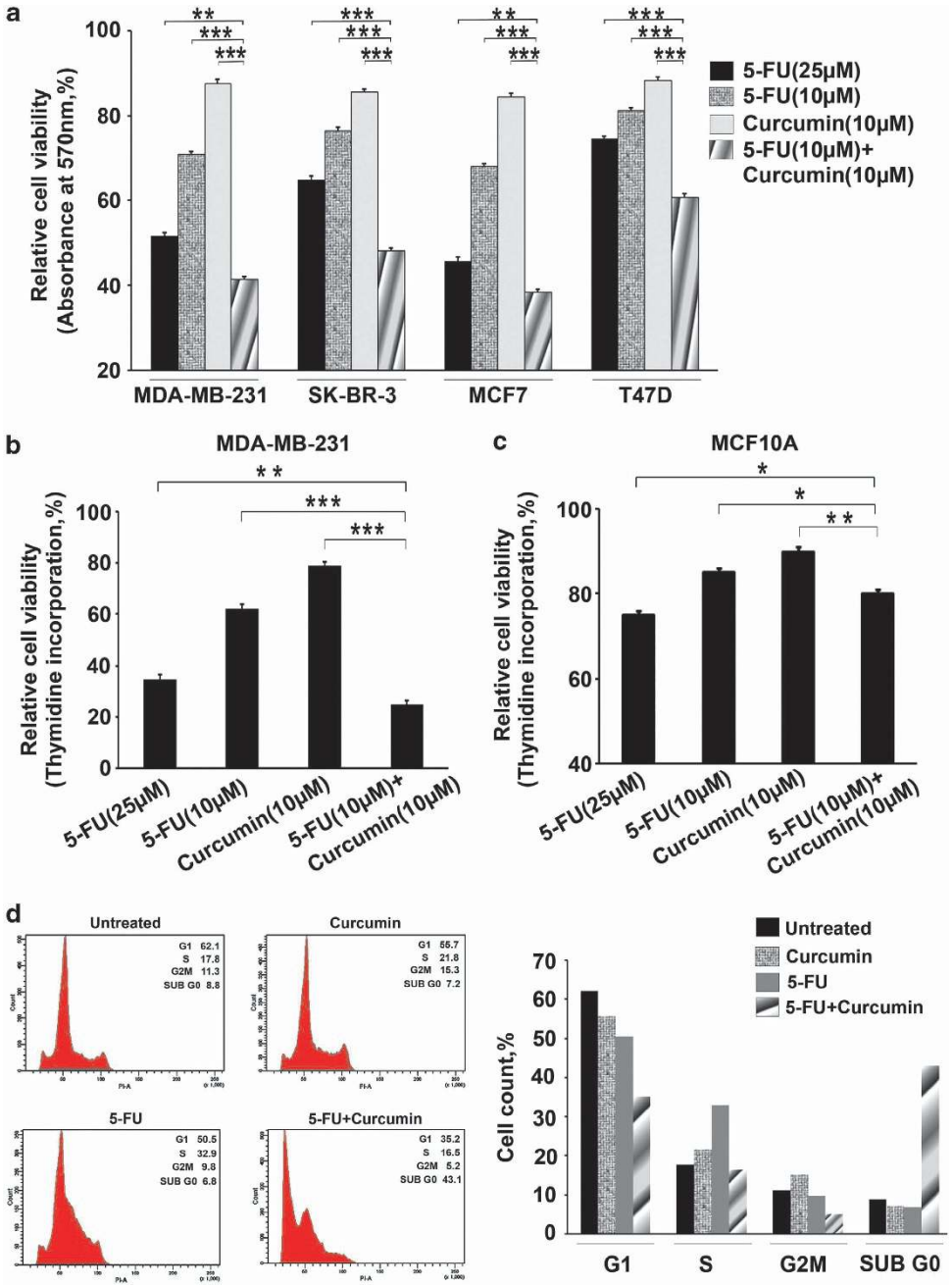

Figure 1 Curcumin sensitizes breast cancer cells to 5-FU-induced apoptosis, while normal breast cells are unaffected. (**a**) Effect of 5-FU and curcumin, alone or in combination, on various breast cancer cells. A total of 5000 cells in triplicates were exposed to the indicated concentrations of the 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 ± S.D. *** and ** represents *P*-values $\leqslant 0.0001$ and $\leqslant 0.001$ 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 $\leqslant 0.0001$ and $\leqslant 0.001$ 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 $\leqslant 0.001$, $\leqslant 0.05$ 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

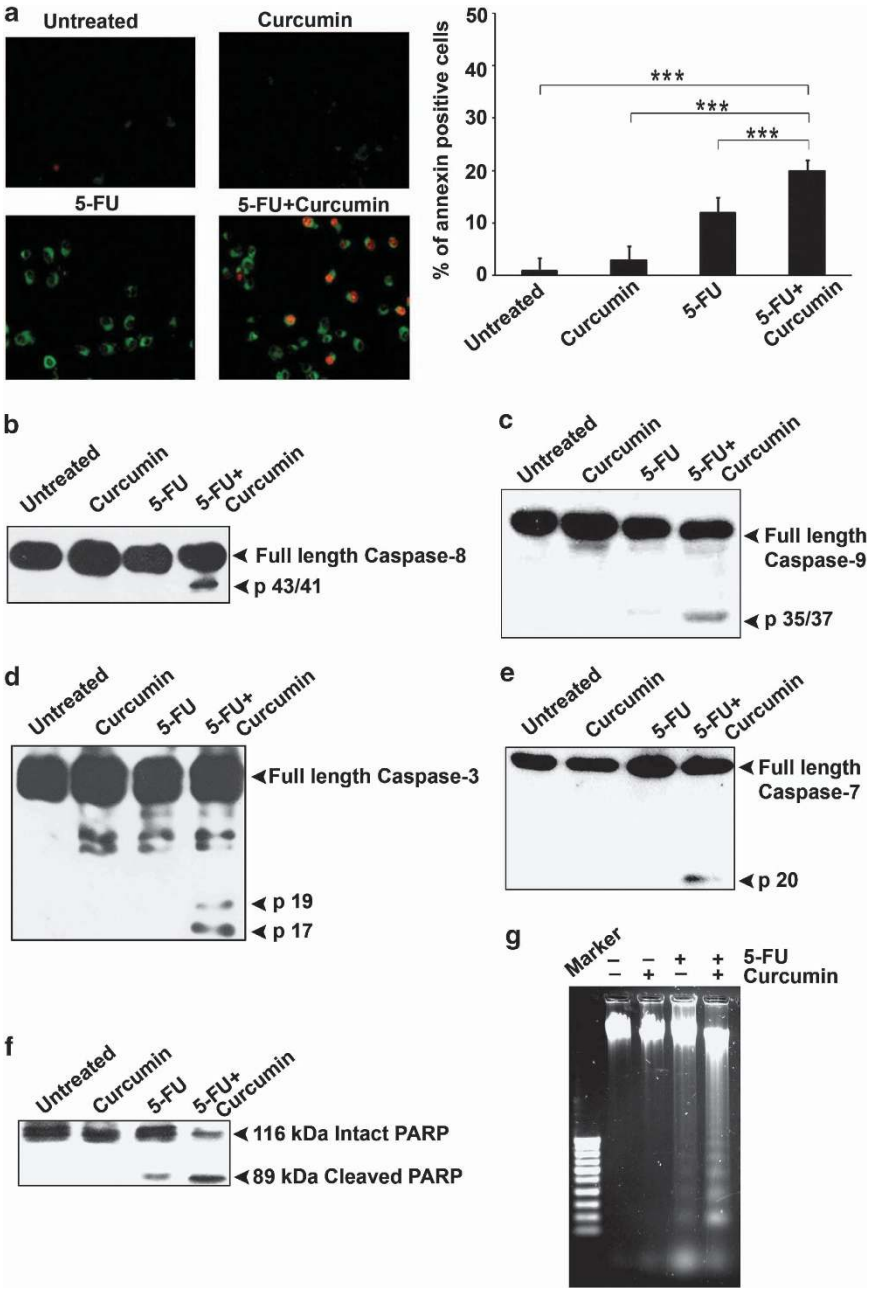

Figure 2 Curcumin potentiates 5-FU-induced membrane flip-flop, caspase activation, poly (ADP-ribose) polymerase (PARP) cleavage and DNA fragmentation. (**a**) MDA-MB-231 cells were treated with 5-FU and/or curcumin for 16 h and stained for Annexin V-propidium iodide (PI) positivity. Annexin V-positive 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 $\leqslant 0.0001$. (**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-231cells.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.

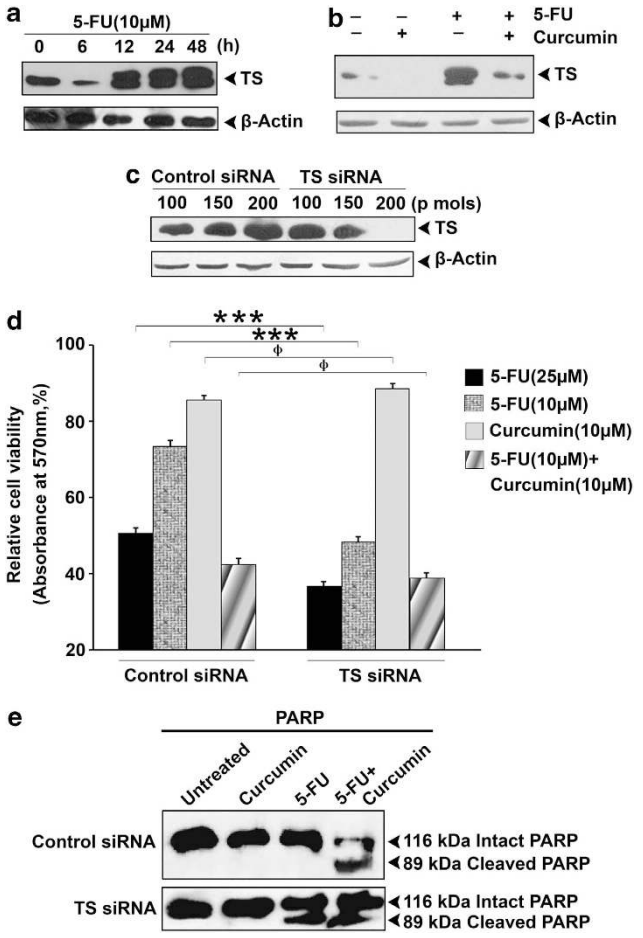

Figure 3 5-FU induces upregulation of TS, which is downregulated by curcumin, and inactivation of TS inhibits the synergism. (**a**) Kinetics of 5-FU-induced activation of TS in MDA-MB-231 cells at different time intervals (0–48 h). The whole cell lysate was immunoblotted against TS antibody and detected 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 ± S.D. *** and $^{\Phi}$ represents *P*-values $\leqslant 0.0001$ 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-κB, degradation of IκBα and phosphorylation of IKK, and the inhibition of NF-κB 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-κB has a pivotal role in regulating the apoptotic program. We found a dose-dependent activation of NF-κB up to 10 μM, which decreases afterwards (Figure 4a), and a time-dependent transient activation of NF-κB up to 2 h by 10 μM 5-FU (Figure 4b) in MDA-MB-231 cells, which was significantly downregulated by curcumin pre-treatment (Figure 4c). The specificity of NF-κB bands was verified by supershift analysis (Figure 4d). The degradation of IκBα (Figure 4e) corresponded to the activation pattern of NF-κB, proving that NF-κB activation by 5-FU is through the classical pathway. Curcumin inhibited the IκBα degradation (Figure 4f) and IKK phosphorylation (Figure 4g) induced by 5-FU. To investigate the role of NF-κB in regulating the synergism, we inhibited NF-κB using the inhibitor SN50 as well as by transient transfection of IκBαDM, in which both the phosphorylation sites are mutated so that IκBα degradation is prevented. The synergism was drastically decreased in the presence of SN50 (Figure 4h) and on IκBα DM transfection (Figure 4i), clearly indicating that NF-κB has a significant role in regulating the synergism. Moreover, NF-κB inhibition sensitized breast cancer cells to 5-FU-induced cytotoxicity, underscoring the importance of NF-κB.

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 μM 5-FU induces phosphorylation of Akt up to 6 h (Figure 5a) and 10 μM 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-κB 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-κB 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-κB and TS in the 5-FU-induced signaling cascade. We checked whether there is any cross-talk between TS, NF-κB, Akt and MAPK pathways in the 5-FU-mediated signaling events. We inhibited NF-κB with SN50 (Figure 6a) and by IκBα DM transfection (Figure 6b), and also checked whether 5-FU can still induce TS upregulation. 5-FU failed to induce IκBα degradation and p65 phosphorylation (Figure 6c) in IκBα DM-transfected cells, confirming the transfection efficiency. It was interesting to note that inhibition of NF-κB by SN50 or by IκBα DM did not prevent the upregulation of TS by 5-FU, indicating that NF-κB is either downstream or independent of

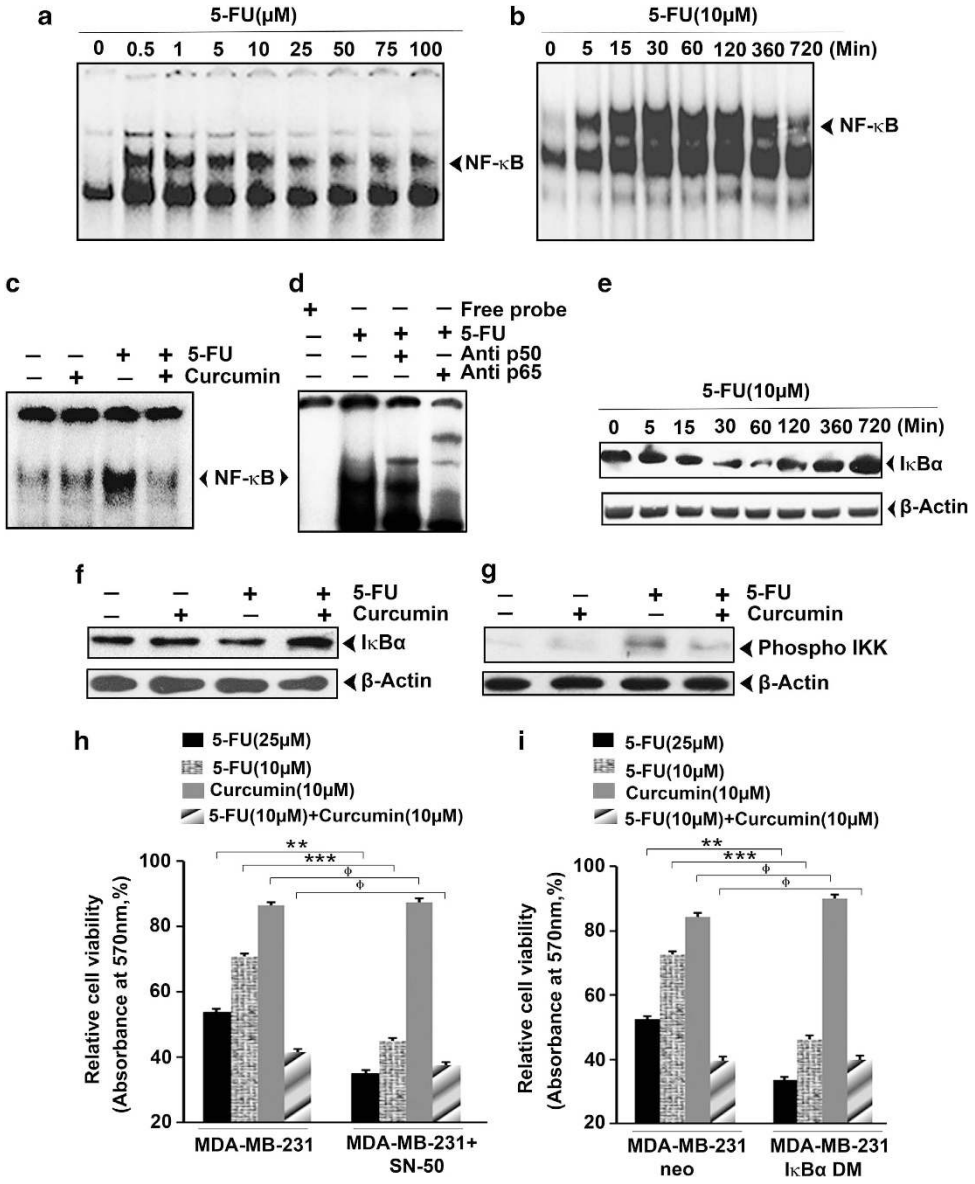

Figure 4 5-FU induces upregulation of NF-κB, which is downregulated by curcumin, and inactivation of NF-κB inhibits the synergism. (**a**) Dose dependence of 5-FU-mediated DNA binding activity of NF-κB in MDA-MB-231 cells. Nuclear extracts prepared from MDA-MB-231 cells exposed to different concentrations of 5-FU (0–100 μM) assayed for NF-κB activation by electrophoretic mobility shift assay (EMSA). (**b**) Kinetics of 5-FU-induced activation of NF-κB in MDA-MB-231 cells. Nuclear extracts were prepared after exposing the cells to 10 μM 5-FU for different time intervals (0–12 h) and NF-κB status was assessed by EMSA. (**c**) Individual and combined effects of 5-FU and curcumin on NF-κB activation in MDA-MB-231 cells compared with untreated controls. NF-κB 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 IκBα degradation corresponding to nuclear translocation of NF-κB. Cytoplasmic extract collected after exposing the cells to 10 μM 5-FU for different time periods were subjected to western blotting using antibody against IκBα. (**f**) Inhibition of 5-FU-induced IκBα 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-IκBα antibody. (**g**) Effect of curcumin on 5-FU-induced phosphorylation of IκB 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 ± S.D. ***, **, and $^{\Phi}$ represents *P*-values $\leqslant$0.0001, $\leqslant$0.001 and $>$0.05, respectively. (**i**) Effect of 5-FU and curcumin, alone or in combination, on empty vector (pcDNA3) or pcDNA3-IκB-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 ± S.D. ***, **, and $^{\Phi}$ represents *P*-values $\leqslant$0.0001, $\leqslant$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 IκBα DM-transfected cells. Probably the cell is trying to upregulate TS through the MAPK pathway when NF-κB is inactivated. We observed a similar pattern of upregulation of the basal expressions of all the IAPs and cyclin D1 in IκBα DM cells as well as Akt DN cells compared with control, in another study too.[29] Supporting this

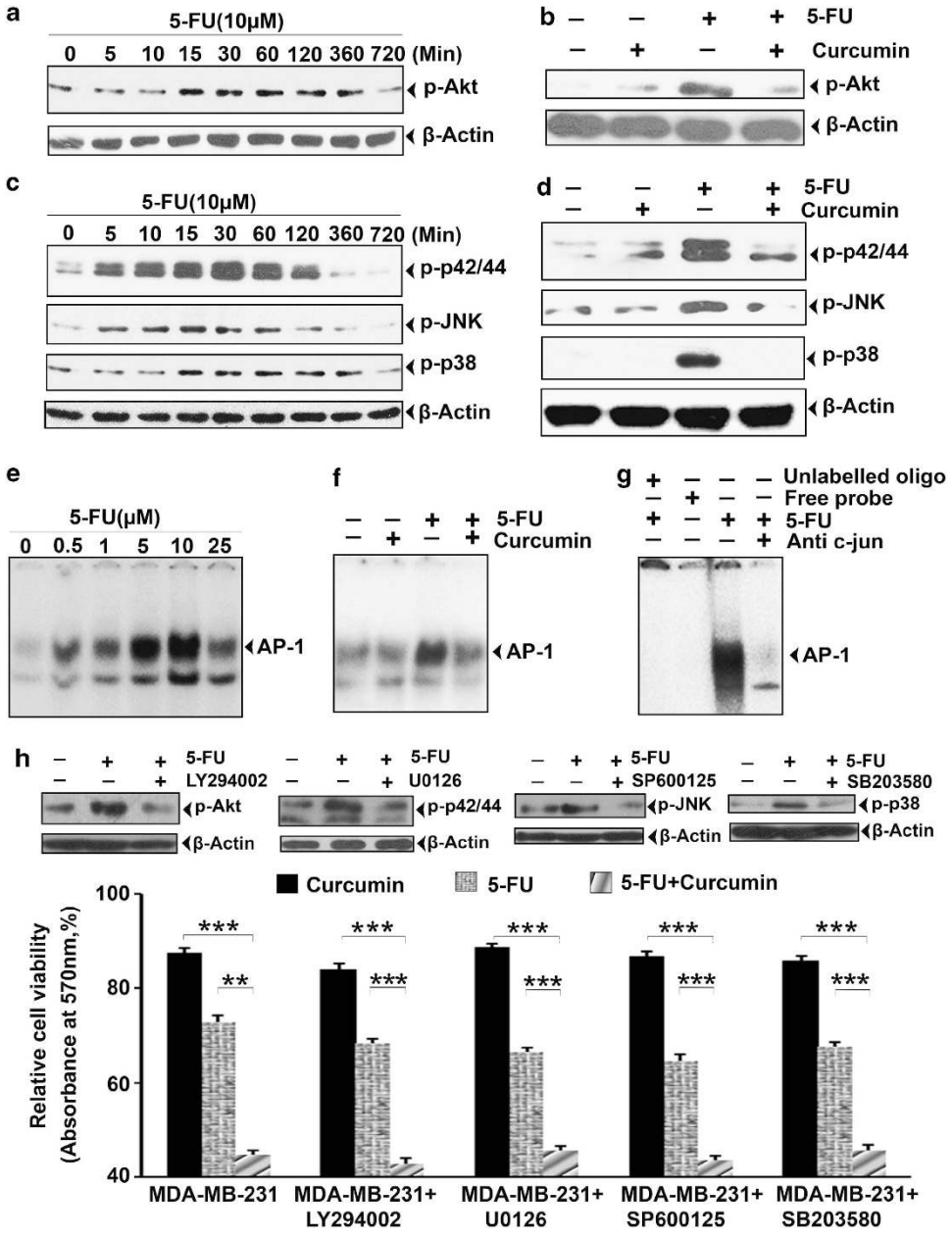

Figure 5 Even though 5-FU induces phosphorylation of Akt and MAPKs in MDA-MB-231 cells and curcumin inhibits this upregulation, the synergism of 5-FU and curcumin is independent of both these survival signals. (**a**) Kinetics of 5-FU-induced activation of Akt in MDA-MB-231 cells after treating them 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 μM 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 μM) 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 μM), U0126 (5 μM), SP600125 (5 μM) and SB203580 (1 μM), 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 $\leqslant$0.0001 and $\leqslant$0.001 respectively. Inhibition status of Akt and various MAPKs were shown in inset

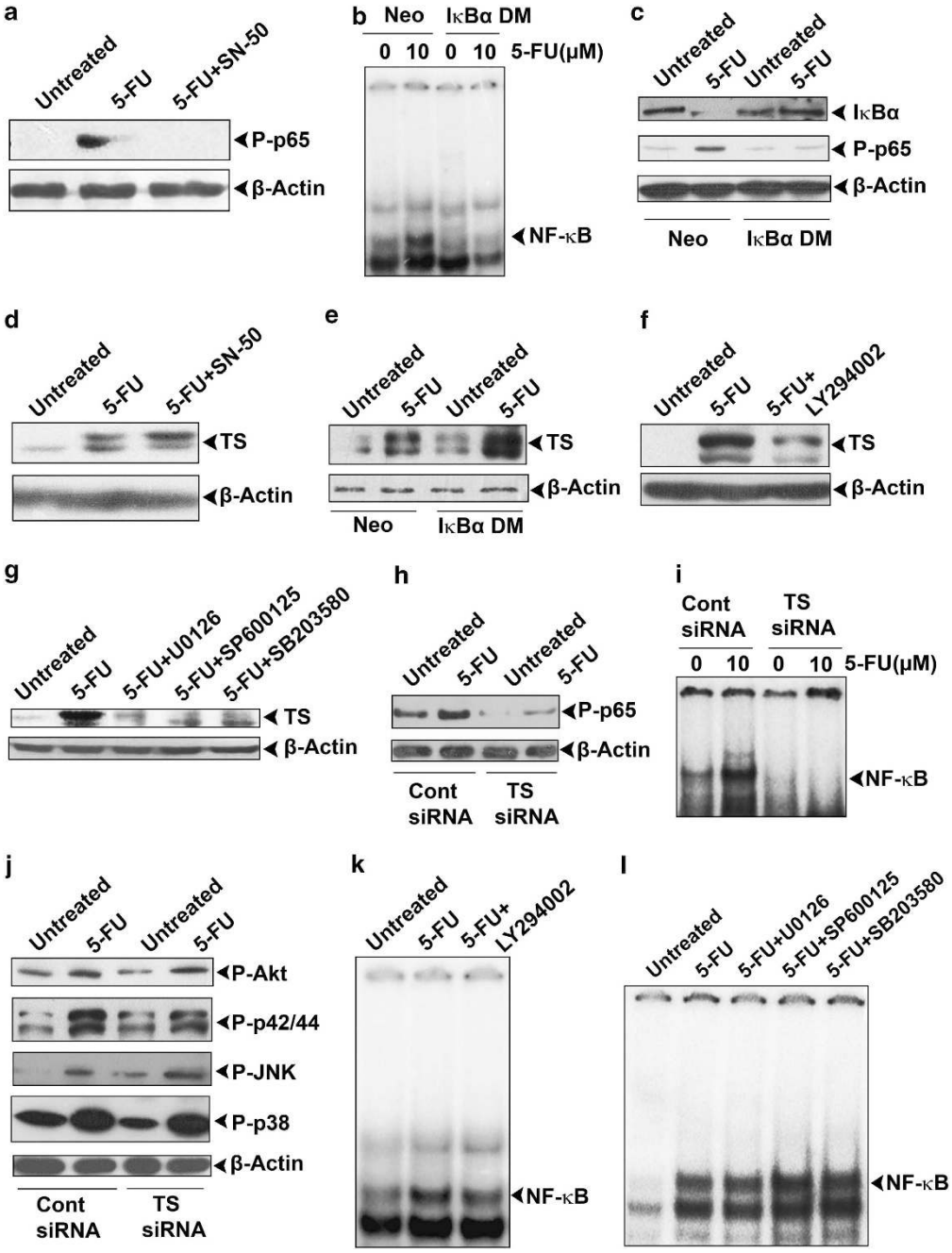

Figure 6 MAPK and Akt are upstream and NF-κB is downstream of TS. (**a**) Inhibition of NF-κB using the peptide inhibitor SN-50. Phospho-p65 status was used to check the inhibition of NF-κB using SN-50. Western blot analysis was carried out using whole cell lysate from MDA-MB-231 cells pre-treated with SN-50 and subsequently to 5-FU. (**b**) 5-FU induced NF-κB activation in MDA-MB-231-Neo cells, while it failed to induce the same in MDA-MB-231-IκB-α DM cells. Nuclear extracts prepared after exposing Neo and IκB-α DM cells to 10 μM 5-FU were subjected to electrophoretic mobility shift assay (EMSA) to check activation of NF-κB. (**c**) 5-FU failed to induce IκBα degradation and p65 phosphorylation in NF-κB-inhibited MDA-MB-231 cells. Western blot analysis was carried out after treating Neo and IκB-α DM cells to 10 μM 5-FU and expression of phospho-p65 and IκB-α was checked. (**d** and **e**) Effect of NF-κB inactivation on 5-FU-induced TS activation. NF-κB expression was inhibited in MDA-MB-231 cells either by SN-50 or by transient transfection using IκB-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 μM), U0126 (10 μM), SP600125 (25 μM) and SB203580 (20 μM) 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-κB 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 μM 5-FU for 30 min and checked for phosphorylation of p65 by western blot and NF-κB 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 μM 5-FU for 30 min and checked for phosphorylation of Akt and MAPKs by western blotting. (**k**) Effect of 5-FU induced NF-κB DNA-binding activity upon silencing of Akt. MDA-MB-231 cells pre-treated with LY294002 (5 μM) were treated with 5-FU for 1 h, nuclear extracts were prepared and EMSA was performed. (**l**) Effect of 5-FU-induced NF-κB DNA-binding activity upon silencing of MAPKs. Nuclear extracts were prepared from MDA-MB-231 cells pre-treated with U0126 (10 μM), SP600125 (50 μM) or SB203580 (40 μM), 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-κB pathway and regulate the synergism.

To locate the position of TS and NF-κB in 5-FU signaling, we transiently silenced TS expression using TS siRNA and checked the phosphorylation and nuclear translocation of NF-κB 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-κB nuclear translocation in TS-silenced cells (Figure 6i). These results clearly prove that NF-κB 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-κB (Figures 6k and l), confirming that 5-FU directly induces TS-mediated activation of NF-κB, independent of Akt and MAPK pathways. Hence, these experiments clearly prove that NF-κB 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-κB 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-κB, 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-κB. We checked whether the synergism exists in other breast cancer cells with different receptor status. Interestingly, 10 μM 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-κB is activated in both the cell lines by 5-FU (Figure 7b) and found that 10 μM curcumin inhibits it almost completely (Figure 7c). We also silenced TS and shutdown NF-κB 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-κB inhibited the synergism almost completely (Figure 7d), establishing that TS and NF-κB 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-κB. Several studies have shown that downregulation of NF-κB by various means could enhance therapeutic efficacy of 5-FU.[15,17] The ability of curcumin to downregulate constitutive and inducible NF-κB, 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-κB, Akt and Bcl-2.[2,23,29] In this study, we observed that curcumin could drastically downregulate 5-FU-induced NF-κB 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

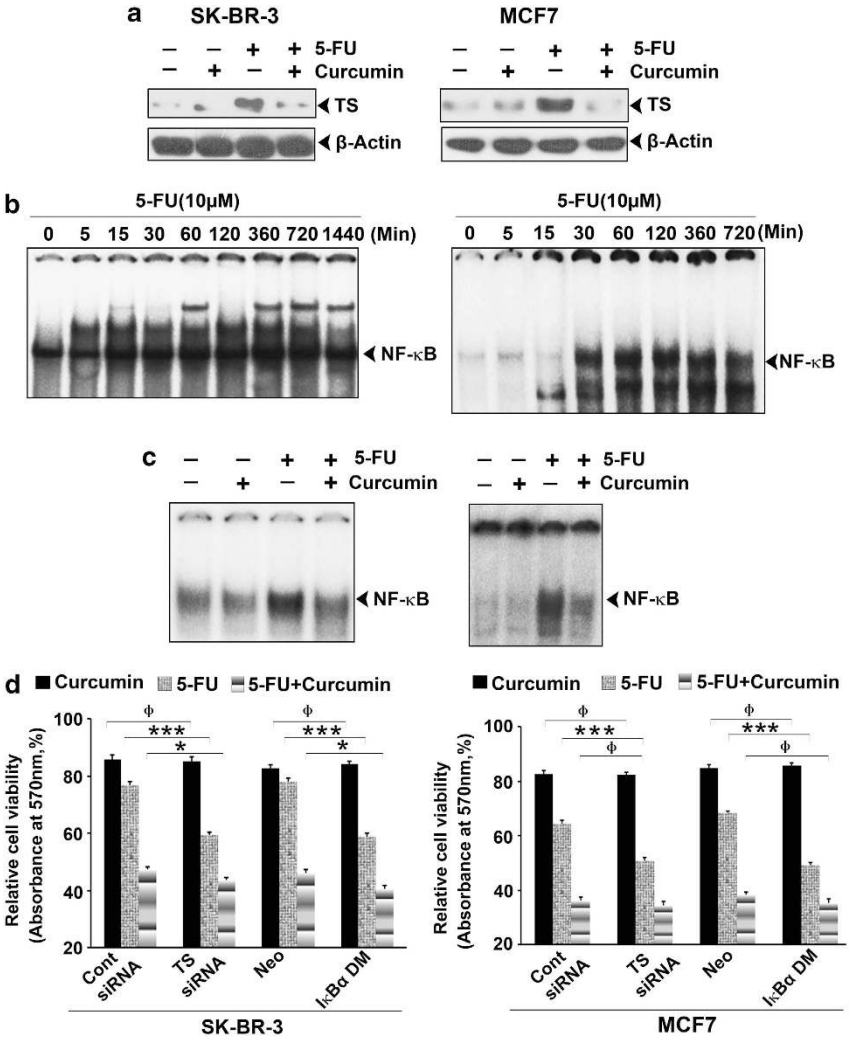

Figure 7 The synergism between 5-FU and curcumin is observed in various breast cancer cells of different receptor status. (**a**) Curcumin-mediated downregulation of 5-FU induced TS activation in SK-BR-3 and MCF7 cells. Cells were pre-treated with curcumin and subsequently exposed to 5-FU for 48 h and subjected to western blotting using TS antibody. (**b**) Kinetics of 5-FU-induced NF-κB activation in SK-BR-3 and MCF7 cells. Nuclear lysates were prepared after exposing the cells to 10 μM 5-FU for different time periods and electrophoretic mobility shift assay (EMSA) was performed. (**c**) Effect of curcumin in 5-FU-induced NF-κB 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-κB-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 ± S.D. ***, * and $^{\Phi}$ represents *P*-values $\leqslant 0.0001$, $\leqslant 0.05$ 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-κB,[29] in this study we found that inhibition of both these pathways did not have an effect on NF-κB (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-κB pathway and regulate the synergism.

Cross-talk between TS and NF-κB pathways in 5-FU therapy has not been reported. However, high NF-κB 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-κB and TS could enhance the efficacy of 5-FU chemotherapy.[43] However, no study has yet reported the interdependence of TS and NF-κB. In this study, we found that silencing of TS considerably inhibits both constitutive and 5-FU-induced NF-κB expression in the breast cancer cell line MDA-MB-231. We also observed that 5-FU upregulates TS and NF-κB, and curcumin downregulates both of them. The inhibition of TS as well as NF-κB 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 μM.[44] As our study indicates that the combined use of curcumin with 10 μM 5-FU can increase the cytotoxic effect of 5-FU to more than double,

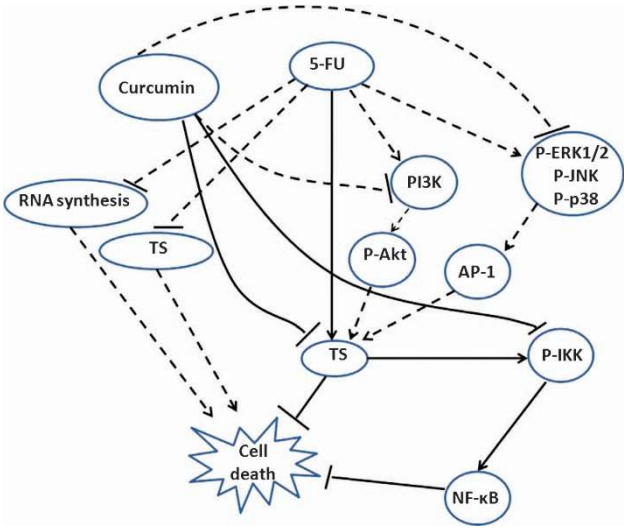

Figure 8 Proposed model for the synergistic effect of 5-FU and curcumin: 5-FU is a well-known inhibitor of TS but also leads to the upregulation of the same on prolonged exposure. It also activates NF-κB, Akt and MAPKs in several cell systems. This study postulates that TS-dependent NF-κB upregulation 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-κB, both directly and also via TS, thereby circumventing 5-FU resistance. The physiologically achievable concentration of curcumin when used orally is around 2 μM.[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 apigenin[50] and tangeretin[51] 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 μM) was added 6 h before 5-FU (10 μM) treatment. The DMSO concentration in all experiments, including controls, was $\leqslant$0.2%.

MTT assay. Proliferative/cytotoxic effect of 5-FU and/or curcumin was determined by MTT assay as described earlier[23] and the relative cell viability percentage is expressed as (Abs_{570} of treated wells/Abs_{570} 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 $^{\Phi}$ represents *P*-values $\leqslant$0.0001, $\leqslant$0.001, $\leqslant$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.

[^{3}H]thymidine incorporation assay. Inhibition of DNA synthesis induced by various drugs was assessed by [^{3}H]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, [^{3}H]thymidine was added (0.2 μCi 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 μg 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-κB or AP-1 as described earlier.[53] In brief, 10 μg of nuclear proteins was incubated for 30 min with ^{32}P-end-labeled double-stranded NF-κB (5′-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-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, IκBαDM-pcDNA3 vector and empty vector using Lipofectamine 2000 reagent

according to the manufacture'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.

Acknowledgements. This work was supported by a program support grant from the Department of Biotechnology, Government of India. We acknowledge Dr. Tessy T Maliekal for meticulously going through the manuscript and Arun Kumar T Thulasidasan for technical help.

1. Aggarwal BB, Shishodia S, Takada Y, Banerjee S, Newman RA, Bueso-Ramos CE *et al.* 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* 2005; **11**: 7490–7498.
2. Sreekanth CN, Bava SV, Sreekumar E, Anto RJ. Molecular evidences for the chemosensitizing efficacy of liposomal curcumin in paclitaxel chemotherapy in mouse models of cervical cancer. *Oncogene* 2011; **30**: 3139–3152.
3. Vinod BS, Maliekal TT, Anto RJ. Phytochemicals as chemosensitizers: from molecular mechanism to clinical significance. *Antioxid Redox Signal* 2012; e-pub ahead of print 9 October 2012; doi:10.1089/ars.2012.4573.
4. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 2003; **3**: 330–338.
5. Chu E, Callender MA, Farrell MP, Schmitz JC. Thymidylate synthase inhibitors as anticancer agents: from bench to bedside. *Cancer Chemother Pharmacol* 2003; **52**(Suppl 1): S80–S89.
6. Ghoshal K, Jacob ST. An alternative molecular mechanism of action of 5-fluorouracil, a potent anticancer drug. *Biochem Pharmacol* 1997; **53**: 1569–1575.
7. Major PP, Egan E, Herrick D, Kufe DW. 5-Fluorouracil incorporation in DNA of human breast carcinoma cells. *Cancer Res* 1982; **42**: 3005–3009.
8. Cho IJ, Chang HJ, Lee KE, Won HS, Choi MY, Nam EM *et al.* A case of Wernicke's encephalopathy following fluorouracil-based chemotherapy. *J Korean Med Sci* 2009; **24**: 747–750.
9. Chu E, Koeller DM, Johnston PG, Zinn S, Allegra CJ. Regulation of thymidylate synthase in human colon cancer cells treated with 5-fluorouracil and interferon-gamma. *Mol Pharmacol* 1993; **43**: 527–533.
10. Swain SM, Lippman ME, Egan EF, Drake JC, Steinberg SM, Allegra CJ. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. *J Clin Oncol* 1989; **7**: 890–899.
11. Lenz HJ, Hayashi K, Salonga D, Danenberg KD, Danenberg PV, Metzger R *et al.* P53 point mutations and thymidylate synthase messenger RNA levels in disseminated colorectal cancer: an analysis of response and survival. *Clin Cancer Res* 1998; **4**: 1243–1250.
12. Rahman L, Voeller D, Rahman M, Lipkowitz S, Allegra C, Barrett JC *et al.* Thymidylate synthase as an oncogene: a novel role for an essential DNA synthesis enzyme. *Cancer Cell* 2004; **5**: 341–351.
13. Wang S, Kotamraju S, Konorev E, Kalivendi S, Joseph J, Kalyanaraman B. Activation of nuclear factor-kappaB during doxorubicin-induced apoptosis in endothelial cells and myocytes is pro-apoptotic: the role of hydrogen peroxide. *Biochem J* 2002; **367**: 729–740.
14. Nakanishi C, Toi M. Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* 2005; **5**: 297–309.
15. Kodach LL, Bos CL, Duran N, Peppelenbosch MP, Ferreira CV, Hardwick JC. Violacein synergistically increases 5-fluorouracil cytotoxicity, induces apoptosis and inhibits Akt-mediated signal transduction in human colorectal cancer cells. *Carcinogenesis* 2006; **27**: 508–516.
16. Wang W, McLeod HL, Cassidy J. Disulfiram-mediated inhibition of NF-kappaB activity enhances cytotoxicity of 5-fluorouracil in human colorectal cancer cell lines. *Int J Cancer* 2003; **104**: 504–511.
17. Wu H, Li W, Wang T, Shu Y, Liu P. Paeoniflorin suppress NF-kappaB activation through modulation of I kappaB alpha and enhances 5-fluorouracil-induced apoptosis in human gastric carcinoma cells. *Biomed Pharmacother* 2008; **62**: 659–666.
18. Jin W, Wu L, Liang K, Liu B, Lu Y, Fan Z. Roles of the PI-3K and MEK pathways in Ras-mediated chemoresistance in breast cancer cells. *Br J Cancer* 2003; **89**: 185–191.
19. Perez-Tenorio G, Stal O. Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. *Br J Cancer* 2002; **86**: 540–545.
20. Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature* 2001; **410**: 37–40.
21. Kunnumakkara AB, Anand P, Aggarwal BB. Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. *Cancer Lett* 2008; **269**: 199–225.
22. Kanai M, Yoshimura K, Asada M, Imaizumi A, Suzuki C, Matsumoto S *et al.* A phase I/II study of gemcitabine-based chemotherapy plus curcumin for patients with gemcitabine-resistant pancreatic cancer. *Cancer Chemother Pharmacol* 2011; **68**: 157–164.
23. Bava SV, Puliappadamba VT, Deepti A, Nair A, Karunagaran D, Anto RJ. 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* 2005; **280**: 6301–6308.
24. Li Y, Revalde JL, Reid G, Paxton JW. Modulatory effects of curcumin on multi-drug resistance-associated protein 5 in pancreatic cancer cells. *Cancer Chemother Pharmacol* 2011; **68**: 603–610.
25. Patel BB, Majumdar AP. Synergistic role of curcumin with current therapeutics in colorectal cancer: minireview. *Nutr Cancer* 2009; **61**: 842–846.
26. Tian F, Fan T, Zhang Y, Jiang Y, Zhang X. Curcumin potentiates the antitumor effects of 5-FU in treatment of esophageal squamous carcinoma cells through downregulating the activation of NF-kappaB signaling pathway *in vitro* and *in vivo. Acta Biochim Biophys Sin (Shanghai)* 2012; **44**: 847–855.
27. Peters GJ, Backus HH, Freemantle S, van Triest B, Codacci-Pisanelli G, van der Wilt CL *et al.* Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism. *Biochim Biophys Acta* 2002; **1587**: 194–205.
28. Jackman AL, Kelland LR, Kimbell R, Brown M, Gibson W, Aherne GW *et al.* Mechanisms of acquired resistance to the quinazoline thymidylate synthase inhibitor ZD1694 (Tomudex) in one mouse and three human cell lines. *Br J Cancer* 1995; **71**: 914–924.
29. Bava SV, Sreekanth CN, Thulasidasan AK, Anto NP, Cheriyan VT, Puliyappadamba VT *et al.* Akt is upstream and MAPKs are downstream of NF-kappaB in paclitaxel-induced survival signaling events, which are down-regulated by curcumin contributing to their synergism. *Int J Biochem Cell Biol* 2011; **43**: 331–341.
30. Peters GJ, van der Wilt CL, van Triest B, Codacci-Pisanelli G, Johnston PG, van Groeningen CJ *et al.* Thymidylate synthase and drug resistance. *Eur J Cancer* 1995; **31A**: 1299–1305.
31. Chu E, Zinn S, Boarman D, Allegra CJ. Interaction of gamma interferon and 5-fluorouracil in the H630 human colon carcinoma cell line. *Cancer Res* 1990; **50**: 5834–5840.
32. Copur S, Aiba K, Drake JC, Allegra CJ, Chu E. Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. *Biochem Pharmacol* 1995; **49**: 1419–1426.
33. Kawate H, Landis DM, Loeb LA. Distribution of mutations in human thymidylate synthase yielding resistance to 5-fluorodeoxyuridine. *J Biol Chem* 2002; **277**: 36304–36311.
34. Ferguson PJ, Collins O, Dean NM, DeMoor J, Li CS, Vincent MD *et al.* Antisense down-regulation of thymidylate synthase to suppress growth and enhance cytotoxicity of 5-FUdR, 5-FU and Tomudex in HeLa cells. *Br J Pharmacol* 1999; **127**: 1777–1786.
35. Bras-Goncalves RA, Pocard M, Formento JL, Poirson-Bichat F, De Pinieux G, Pandrea I *et al.* Synergistic efficacy of 3n-butyrate and 5-fluorouracil in human colorectal cancer xenografts via modulation of DNA synthesis. *Gastroenterology* 2001; **120**: 874–888.
36. Di Gennaro E, Bruzzese F, Pepe S, Leone A, Delrio P, Subbarayan PR *et al.* Modulation of thymidilate synthase and p53 expression by HDAC inhibitor vorinostat resulted in synergistic antitumor effect in combination with 5FU or raltitrexed. *Cancer Biol Ther* 2009; **8**: 782–791.
37. Jin HS, Lee DH, Kim DH, Chung JH, Lee SJ, Lee TH. cIAP1, cIAP2, and XIAP act cooperatively via nonredundant pathways to regulate genotoxic stress-induced nuclear factor-kappaB activation. *Cancer Res* 2009; **69**: 1782–1791.
38. Ferguson PJ, DeMoor JM, Vincent MD, Koropatnick J. Antisense-induced down-regulation of thymidylate synthase and enhanced cytotoxicity of 5-FUdR in 5-FUdR-resistant HeLa cells. *Br J Pharmacol* 2001; **134**: 1437–1446.
39. Okabe T, Okamoto I, Tsukioka S, Uchida J, Iwasa T, Yoshida T *et al.* Synergistic antitumor effect of S-1 and the epidermal growth factor receptor inhibitor gefitinib in non-small cell lung cancer cell lines: role of gefitinib-induced down-regulation of thymidylate synthase. *Mol Cancer Ther* 2008; **7**: 599–606.
40. Johnston PG, Mick R, Recant W, Behan KA, Dolan ME, Ratain MJ *et al.* Thymidylate synthase expression and response to neoadjuvant chemotherapy in patients with advanced head and neck cancer. *J Natl Cancer Inst* 1997; **89**: 308–313.
41. Pestalozzi BC, Peterson HF, Gelber RD, Goldhirsch A, Gusterson BA, Trihia H *et al.* Prognostic importance of thymidylate synthase expression in early breast cancer. *J Clin Oncol* 1997; **15**: 1923–1931.
42. Wang W, Cassidy J. Constitutive nuclear factor-kappa B mRNA, protein overexpression and enhanced DNA-binding activity in thymidylate synthase inhibitor-resistant tumour cells. *Br J Cancer* 2003; **88**: 624–629.
43. Wang HE, Wu HC, Kao SJ, Tseng FW, Wang YS, Yu HM *et al.* Modulation of 5-fluorouracil cytotoxicity through thymidylate synthase and NF-kappaB down-regulation and its application on the radiolabelled iododeoxyuridine therapy on human hepatoma cell. *Biochem Pharmacol* 2005; **69**: 617–626.
44. Clarke R, Morwood J, van den Berg HW, Nelson J, Murphy RF. Effect of cytotoxic drugs on estrogen receptor expression and response to tamoxifen in MCF-7 cells. *Cancer Res* 1986; **46**: 6116–6119.
45. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS *et al.* Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res* 2001; **21**: 2895–2900.

46. Deepa G, Thulasidasan AK, Anto RJ, Pillai JJ, Kumar GV. Cross-linked acrylic hydrogel for the controlled delivery of hydrophobic drugs in cancer therapy. *Int J Nanomed* 2012; **7**: 4077–4088.
47. Nair KL, Thulasidasan AK, Deepa G, Anto RJ, Kumar GS. Purely aqueous PLGA nanoparticulate formulations of curcumin exhibit enhanced anticancer activity with dependence on the combination of the carrier. *Int J Pharm* 2012; **425**: 44–52.
48. Li L, Ahmed B, Mehta K, Kurzrock R. Liposomal curcumin with and without oxaliplatin: effects on cell growth, apoptosis, and angiogenesis in colorectal cancer. *Mol Cancer Ther* 2007; **6**: 1276–1282.
49. Narayanan NK, Nargi D, Randolph C, Narayanan BA. Liposome encapsulation of curcumin and resveratrol in combination reduces prostate cancer incidence in PTEN knockout mice. *Int J Cancer* 2009; **125**: 1–8.
50. Choi EJ, Kim GH. 5-Fluorouracil combined with apigenin enhances anticancer activity through induction of apoptosis in human breast cancer MDA-MB-453 cells. *Oncol Rep* 2009; **22**: 1533–1537.
51. Meiyanto E, Hermawan A. Anindyajati. Natural products for cancer-targeted therapy: citrus flavonoids as potent chemopreventive agents. *Asian Pac J Cancer Prev* 2012; **13**: 427–436.
52. Chou TC. Drug combination studies and their synergy quantification using the Chou–Talalay method. *Cancer Res* 2010; **70**: 440–446.
53. Puliyappadamba VT, Cheriyan VT, Thulasidasan AK, Bava SV, Vinod BS, Prabhu PR *et al.* Nicotine-induced survival signaling in lung cancer cells is dependent on their p53 status while its down-regulation by curcumin is independent. *Mol Cancer* 2010; **9**: 220.

Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)