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Inhibition of RelA phosphorylation sensitizes apoptosis in constitutive NF-kappaB-expressing and chemoresistant cells   - Retracted -

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The compound 5-(4-methoxyarylimino)-2-*N*-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine (P₃-25) is known to possess anti-bacterial, anti-fungal, anti-tubercular, and local anesthetic activities. We studied the anti-tumorigenic activity of P₃-25 and the role of nuclear transcription factor kappaB (NF-κB) in this process. In constitutive NF-κB-expressing cells, treatment with P₃-25 inhibited the expression of NF-κB-dependent reporter gene, adhesion molecules, and cyclooxygenase. It downregulated phosphorylation of p65 by inhibiting upstream kinases, such as protein kinase A and casein kinase II, but did not alter NF-κB DNA-binding activity. Alone, P₃-25 induced apoptosis in NF-κB-expressing and doxorubicin-resistant breast cancer cells, and in the presence of other chemotherapeutic agents, it potentiated apoptosis. Overall, our results suggest that P₃-25 exerts antitumorigenic activity by inhibiting phosphorylation of p65, the transcriptionally active subunit of NF-κB by inhibiting its upstream kinases, and potentiates apoptosis mediated by chemotherapeutic agents. These results suggest novel approaches for designing of anticancer drugs for combination chemotherapy.
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Nuclear transcription factor kappa B (NF-κB) has a role in oncogenesis and its regulation is important in cancer therapy. Overexpression, amplification, and rearrangements of different genes related to NF-κB have been observed in tumors.[1] NF-κB is activated in response to various inflammatory stimuli including cytokines, mitogens, bacterial products, viral proteins, and apoptosis-inducing agents.[2,3] Although the primary level of NF-κB activation lies in the removal of the inhibitory subunit of NF-κB (inhibitory subunit of kappa B (IκBα)), the phosphorylation of p65 is required to recruit the transcriptional apparatus and to stimulate expression of several genes including cyclin D1, adhesion molecules, cyclooxygenase (Cox)-2, and matrix metalloproteinases. The activity of casein kinase II (CKII), known to be induced by several growth stimuli, is elevated in a variety of human cancers.[4] Cytoplasmic NF-AT (cytoplasmic nuclear factor of activated T cells (NF-Atc)) is phosphorylated by CKII and translocated into the nucleus.[5] RelA (p65) is phosphorylated at multiple serine residues such as Ser529,[6,7] Ser536,[8] and Ser276 [9] by CKII, IκB kinase (IKK), and protein kinase A (PKA)/mitogen and stress-activated protein kinase (MSK), respectively. PKA catalytic subunit binds with IκBα of NF-κB/IκB complex and remains inactive. When IκB is degraded, PKA becomes activated and phosphorylates p65 at Ser276.[10] PKA activates another

transcription factor cyclic AMP responsive element-binding protein (CREB) directly or via p38 MAPK and MSK1 pathways.[11]

Higher activity of NF-κB is a common characteristic of many tumor cells.[1,2,12,13] HuT-78, a cutaneous lymphoid T cell line that constitutively expresses NF-κB, is resistant to TNF-induced apoptosis.[14,15] Constitutive activity of IKK, mediated by different factors like interleukin-1, epidermal growth factor, heregulin, and so on, has been shown in constitutive NF-κB-expressing cells.[16] BAY 11-7082 (BAY) is known to inhibit IKK, and thereby inhibits IκBα phosphorylation and NF-κB activation.[17,18] Constitutive expression of NF-κB leads to activation of several factors involved in cell cycle progression and cell differentiation for cancer metastasis. Inhibiting NF-κB activity in tumor cells dramatically reduced the cell growth *in vitro* and *in vivo*.[19] NF-κB, possibly through the activation of the antiapoptotic genes, plays a key role in the protection of cells against inducers of apoptosis including chemotherapeutic drugs.[13] Several mechanisms including increased expression of NF-κB proteins, mutations and/or deletions in IκBα gene, and increased IκBα turnover, and so on, are involved in NF-κB activation in tumor cells.[1,20] In spite of the growing evidence of the important role of NF-κB in tumorigenesis and its resistance to chemotherapy, only few attempts have been made to understand the mechanisms of the constitutive

activity of NF-κB in tumor cells and its regulation for successful therapy.

The thiazolidones and thiadiazolie have drawn considerable attention for their anti-bacterial,[21] anti-fungal,[21] and anti-inflammatory [22] activities. Considering these effects and NF-κB's role in inflammation and tumor biology, newer derivatives of 1,2,4-thiadiazolidines, including 3-oxo-1,2,4-thiadiazolidines,[23] were synthesized by using the oxidative debenzylation technique and their biological effects were studied.[24] The dichlorophenyl derivatives of 1,2,4-thiadiazolidine were shown to inhibit TNF-induced activation of NF-κB through the inhibition of IKK activation.[25]

The aim of this study is to understand the molecular mechanisms underlying the resistance against chemotherapeutic agents due to basal NF-κB activation in different cancer cells. We have tested the role of upstream kinases for p65 phosphorylation and, thereby, the NF-κB-dependent genes transcription in those tumor cells that constitutively expressed NF-κB. Our results indicate that NF-κB is constitutively activated in different tumor cells such as HuT-78, HT-29, DU145, and PC3, but not Jurkat cell lines. The high level of phosphorylated p65 and its upstream kinases such as PKA and CKII in those cells led to the activation of several NF-κB-dependent genes. In this report, first the time we provide evidence that the dimethyl form of 1,2,4 thiadiazolidine inhibited PKA and CKII activities and consequently decreased phosphorylation of p65 and this resulted in the downregulation of transcription of several NF-κB-dependent genes involved in cell proliferation and metastasis. This compound potentiated cell death, mediated by chemotherapeutic agents in those constitutive NF-κB-expressing cells. Taken together, this study helps to understand the importance of phosphorylation of p65 in constitutive NF-κB-expressing cells for its tumorigenic activities. Downregulation of these kinases leads to the potentiation of apoptotic responses mediated by chemotherapeutic agents, which may help to further design oxo-1,2, 4-thiadiazolidine compounds as potential antitumor drugs for combination therapy.

Results

In this study, we examined the effect of 5-(4-methoxyarylimino)-2-*N*-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine (designated as P$_3$-25 throughout the study) in NF-κB-expressing cells on phosphorylation of p65 and DNA-binding activity of NF-κB. Cell lines such as HuT-78, HT-29, DU145, and PC3 have constitutive activity of NF-κB. Jurkat cells, pulsed with 10 pM TNF for 1 h (indicated as Jkt/TNF), maintained high NF-κB activity for at least the duration of the experiments. All these cells were used as NF-κB-expressing cells for the entire study. P$_3$-25 was chemically synthesized in the laboratory as described previously[24] and was used as a solution in DMSO at 10 mM concentration. Further dilution was carried out in cell culture medium. The concentrations and duration of exposure of P$_3$-25 employed in these studies had no effect on cytolysis as detected by the lactate dehydrogenase (LDH) assay from culture supernatant of treated cells (Culture supernatant from 100 nM P$_3$-25-treated HuT-78 cells for 0, 6, 12, and 24 h when incubated with substrate solution (0.23 M sodium pyruvate and 5 mM NADH in 0.1 M phosphate buffer, pH 7.5)

showed 1.214 ± 0.102, 1.202 ± 0.092, 1.192 ± 0.124, and 1.172 ± 0.114 absorbances, respectively, at 340 nm).

P$_3$-25 blocks NF-κB-dependent reporter gene expression. NF-κB-expressing tumor cells, Jurkat, HuT-78, HT-29, DU145, and PC3 cells were cotransfected with a reporter plasmid containing an NF-κB-binding site (wild or mutant type) cloned upstream of heat-stable secretory alkaline phosphatase (SEAP) designated as NF-κB-SEAP or NF-κB(mut)-SEAP and green fluorescent protein (GFP) constructs for 3 h using SuperFect reagent (Qiagen, Germany). Cells were cultured for 12 h. Transfection efficiencies for Jurkat, HuT-78, HT-29, DU145, and PC3 cells were found to be 24, 25, 28, 27, and 29%, respectively, measured by GFP-positive cell count. Jurkat cells, stimulated with TNF (10 pM) for 1 h (Jkt/TNF), were cultured for 12 h with other cells. Then the cells were treated with P$_3$-25 for different time periods or 2% DMSO for 12 h. The SEAP activity was assayed in culture supernatant. Results, expressed as fold increase in activity over the nontransfected control, in Figure 1a showed that Jkt/TNF, HuT-78, HT-29, DU145, and PC3 cells induced an 8–9-fold increase in SEAP activity, indicating the activation of NF-κB-dependent SEAP reporter gene. Treatment with DMSO (2% for 12 h, control) did not interfere with the basal SEAP activity in these cells. NF-κB(mut)-SEAP-transfected cells showed no basal SEAP activity in all these cells. P$_3$-25 treatment decreased the basal activity of SEAP in a time-dependent manner in all these cells, suggesting P$_3$-25-mediated downregulation of NF-κB-dependent reporter gene in different cell types.

P$_3$-25 does not inhibit DNA-binding activity of NF-κB. NF-κB-expressing cells were treated with 100 nM P$_3$-25 for different times and then the DNA-binding activity of NF-κB was measured by a gel shift assay from nuclear extracts. TNF-induced NF-κB binding decreased partially on treatment with P$_3$-25 in a time-dependent manner without interfering with the basal DNA-binding activity of NF-κB (Figure 1b). These data suggest that P$_3$-25 decreases the expression of NF-κB-dependent reporter gene without altering constitutive NF-κB DNA binding.

Nuclear extracts from HuT-78 or DU145 cells were incubated with antibodies (Abs) to p50 and p65 alone or in combination, and then the gel shift assay was conducted to study the specificity and composition of the retarded band. Abs to either subunit of NF-κB shifted the band to a higher molecular weight (Figure 1c), thus suggesting that the retarded complex consisted of both p50 and p65 subunits. Neither preimmune serum nor irrelevant Abs such as anti-c-Rel had any effect on the mobility of NF-κB. This complex completely disappeared in the presence of a 50-fold molar excess of cold NF-κB and was unable to bind with mutant oligonucleotides, indicating its specificity.

P$_3$-25 does not inhibit AP-1-dependent reporter gene expression. HuT-78 and DU145 cells were transfected with plasmids for AP-1 promoter DNA that linked to luciferase (AP-1-Luciferase) and GFP for 3 h. Cells were cultured for 12 h and transfection efficiency determined by GFP-positive

cell count was found to be 26 and 27% for HuT-78 and DU145 cells, respectively. Cells were treated with P_3-25 for different time periods and with curcumin or DMSO for 12 h. The luciferase activity was assayed in cell extracts. Results, expressed as fold activity over the vector-transfected cells, showed that P_3-25 partially inhibited the basal activation of luciferase activity in HuT-78 or DU145 cells (Figure 1d). Curcumin potently inhibited the basal activation of luciferase activity, and DMSO did not interfere in the basal luciferase activity in these cells.

P_3-25 blocks ICAM1 and Cox-2 expression. Jkt/TNF, HuT-78, HT-29, DU145, and PC3 cells were treated with P_3-25 (100 nM) for different time periods and the amounts of NF-κB-dependent gene products, intercellular adhesion molecule (ICAM) 1 and Cox-2, were analyzed by Western blot technique. The amounts of ICAM1 (Figure 2a) and Cox-2 (Figure 2b) were found to decrease with increasing time of incubation with P_3-25. These cells, transfected with Cox-2-luciferase and GFP constructs, showed decreased luciferase activity on treatment with P_3-25 in a time-dependent manner (Figure 2c). The amounts of mRNA of Cox-2 and ICAM1 decreased with increasing time of P_3-25 treatment in HuT-78 and DU145 cells (Figure 2d and e). Treatment for 12 h with BAY, a selective inhibitor of IκBα kinase (IKK) decreases NF-κB, which completely inhibited the basal expression of ICAM1 or Cox-2, Cox-2-dependent expression of luciferase, and basal levels of Cox-2 or ICAM1 mRNA. All these data suggest that P_3-25 downregulates the expression of NF-κB-dependent genes.

P_3-25 induces cell death. NF-κB-expressing cells were treated with P_3-25 for 72 h and then cell viability was tested by the MTT (3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay. The cell viability decreased with increasing concentrations of P_3-25 in NF-κB-expressing cells (Figure 3a). Treatment with BAY alone showed 25–35% and in combination with P_3-25 (100 nM) showed it 70–80% cell death in these cell types. HuT-78 cells were treated with different concentrations of P_3-25 or 5 μM BAY and the cell viability was tested by the 'Live & Dead' cell assay. The percentage of dead cells (in red color) increased in a dose-dependent manner on treatment with P_3-25 (Figure 3b). These results suggest that P_3-25 induces cell death in NF-κB-expressing cells.

P_3-25 induces lipid peroxidation and poly-(ADP)-ribose polymerase cleavage. We also examined the effect on lipid peroxidation and poly ADP-ribose polymerase (PARP)

cleavage under conditions of P_3-25-induced cell death. Constitutive NF-κB-expressing cells were treated with 100 nM P_3-25 for different times or 5 μM BAY for 12 h and then lipid peroxidation was assayed in whole-cell extracts. Treatment with P_3-25 induced lipid peroxidation in HuT-78, HT-29, DU145, and PC3 cells in a time-dependent manner (Figure 3c). Cells treated with BAY did not increase lipid peroxidation significantly. P_3-25 treatment induced PARP cleavage, as 116 kDa PARP cleaved into 85 kDa protein fragment in a time-dependent manner (Figure 3d). The same blot when reprobed with anti-tubulin antibody showed an equal intensity of bands in different lanes suggesting equal loading of extract proteins. These data suggest that P_3-25 is a potent inducer of apoptosis for NF-κB-expressing cells.

P_3-25 inhibits PKAα or CKII but not IKK activity. The Jkt/TNF, HuT-78, HT-29, DU145, and PC3 cells were treated with 100 nM P_3-25 for different times and then activities of IKK, PKAα, and CKII were measured. The IKK activity marginally decreased in Jkt/TNF cells at 6 h of treatment by P_3-25. In other cells, high basal activity of IKK remained unaltered on P_3-25 treatment (Figure 4a). BAY-treated Jkt/TNF or HuT-78 cells showed complete inhibition of IKK activity. The activity of PKAα, assayed either by *in vitro* phosphorylation of GST-p65 protein (Figure 4b) or by kemptide as substrate, decreased in all these cell types (Figure 4c). Pretreatment with BAY did not have an effect on PKA activity in Jkt/TNF or HuT-78 cells. The protein concentrations of PKAα remained unchanged in these cells after P_3-25 treatment. The activity of CKII decreased on P_3-25 treatment in a time-dependent manner in these cells without altering the amounts of its protein (Figure 4d). BAY treatment did not decrease the activity of CKII in Jkt/TNF or HuT-78 cells. All these constitutively NF-κB-expressing cells had high basal IKK, PKAα, and CKII activities. These results indicate that the treatment of these cells with P_3-25 decreases activities of PKAα or CKII, but not of IKK.

P_3-25 decreases phospho-p65 levels. The concentrations of phospho-p65 were measured in whole-cell extracts using anti-phospho-p65 (Ser[276]) or -phospho-p65 (Ser[536]) antibody by Western blot in HuT-78 and DU145, after treatment with BAY for 6 h or P_3-25 for different time intervals. Both forms of phospho-p65 decreased with increasing time of P_3-25 incubation (Figure 4e1 and e2). BAY did not affect phospho-p65 in any of these cells. Upon reprobing those blots with anti-p65 antibody, the amounts of p65 protein were found to be unchanged.

Figure 1 P_3-25 inhibits reporter gene expression dependent on NF-κB-, but not AP-1, and also NF-κB DNA-binding activity in NF-κB-expressing cells. (**a**) Jurkat, HuT-78, HT-29, DU145, and PC3 cells were transfected with Qiagen superfect transfection reagent for 3 h with plasmids for NF-κB promoter (either wild or mutant) DNA that had been linked to SEAP (NF-κB-SEAP or NF-κB(mut)-SEAP) and GFP. After washing, Jurkat cells were stimulated with 10 pM TNF and then all cells were cultured for 12 h. The GFP-positive cells were counted and transfection efficiency was calculated. Cells were treated with P_3-25 (100 nM) for different times or 2% DMSO for 12 h and culture supernatant was assayed for SEAP activity. The results are represented as fold of activation over the nontransfected control. (**b**) Jkt/TNF (Jurkat cells were stimulated with 10 pM TNF for 1 h), HuT-78, HT-29, DU145, and PC3 cells were treated with P_3-25 (100 nM) for different times or 2% DMSO (for Jkt/TNF and HuT-78 cells) for 12 h. The nuclear extracts were prepared and assayed for NF-κB by the gel shift assay. (**c**) Supershift of NF-κB band. Nuclear extracts, prepared from HuT-78 or DU145 cells, were incubated with different antibodies and cold NF-κB (wild or mutant, 200 ng each) oligonucleotides for 15 min and then assayed for NF-κB. (**d**) HuT-78 and DU145 cells, transfected with Qiagen superfect transfection reagent for 3 h with plasmids for AP-1 promoter DNA that linked to luciferase reporter gene and GFP, were cultured for 12 h. GFP-positive cells were counted. Cells were treated with P_3-25 (100 nM) for different times and curcumin (10 μM) or 2% DMSO for 12 h and the pellet was extracted and assayed for luciferase activity

PKAα or CKII does not phosphorylate GST. The extracts of HuT-78 cells, untreated or treated with P$_3$-25 for 6 h, were immunoprecipitated with anti-PKAα or -CKII antibody. The concentration of GST was determined by Western blot technique from one half of immunoprecipitated beads incubated with GST or GST-p65 substrate in the presence of ATP. Bands appeared at 93 kDa (for full length p65 fused with GST), 45 kDa (for IgG, light chain), and 28 kDa (for GST
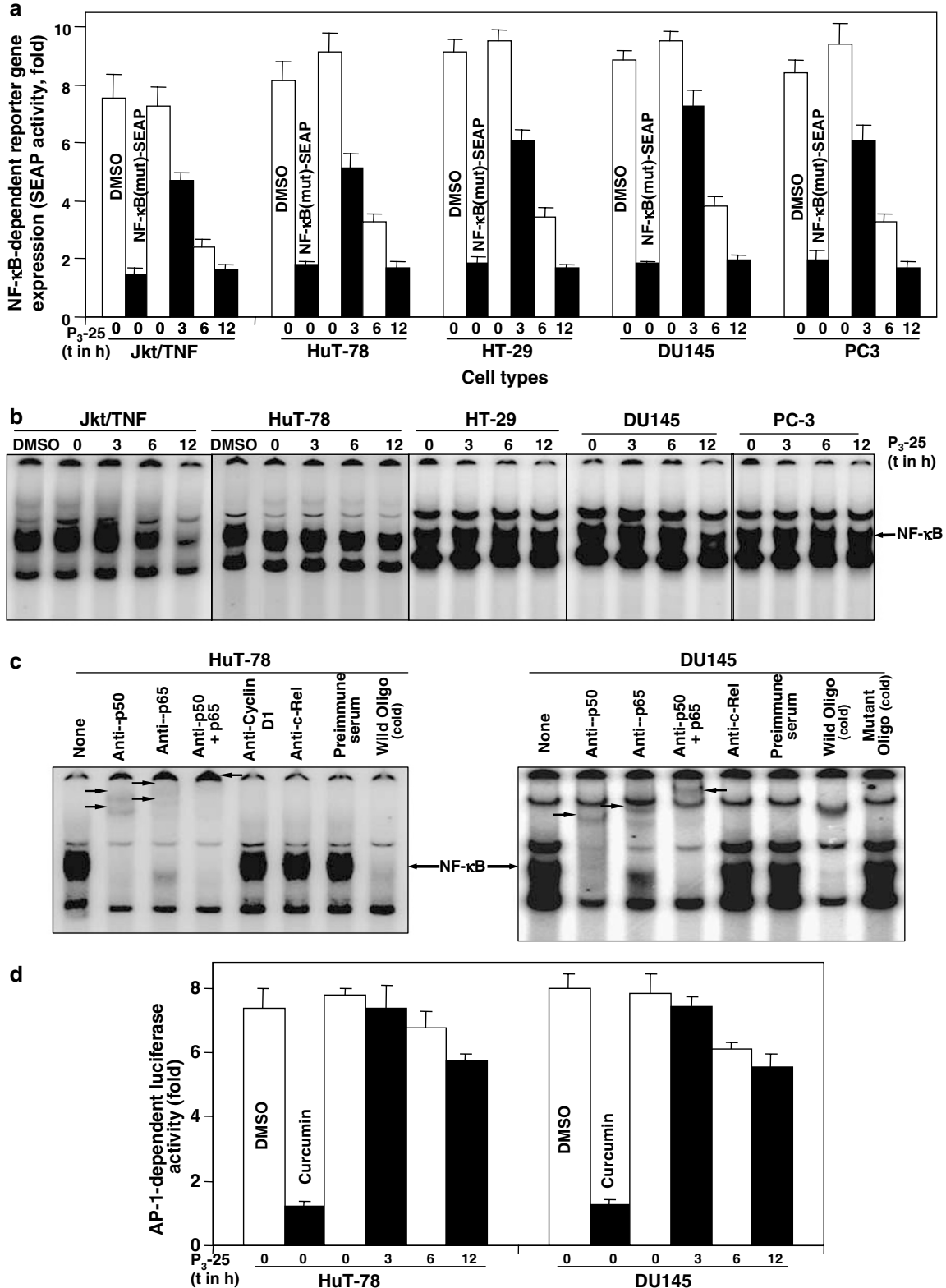
alone) positions for those samples immunoprecipitated with anti-PKAα or -CKII antibody (Figure 4f1 and f2, upper panels). The p65 bands appeared at 93 kDa position by reprobing the same blot (Figure 4f1 and f2, middle panels). The other half of the immunoprecipitated beads, used for assay of PKAα or CKII *in vitro*, showed no phosphorylation of GST but phosphorylated GST-p65. Treatment with P$_3$-25 inhibited this phosphorylation (Figure 4f1 and f2, lower panels). These results suggest that the PKAα or CKII phosphorylates *in vitro* GST-p65 (full length), but not GST alone.

P$_3$-25 inhibits NF-ATc or CREB activity. As P$_3$-25 treatment decreased activities of PKAα and CKII, the DNA-binding activities of NF-AT and CREB were measured from nuclear extracts obtained from P$_3$-25-treated HuT-78 and DU145 cells. NF-AT DNA-binding activity decreased with increasing time of P$_3$-25 treatment (Figure 4g1). The luciferase activity decreased in NF-AT-luciferase construct-

transfected HuT-78 and DU145 cells with increasing time of P$_3$-25 treatment (Figure 4g2), suggesting P$_3$-25-mediated downregulation of NF-AT. Similarly, CREB DNA-binding activity also decreased with increasing time of P$_3$-25 treatment in HuT-78 and DU145 cells (Figure 4h). Collectively, these results indicate the inhibition of PKAα- and CKII-mediated cellular activities on treatment of cells with P$_3$-25.

P$_3$-25 inhibits expression of NF-κB-dependent reporter gene and activation of PKAα and induces cell death in Jurkat cells. So far, all experiments were carried out in NF-κB-expressing cells. The effect of P$_3$-25 was also tested in cells such as Jurkat cells that showed very low expression of NF-κB and PKAα activity. Jurkat and HuT-78 cells were treated with P$_3$-25 for different time periods and then the DNA-binding activity of NF-κB was assayed. Treatment with P$_3$-25 did not affect DNA binding by NF-κB in HuT-78 cells, but decreased PKAα activity (Figure 5a). A marginal
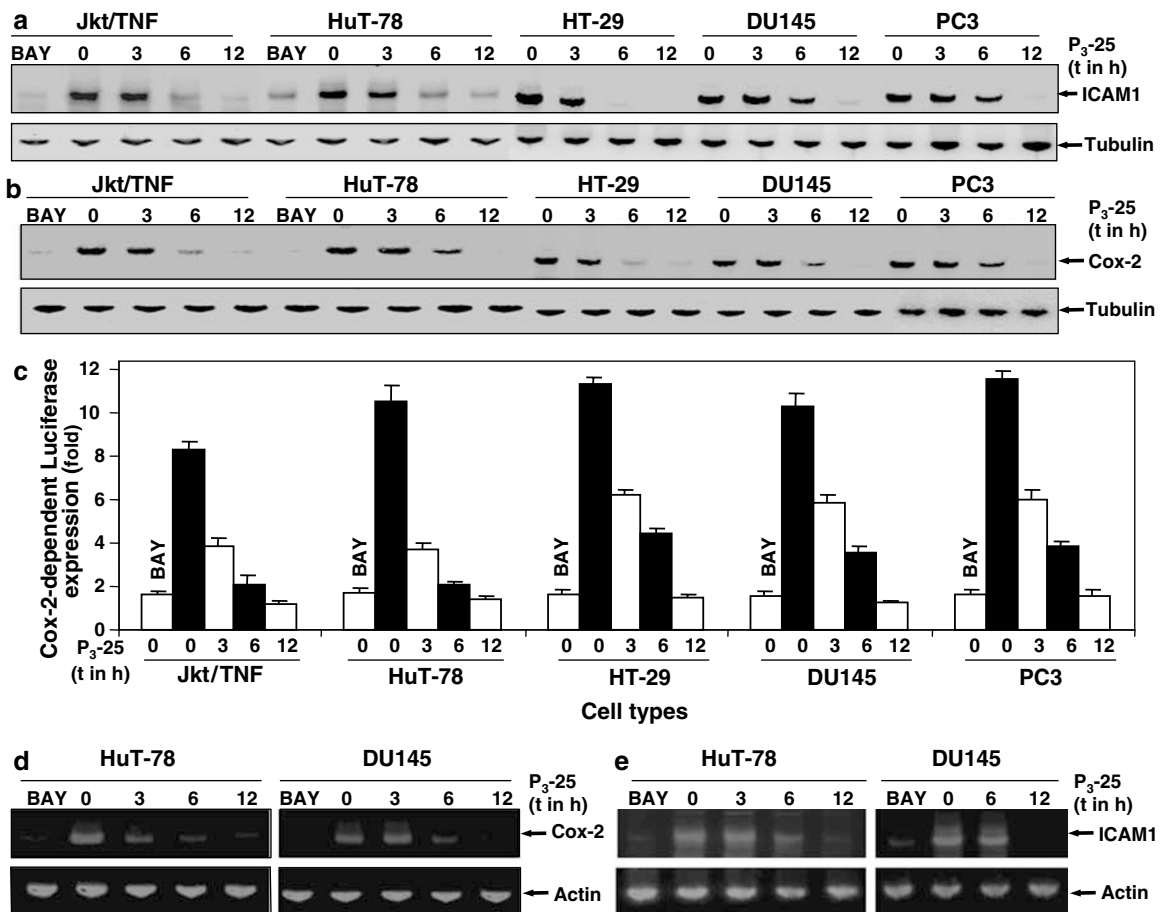

Figure 2 P$_3$-25 inhibits expression of ICAM1 and Cox-2. (**a** and **b**) Jkt/TNF, HuT-78, HT-29, DU145, and PC3 cells were treated with P$_3$-25 (100 nM) for different times at 37°C. Jkt/TNF and HuT-78 cells were separately treated with 5 μM BAY 11-7082 (designated as BAY) for 12 h. ICAM1 (**a**) or Cox-2 (**b**) was detected from whole-cell extract proteins (100 μg) by Western blot. Tubulin was detected by reprobing both blots. (**c**) All these cells were cotransfected with vector, Cox-2-Luciferase, and GFP constructs using SuperFect reagent for 3 h. Cells were washed, cultured for 12 h, and counted for GFP-positive cells. Luciferase activity was assayed from whole-cell extracts prepared from P$_3$-25- (for different times) or BAY- (for 12 h) treated cells, considering vector alone value as one fold. (**d** and **e**) HuT-78 or DU145 cells were treated with 100 nM P$_3$-25 for different times or 5 μM BAY for 12 h. Total RNA was isolated and Cox-2, ICAM1, and actin were detected by RT-PCR followed by PCR using Cox-2, ICAM1, and actin-specific primers at 305, 447, and 496 bp bands, respectively

activation of PKAα was observed in Jurkat cells, which was inhibited by P$_3$-25 in a time-dependent manner. It also inhibited the marginal activation of NF-κB-dependent SEAP activity in Jurkat cells transfected with NF-κB-SEAP construct (Figure 5b). As shown earlier, P$_3$-25 inhibited NF-κB-dependent SEAP activity in HuT-78 cells in a time-dependent manner (Figure 5b). The cell viability, tested by MTT dye uptake, was decreased in a time-dependent manner in both Jurkat and HuT-78 cells (Figure 5c). The results suggest that P$_3$-25 also exerts its effect in cell lines having low activity of NF-κB.

P$_3$-25 potentiates cell death mediated by chemotherapeutic agents. HuT-78, HT-29, DU145, and PC3 cells were treated with 100 nM P$_3$-25 for 4 h and then with chemotherapeutic agents, doxorubicin, taxol, cisplatin, or vincristine (1 μM each), for 72 h. Cell viability was assayed by the MTT assay. Alone, P$_3$-25 or the chemotherapeutic agents induced about 60% cell death in these cell types.

P$_3$-25-pretreated cells showed almost 90% cell death in combination with doxorubicin, taxol, cisplatin, or vincristine (Figure 6a). It appears that P$_3$-25 exerts an additive effect on cell death. P$_3$-25 alone or in combination with different chemotherapeutic agents showed almost similar cell viability in HuT-78 cells as detected by the 'Live & Dead' cell assay (Figure 6b).

PC3 and DU145 cells were treated with P$_3$-25 (100 nM) for 4 h followed by incubation with doxorubicin, taxol, cisplatin, or vincristine (1 μM each) for 72 h. The PARP cleavage was detected by Western blot. These agents and P$_3$-25 alone increased the cleaved fragment of PARP at 85 kDa. In P$_3$-25-pretreated cells, the intensity of cleaved fragment increased more than with agents alone in PC3 (Figure 6c) and DU145 cells (Figure 6d), indicating an additive effect in combination with chemotherapeutic agents. The cisplatin-mediated cleavage of PARP protein also showed an additive effect when Jkt/TNF, HuT-78, HT-29, DU145, and PC3 cells were pretreated with P$_3$-25 (Figure 6e). The equal intensity of
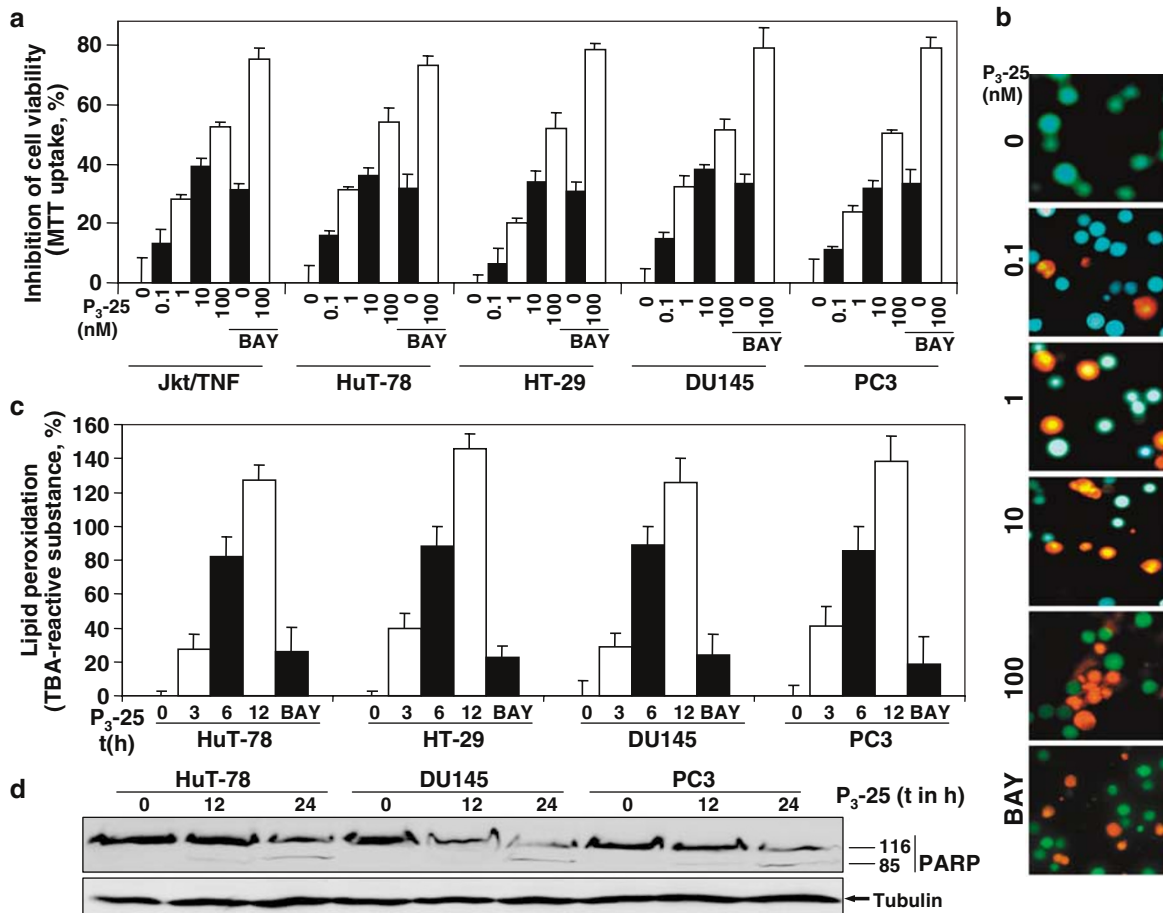
Figure 3 P$_3$-25 induces apoptosis. (**a**) Jkt/TNF, HuT-78, HT-29, DU145, or PC3 cells (5000/well of 96-well plate), cultured for 12 h, were treated with varying concentrations of P$_3$-25 or 5 μM BAY for 72 h at 37°C, CO$_2$ incubator in triplicate. Then MTT was assayed and absorbance was taken at 570 nm. The results are expressed as inhibition of cell viability measured from the values of mean absorbance of triplicate samples. (**b**) HuT-78 cells were treated with varying concentrations of P$_3$-25 or 5 μM BAY for 72 h and percentage of dead cells (red color) were counted using the 'Live & Dead' cell assay kit and indicated in mean percentage in bracket from triplicate assays. (**c**) HuT-78, HT-29, DU145, and PC3 cells were treated with 100 nM P$_3$-25 for different times or 5 μM BAY for 12 h. Cell extract (0.5 mg) protein, obtained from the pellet by three times freeze–thaw method with water, was used to measure malondialdehyde by TBA-SDS buffer. The data represented increased production of TBA-reactive substances in percentage from one out of three independent experiments. (**d**) HuT-78, DU145, or PC3 cells were treated with 100 nM P$_3$-25 for different times at 37°C. Cell extracts were prepared and 50 μg proteins were used to detect PARP

tubulin from those blots indicated equal loading of the proteins for different treatments. These data suggest that P$_3$-25 shows an additive effect in combination with different chemotherapeutic agents on cell death.

P$_3$-25 downregulates the activation of NF-κB-dependent genes, PKA- or CKII-dependent p65 phosphorylation, IKK activity, and cell death in doxorubicin-resistant MCF-7 cells.

Doxorubicin-resistant MCF-7 cells differ from the wild-type cells in having higher expression of caspase 3 and tissue transglutaminase.[26] We tested the sensitivity of these cells to P$_3$-25 with respect to the properties of expression of NF-κB-dependent genes – activities of PKAα, CKII, and IKK, and apoptosis. The increased activities of NF-κB-dependent SEAP (Figure 7a) and Cox-2-lucifearse (Figure 7d) in doxorubicin-resistant cells were progressively downregulated with time of incubation in the presence of P$_3$-25. The levels of ICAM1 (Figure 7b) and Cox-2 (Figure 7c) also decreased with time of incubation in doxorubicin-resistant cells. The high basal activities of PKAα and CKII, but not of IKK, observed in doxorubicin-resistant cells, decreased on treatment with P$_3$-25 in a time-dependent manner (Figure 7d). Both methods of MTT assay (Figure 7e) and PARP cleavage (Figure 7f) indicated time-dependent cytotoxicity of P$_3$-25 in doxorubicin-resistant cells. Thus, P$_3$-25 acts by the inhibiting activation of NF-κB-dependent genes and cell death mediated by it follows a pathway different from that of doxorubicin.

Discussion

The present study reinforces the concept that high levels of NF-κB and expression of genes dependent on it confer viability in tumor cells. Indeed, these are the likely factors in the development of their resistance against chemotherapeutic drugs. High basal activities of PKA, CKII, and IKK result in the constitutive expression of NF-κB and other genes involved in tumorigenesis. The thiadiazolidine derivative P$_3$-25, known to block TNF-induced NF-κB activation,[25] also blocked the expression of NF-κB-dependent genes without interfering with the consensus DNA-binding activity in NF-κB-expressing cells; it was also found that P$_3$-25 is a potent inhibitor of upstream kinases PKAα and CKII required for phosphoryla-

tion of p65. These important findings suggested that P$_3$-25 achieves these effects by controlling phosphorylation activities of upstream kinases and, thereby, expression of NF-κB-dependent genes.

Most of the tumor cells have basal activity of NF-κB. Cells lacking NF-κB cannot survive for long, as NF-κB is required for the function of normal cell cycle. Although the activities of PKAα and NF-κB were very low in Jurkat cells, downregulation of this low basal activity of PKAα and expression of NF-κB-dependent SEAP gene were observed on treatment with P$_3$-25. Involvement of any other pathway in P$_3$-25-mediated cell death in low NF-κB-expressing cells needs further investigation.

Our results indicate that activation of NF-κB-dependent genes is linked with apoptosis. How constitutively NF-κB-expressing cells overcome the apoptotic effect, mediated by several apoptotic inducers including chemotherapeutic agents, is not well understood. Several genes regulated by the activation of NF-κB, including those of the zinc-finger protein A20,[27] manganese superoxide dismutase, [28,29] and cIAP2 (cellular inhibitor for apoptosis), [30] are known to be involved in the inhibition of apoptosis. It is possible that these genes are constitutively expressed in different cancer cells, thus leading to inhibition of apoptosis. Mice, lacking the NF-κB p65 gene, die early in embryonic development from massive cellular death of hepatic parenchyma.[31] The antiapoptotic role of NF-κB was also supported by the observation that embryonic fibroblasts from IκB-knockout mice are resistant to TNF.[32] Similarly, transfection of a dominant negative form of IκBα complememtary DNA (cDNA) (IκBα-DN) prevented the TNF-induced cell death.[33,34] A sustained level of IKKs is required for constitutive activation of NF-κB.[35,36] Although BAY completely inhibited IKK activity in NF-κB-expressing cells, it showed 30% cell death alone at 72 h of treatment. Lipid peroxidation is an important marker of cell death, which occurs by excessive production of ROS mainly via the mitochondrial pathway. BAY induced cell death by inhibiting IKK and thereby NF-κB without inducing lipid peroxidation, which indicates that BAY may not use the mitochondrial pathway to induce apoptosis. P$_3$-25 and BAY in combination induced almost 75% cell death, indicating that P$_3$-25 works in some way other than inhibition of IKK. In this study, we show that PKAα and CKII are involved in cell survival in NF-κB-

Figure 4 P$_3$-25 inhibits activities of PKAα, CKII, NF-AT, and CREB, but not of IKK in NF-κB-expressing cells. Jkt/TNF, HuT-78, HT-29, DU145, and PC3 cells were treated with 100 nM P$_3$-25 for different times. Jkt/TNF and HuT-78 cells were treated with 5 μM BAY for 6 h. (**a**) The whole-cell extracts (300 μg) protein was immunoprecipitated with anti-IKKα and IKKβ (1 μg each) antibodies and kinase assayed using GST-IκBα as substrate. The IKKα was assayed from 100 μg protein extract by Western blot. (**b–d**) The whole-cell extracts (400 μg protein) were immunoprecipated with anti-PKAα or -CKII antibody (1 μg each) and immune complex was used to assay PKAα (**b**) or CKII (**d**) using GST-p65 protein (3 μg/lane) as substrate. The levels of PKAα or CKII were assayed from the extract (100 μg protein) by Western blot. The anti-PKAα-immunoprecipitated samples were assayed for PKAα activity using kemptide as substrate (**c**). (**e**) The whole-cells extracts (100 μg) from HuT-78 and DU145 were analyzed in 10% SDS-PAGE and detected for phospho-p65 (Ser^{276} or Ser^{536}) using specific phospho-p65 (Ser^{276}) (**e1**) or -p65 (Ser^{536}) (**e2**) antibody. Blots were reprobed with anti-p65 antibody. (**f**) HuT-78 cells were treated without or with 100 nM P$_3$-25 for 6 h and extracts were immunoprecipitated against anti-PKAα (**f1**) or CKII (**f2**) antibody and one half of immunoprecipitated beads were incubated with a mixture containing GST or GST-p65 substrate and ATP. Samples were analyzed in 10% SDS-PAGE followed by Western blots against anti-GST antibody (upper panels). These blots were reprobed with anti-p65 (C-terminal) antibody (middle panels). The other half of immunoprecipitated beads was used for PKAα or CKII assay using GST or GST-p65 substrate and the same extract (100 μg protein) was tested for amounts of PKAα or CKII presented by Western blots (lower panels). (**g**) HuT-78 and DU145 cells were treated with 100 nM P$_3$-25 for different times and NF-AT was assayed from the nuclear extract (**g1**). HuT-78 and DU145 cells, transfected with vector, NF-AT-luciferase, and GFP constructs for 3 h by Qiagen SuperFect reagent were cultured for 12 h and GFP-positive cells were counted. Cells were treated with 100 nM P$_3$-25 for different times or 2% DMSO for 24 h. The cell extracts were assayed for luciferase activity. Luciferase activity was expressed as fold of activation (**g2**), considering vector-transfected cells value as one. (**h**) HuT-78 and DU145 cells were treated with 100 nM P$_3$-25 for different times or 2% DMSO for 6 h. Nuclear extract (10 μg protein) was assayed for CREB by the gel shift assay. CREB was assayed in presence of 200 ng unlabeled (cold) oligonucleotide
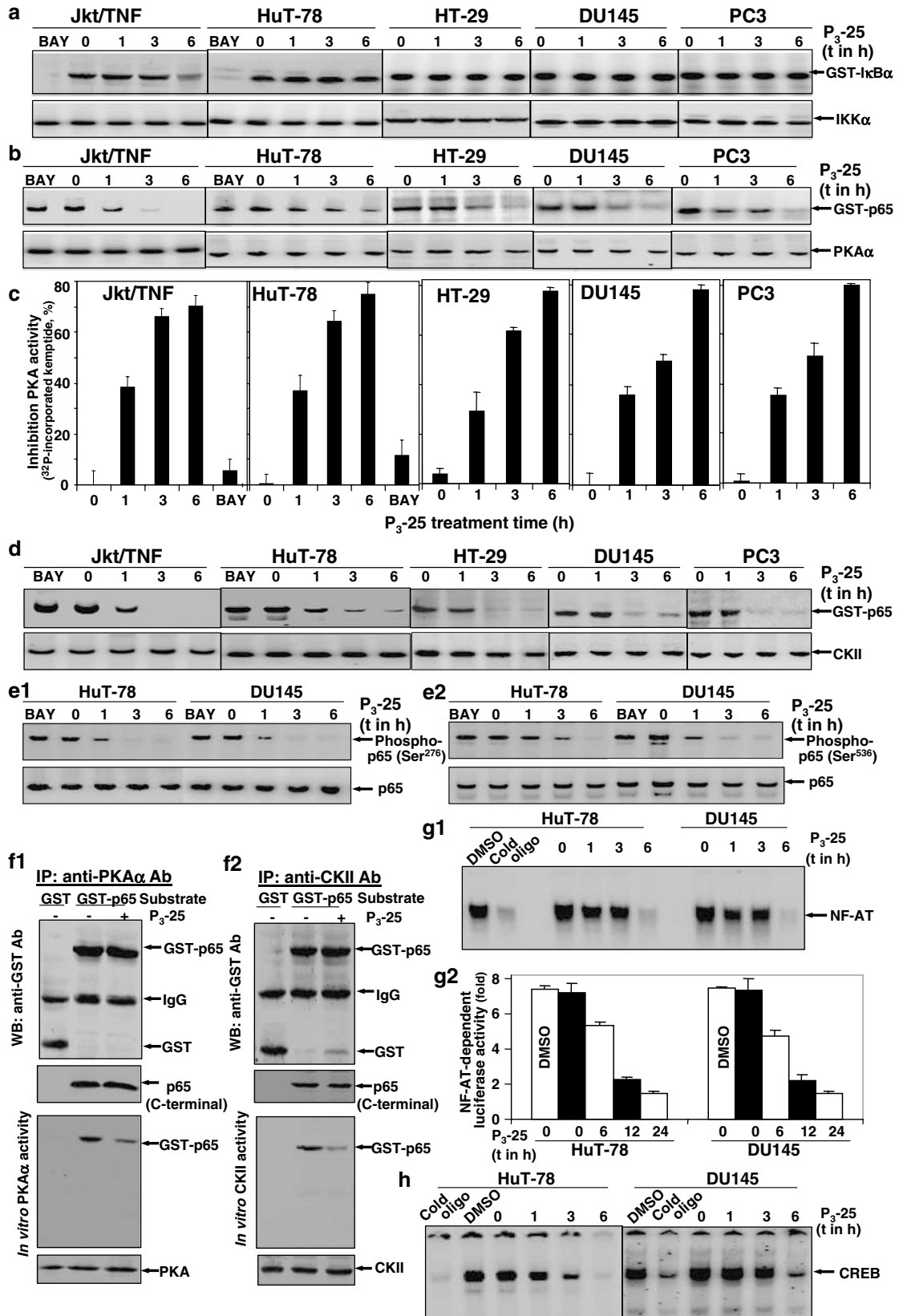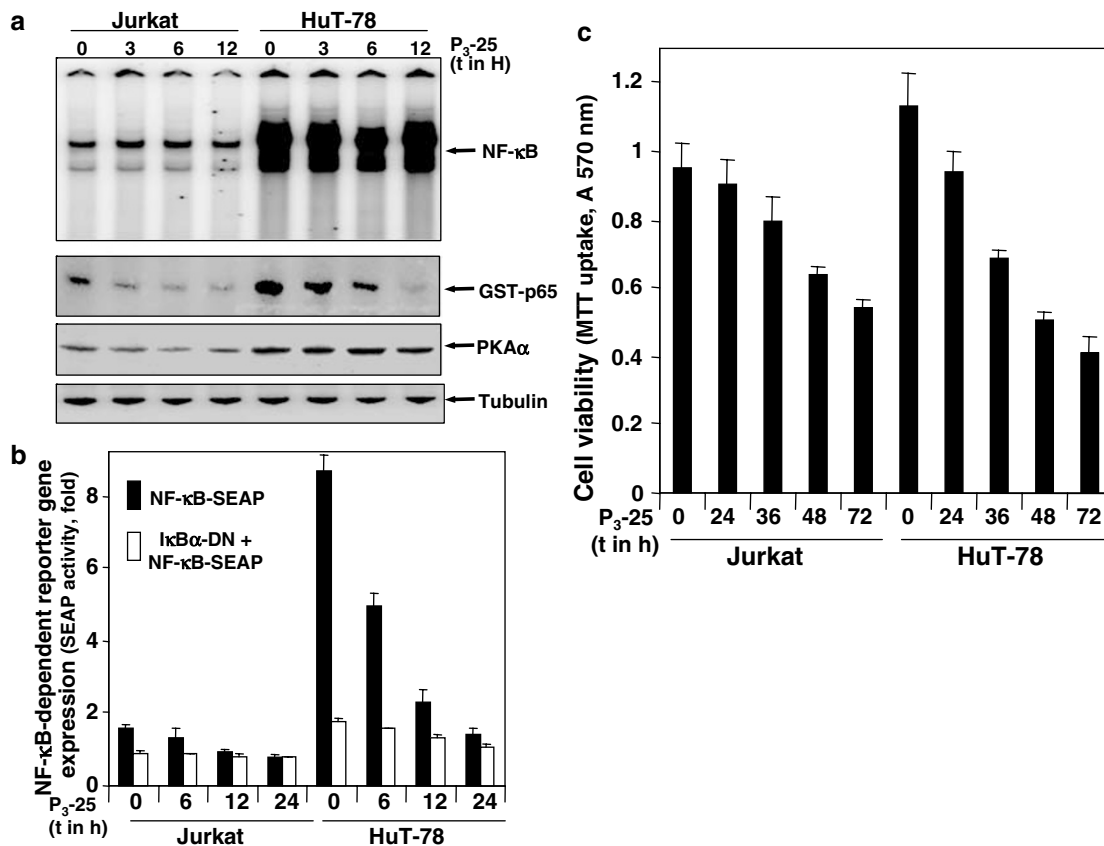
Figure 5 P_3-25 inhibits marginal activation of PKAα and induces cell death in Jurkat cells. Jurkat and HuT-78 cells were treated with 100 nM P_3-25 for different times and DNA-binding activity of NF-κB was assayed from nuclear extracts, and PKAα activation was assayed from whole-cell extracts by using GST-p65 *in vitro* (**a**). Jurkat and Hut-78 cells were transfected with NF-κB-SEAP, IκBα-DN, and GFP constructs. Cells were treated with P_3-25 for different times; SEAP activity was assayed and indicated as fold activation (**b**). Cells were treated in triplicate with 100 nM P_3-25 for different times and cell viability was assayed by MTT uptake (**c**)
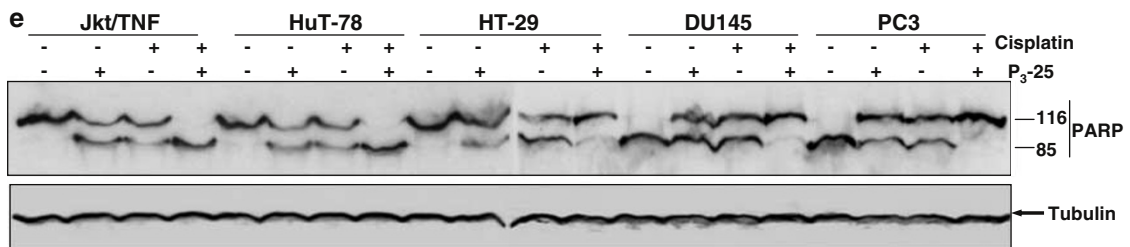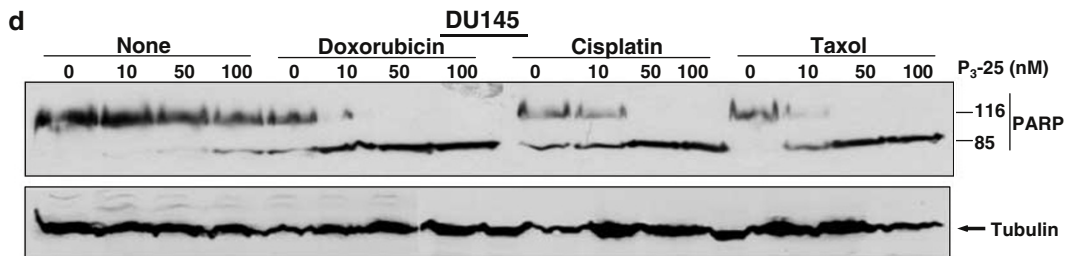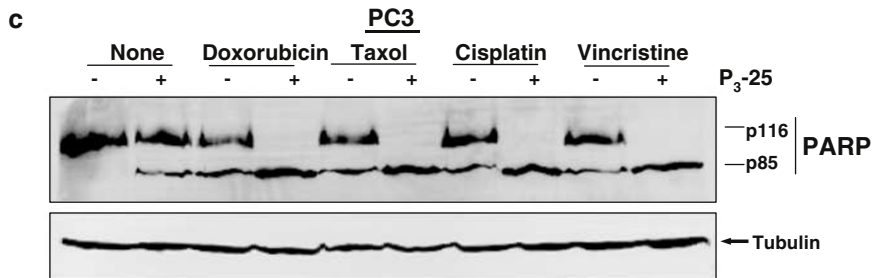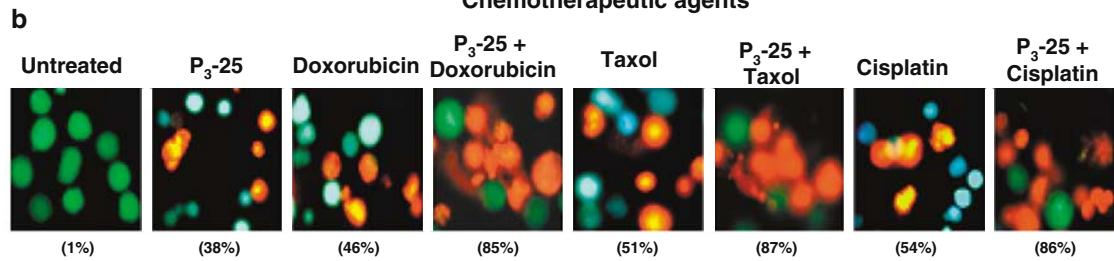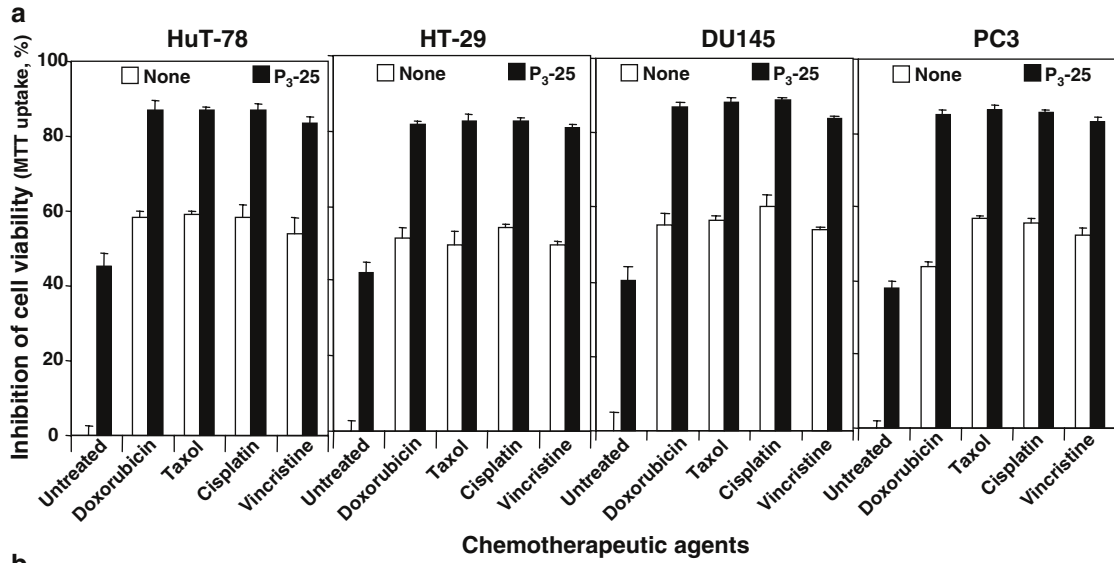
expressing or chemotherapeutically resistant cells, and are downregulated by P_3-25, thus leading to cell death. How P_3-25 downregulates PKAα and CKII needs to be studied further. Several reports indicate that constitutive or induced NF-κB gives resistance to apoptosis stimulated by a wide variety of agents.[29,37–39] It is possible that the cytotoxic effect of P_3-25 on tumor cells is mediated through its suppression of NF-κB. Surprisingly, the NF-κB DNA-binding ability was not altered by P_3-25 in constitutively NF-κB-expressing cells. The upstream kinase IKK complex, involved in the activation of NF-κB, was not inhibited by P_3-25.
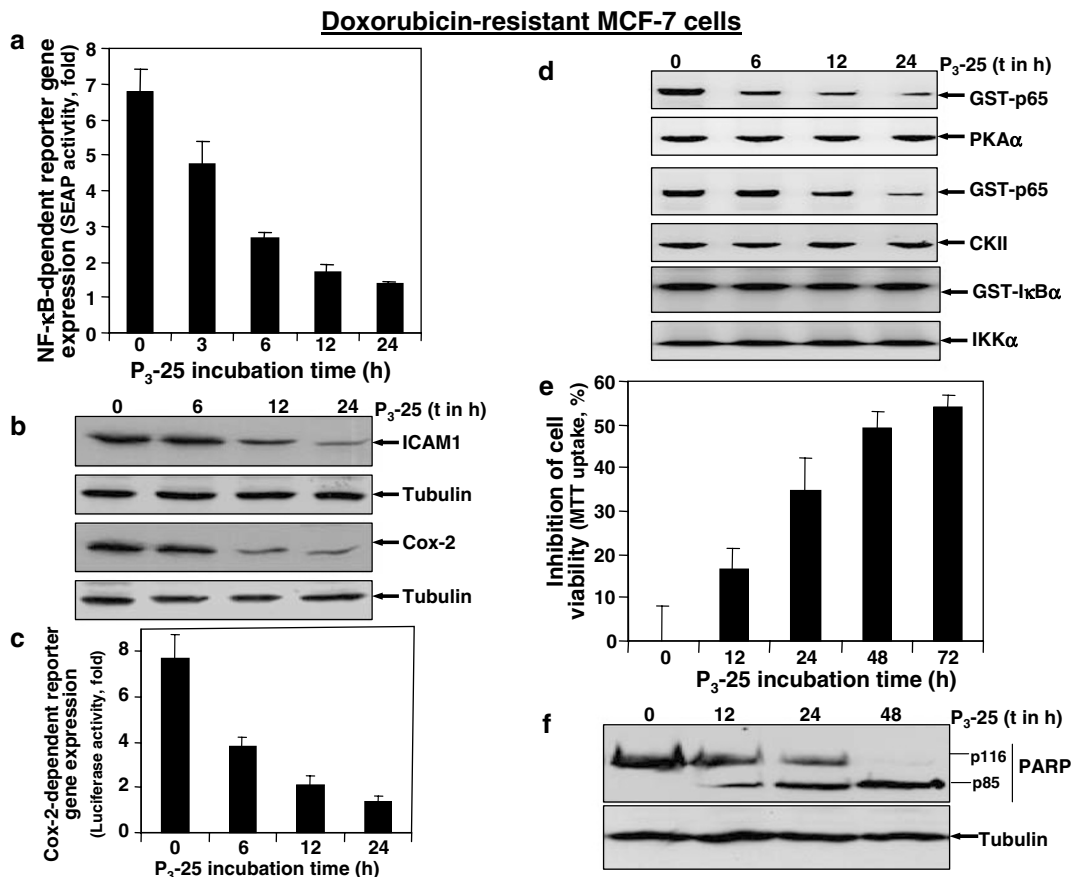
Several genes involved in tumor promotion, such as those of cytokines, Cox-2, metalloproteases, urinary plasminogen activator, and cell surface adhesion molecules, are regulated by NF-κB.[40–42] By blocking expression of NF-κB-dependent gene, P_3-25 plays a critical role in exhibiting anticarcinogenic and anti-inflammatory effects. Inhibiting NF-κB by adenoviral IκBα or proteasome inhibitors was found to overcome resistance induced after chemotherapy.[37] Therefore, the ability of P_3-25 to suppress NF-κB could be exploited by combination with chemotherapy, as recurrence of tumor is common in cancer patients after chemotherapy.

Aspirin was found to be beneficial for preventing colon cancer because of its ability to suppress Cox-2 through NF-κB.[43] Similarly, P_3-25 may also be beneficial for colon cancer as it inhibits NF-κB-dependent gene expression and the products required for metastasis. NF-κB-inhibitory activity of P_3-25 may also have applications for various tumors and

Figure 6 P_3-25 potentiates apoptosis mediated by chemotherapeutic agents. (**a**) HuT-78, HT-29, DU145, and PC3 cells (5×10^3/well of 96-well plate), incubated for 12 h, were treated with 100 nM P_3-25 for 4 h followed by doxorubicin, taxol, cisplatin, or vincristine (1 μM each) for 72 h at 37°C, in a CO_2 incubator. Then, MTT uptake was assayed by absorbance at 570 nm. Inhibition of cell viability was calculated from the mean absorbance values of triplicate samples. (**b**) HuT-78 cells were treated with 100 nM P_3-25 for 4 h followed by treatment with doxorubicin, taxol, or cisplatin (1 μM each) for 72 h. The number of dead cells (red color) were counted using the 'Live & Dead' cell assay kit and indicated in percentage in brackets. (**c**) PC3 cells were pretreated with 100 nM P_3-25 for 4 h and then treated with doxorubicin, taxol, cisplatin, or vincristine (1 μM each) for 72 h. The cell extracts (50 μg protein) were used to detect PARP by Western blot. The blot was reprobed to detect tubulin by Western blot. (**d**) DU145 cells were pretreated with varying concentrations of P_3-25 for 4 h and then treated with doxorubicin, cisplatin, or taxol (1 μM each) for 72 h. The PARP was detected from cells extract. Blot was reprobed to detect tubulin by Western blot. (**e**) Jkt/TNF, HuT-78, HT-29, DU145, and PC3 cells were treated with 100 nM P_3-25 for 4 h and then treated with cisplatin (1 μM) for 72 h. The PARP was detected from the extracts (50 μg proteins) by Western blot. The tubulin was detected after reprobing with anti-tubulin antibody from the same blot

a

HuT-78 | HT-29 | DU145 | PC3

Inhibition of cell viability (MTT uptake, %)

None | P$_3$-25

Chemotherapeutic agents

Untreated, Doxorubicin, Taxol, Cisplatin, Vincristine

b

Untreated | P$_3$-25 | Doxorubicin | P$_3$-25 + Doxorubicin | Taxol | P$_3$-25 + Taxol | Cisplatin | P$_3$-25 + Cisplatin

(1%) | (38%) | (46%) | (85%) | (51%) | (87%) | (54%) | (86%)

c

PC3

None | Doxorubicin | Taxol | Cisplatin | Vincristine
- | + | - | + | - | + | - | + | - | + | P$_3$-25

p116 / p85 PARP

Tubulin

d

DU145

None | Doxorubicin | Cisplatin | Taxol
0 | 10 | 50 | 100 | 0 | 10 | 50 | 100 | 0 | 10 | 50 | 100 | 0 | 10 | 50 | 100 | P$_3$-25 (nM)

116 / 85 PARP

Tubulin

e

Jkt/TNF | HuT-78 | HT-29 | DU145 | PC3
- | - | + | + | - | - | + | + | - | - | + | + | - | - | + | + | - | - | + | + | Cisplatin
- | + | - | + | - | + | - | + | - | + | - | + | - | + | - | + | - | + | - | + | P$_3$-25

116 / 85 PARP

Tubulin

Doxorubicin-resistant MCF-7 cells

Figure 7 P$_3$-25 inhibits the expression of NF-κB-dependent genes, activation of PKA or CKII, and induces apoptosis in doxorubicin-resistant MCF-7 cells. (**a**) Doxorubicin-resistant MCF-7 cells, cultured in the presence of 2 μM doxorubicin, transfected with vector, NF-κB-SEAP, and GFP constructs were cultured for 12 h. GFP-positive cells were counted. Cells were treated with P$_3$-25 (100 nM) for different times and the culture supernatant was assayed for SEAP activity. The results represented as fold of activation over the vector-transfected cells. (**b**) Doxorubicin-resistant cells, cultured in the presence of 2 μM doxorubicin, were treated with 100 nM P$_3$-25 for different times. The amounts of ICAM1 and Cox-2 were measured from cell extracts by Western blot. As loading control, tubulin was detected by reprobing both blots. (**c**) Doxorubicin-resistant cells, cotransfected with Cox-2-Luciferase and GFP constructs, were cultured for 12 h and treated with 100 nM P$_3$-25 for different times. Luciferase activity assayed from whole-cell extracts. (**d**) Doxorubicin-resistant cells were treated with P$_3$-25 for different times and PKAα or CKII was assayed from cell extracts using GST-p65 as substrate; IKK was assayed using GST-IκBα as substrate. From the same extracts, the levels of PKAα, CKII, or IKKα were detected by Western blot. (**e**) Doxorubicin-resistant cells were treated with 100 nM P$_3$-25 for different times and then cell viability was assayed by MTT dye uptake. Results are represented as inhibition of cell viability in percentage. (**f**) Doxorubicin-resistant cells were treated with 100 nM P$_3$-25 for different times and PARP was detected from cell extracts by Western blot. The same blot was reprobed for tubulin

may also be useful for other diseases including inflammation and arthritis, where its activation has been shown to mediate pathogenesis.

Materials and Methods

Materials. Glycine, 4-methyl umbelliferyl phosphate, paclitaxol, doxorubicin, cisplatin, vincristine, MTT, kemptide, and anti-tubulin antibody were obtained from Sigma-Aldrich Chemicals (St Louis, MO, USA). Penicillin, streptomycin, neomycin, RPMI-1640 medium, and fetal bovine serum (FBS) were obtained from Life Technologies. Antibodies against p65, IKKα, IKKβ, PKAα, ICAM1, PARP, CKII, and Cox-2 and double-stranded CREB and NF-ATc oligonucleotides were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The 'Live & Dead' cell assay kit was obtained from Molecular Probes (Eugene, OR, USA). Plasmids for NF-κB-SEAP, NF-κB(mut)-SEAP, Cox-2-Luciferase, and GFP were kindly supplied by Professor BB Aggarwal of the University of Texas MD Anderson Cancer Center (Houston, TX, USA). P$_3$-25 was prepared from 1-(4-methoxyaryl) thiocarbamide as described previously.[25]

Cell lines. The cell lines used in this study were as follows: Jurkat (T cell), HuT-78 (modified T cell), HT-29 (colorectal adenocarcinoma), DU145 (prostatic

adenocarcinoma), and PC3 (prostatic adenocarcinoma). These cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Doxorubicin-resistant MCF-7 cells were obtained from Professor Kapil Mehta, MD Anderson Cancer Center, Houston, USA. Cells were cultured in RPMI-1640 medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were free from mycoplasma, as detected by the Gen-Probe mycoplasma rapid detection kit (Fisher Scientific, PA, USA).

NF-κB, NF-AT, or CREB activation assay. Activation of NF-κB was determined by EMSA.[29] Briefly, cells, after different treatments, were used to prepare cytoplasmic and nuclear extracts. Nuclear extract proteins (8 μg) were incubated with ^{32}P end-labeled double-stranded NF-κB oligonucleotide of HIV-LTR, 5'-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3', for 30 min at 37°C, and the DNA–protein complex was separated from free oligonucleotide on 6.6% native PAGE. Similarly, NF-AT and CREB binding were assayed using specific double-stranded labeled oligonucleotides. The visualization of radioactive bands was carried out in a PhosphorImager (Fuji, Japan).

NF-κB-dependent SEAP reporter gene assay. Cells were transiently transfected with Qiagen SuperFect transfection reagent (Hilden, Germany) with reporter plasmid containing NF-κB-binding site cloned upstream of heat-stable

SEAP designated as NF-κB-SEAP and GFP (0.5 μg. each) constructs for 3 h and cultured for 12 h. GFP-positive cells were counted and indicated for transfection efficiency. Cells were treated with P$_3$-25 for different times. Cell culture-conditioned medium (25 μl) was analyzed for SEAP activity essentially as per the Clontech protocol (Palo Alto, CA) and reported as fold activation with respect to vector-transected cells, as described previously.[44]

Cox-2-, AP-1-, or NF-AT-dependent luciferase gene transcription assay. The expression of Cox-2-, AP-1-, or NF-AT-dependent luciferase reporter gene was carried out as described previously.[45] Cells were transiently transfected with SuperFect transfection reagent containing 0.5 μg of each reporter plasmid containing Cox-2-, AP-1-, or NF-AT-binding site cloned upstream of luciferase (designated as Cox-2-, AP-1-, or NF-AT-Luciferase, respectively) and GFP constructs. After 3 h of transfection, cells were washed and cultured for 12 h followed by treatment with P$_3$-25 for different times. GFP-positive cells were counted. The cell pellets were extracted with lysis buffer (part of the Luciferase assay kit from Promega) and the extracts were incubated with the firefly luciferin (substrate, Promega). Light emission was monitored with a Luminometer and values were calculated as fold of activation over vector-transfected value.

Detection of ICAM1 and Cox2 by semiquantitative RT-PCR. After treatment, total RNA was extracted using TRIzol (Gibco BRL) and 1 μg of total RNA was reverse-transcribed using poly-T oligonucleotide and M-MuLV reverse transcriptase (Invitrogen). The PCR was performed using primers for ICAM1 (5′-AGGGAGGCTCCGTGCTGGTGA-3′, sense primer and 5′-TCAGTGCGGCACGAG AAATTG-3′, antisense primer),[46] Cox-2 (5′-TTCAAATGAGATTGTGGGAAAAT-3′, sense primer and 5′-AGATCATCTCTGCCTGAGTATCTT-3′, antisense primer),[47] and actin. Following PCR, the amplicons were analyzed by gel electrophoresis with ethidium bromide staining.

IKK assay. The IKK assay was performed by a method described previously.[44] Briefly, IKK complex from whole-cell extract (300 μg) was precipitated with anti-IKKα and anti-IKKβ antibodies (1 μg each), followed by incubation with protein A/G-Sepharose beads (Pierce, Rockford, IL). After 2 h of incubation, the beads were washed with lysis buffer and assayed for IKK activity using 2 μg of GST-IκBα (aa 1–54) substrate protein.

Purification of GST-p65 and assay of PKAα and CKII. A GST-p65 (1656 bp) full-length fusion protein was prepared as follows. A double stranded cDNA encoding the p65 full-length flanked by a 5′ BamHI restriction and 3′ stop codon with a XhoI restriction site was synthesized by PCR using the HeLa cDNA library as template and the following oligonucleotide primers: 5′-ATCGGGGATCC GBATGGACGAA CTGTTCCCCCTCATC-3′ and 5′-TAGACTCTCGAGTTAGGA GCTGATCTGACTCAGCAG GG-3′. The amplified product was purified and ligated into BamHI- and XhoI-digested vector pET41b (Novagen). The GST-p65 protein was expressed in expression strain Rossetta (Novagen).

The whole-cell extracts from different treatments were immunoprecipitated with anti-PKAα or -CKII antibody (1 μg each). The immune complex was washed, incubated with 3 μg of GST-p65 substrate protein and ^{32}P-ATP in kinase assay buffer for 30 min at 37°C, and analyzed in 8% SDS-PAGE. The gel was dried and exposed to phosphorimager screen and radioactive bands were detected in PhosphorImager. Immunoprecipitated PKA from cell extracts was incubated with 20 μl of substrate cocktail containing 100 μM kemptide. The reaction was started by adding 5 μl of the mixture containing 0.5 mM ATP and 10 μCi of ^{32}P-ATP. After incubation, the mixture was spotted onto a streptavidin-coated membrane, washed repeatedly, dried, and counted in a Beta Counter with scintillation fluid.

Cytotoxicity assay

MTT assay. The cytotoxicity was measured by the MTT assay.[44] Briefly, cells (1 × 10^4 cells/well of 96-well plate), cultured for 12 h, were treated with different agents for 72 h and thereafter 25 μl of MTT solution (5 mg/ml in PBS) was added. After 2 h of incubation, 100 μl extraction buffer (20% SDS in 50% dimethylformamide) was added. After an overnight incubation at 37°C, absorbance was read at 570 nm.

Live & dead assay. The cytotoxicity was determined by the Live/Dead assay [44] (Molecular Probes, Eugene, OR, USA). Briefly, after different treatment, 1 × 10^5 cells were stained with 'Live & Dead' cell assay reagent (5 μM ethidium homodimer, 5 μM calcein-AM). Red (as dead) and blue (as live) cells were analyzed under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan).

Western blot of PARP. Apoptosis was examined by proteolytic cleavage of PARP.[14] Briefly, 50 μg whole-cell extracts were resolved on 7.5% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, blotted with mouse anti-PARP antibody, and then detected by chemiluminescence (Amersham Pharmacia Biotech, NJ, USA).

Determination of lipid peroxidation. Lipid peroxidation was determined by measuring thiobarbituric acid-reactive malondialdehyde (MDA), an end product of peroxidation of polyunsaturated fatty acids, as described previously.[15,25]

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Retraction

Retraction: Inhibition of RelA phosphorylation sensitizes chemotherapeutic agents-mediated apoptosis in constitutive NF-kappaB-expressing and chemoresistant cells

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Retraction to: *Cell Death and Differentiation* (2007) **14**: 158–170, Subsequent 'Corrigendum' in *Cell Death and Differentiation* (2012) **19:** 1572

The corresponding author wishes to retract the above paper for the reason that the images in some of the Figure panels in the paper could not unambiguously be attributed in the laboratory notebooks. Thus, the results presented in this paper are not fully supported by the available laboratory records. All the authors have agreed to sign this retraction notice. We deeply regret this circumstance and apologize for any adverse consequences that this retraction might cause to the scientific community.