Abstract
Breast cancer is the most commonly diagnosed disease among women. In recent years, chemotherapeutic drugs such as gemcitabine, erlotinib, etc. have been developed for the treatment of breast cancer. Breast cancers relapse due to the generation of chemoresistance that makes therapeutic drugs ineffective. Hence, agents that can reduce the chemoresistance to the therapeutic drugs are being developed for better advancement of cancer therapy. The present study evaluates the efficiency of curcumin, in suppression of gemcitabine induced NF-κB activity and in the enhancement of antitumor effect of gemcitabine on MCF-7 and MDA MB-231 breast cancer cells. 10 or 20 µM of curcumin and 10 or 100 µM of gemcitabine, alone or in combination, were used. Cell proliferation by MTT assay, apoptotic effects by Live/Dead assay, nuclear factor-kB (NF-κB) activation or suppression by EMSA were determined. The results indicated a decrease in cell proliferation of up to 61% (p < 0.01) and 45% (p < 0.01) at 20 µM curcumin and 100 µM of gemcitabine in MCF-7 and MDA MB-231, respectively. Whereas 20 µM curcumin potentiated the apoptotic effects of gemcitabine (100µM) predominantly in MCF-7 by 61% and in MDA MB-231 by 46% which was determined by using Live/Dead assay. However, curcumin (20 µM) significantly (p ≤ 0.05) suppressed NF-κB activation by 80% which was induced by gemcitabine (100µM) in both cell lines. The data obtained from the present investigation shows the dose dependent changes in MCF-7 and MDA MB-231. The combined results revealed the beneficial role of curcumin in potentiating the anti-tumor effects of gemcitabine through NF-κB suppression and apoptotic effects.

Keywords: Apoptosis, Curcumin, Gemcitabine, NF-κB

Introduction
Nowadays, breast cancer is a major risk factor among women worldwide. In Western countries, the mean 5-year relapse-free survival rate of breast cancer patients is approximately 60%, but this value differs significantly across individuals (1-3). Breast cancer is caused due to various potent chemical drugs or epigenetic factors, including regulation of transcription factors, growth factors, etc (4). Various reports denote increased cell proliferation, invasion, suppression of apoptosis and chemoresistance in multiple tumours (4). Several reports indicate that such detrimental effects might be linked with transcription factor NF-κB, which plays a major role in regulating the cell proliferation and chemoresistance of breast cancer (5, 6).

At present, gemcitabine (2’2’-difluorodeoxycytidine), a novel nucleoside analogue of deoxycytidine is employed as a chemotherapeutic drug for the treatment of various cancers which induces NF-κB activity (7, 8). This drug is inactive in the parental form,
although it is progressively phosphorylated to its active diphosphate and triphosphate metabolites via kinases in intracellular compartments (9). In addition, its active diphosphate form inhibits ribonucleotide reductase apart from incorporation of its triphosphate into DNA as a fraudulent base in competition with dCTP. Such incorporation into DNA resulted in DNA chain termination during replication and the mimic base is relatively resistant to excision repair. However, gemcitabine treatment might be associated with multiple adverse effects and drug resistance which results in an objective tumour response rate of <10% with a marginal survival advantage (8, 9). Breast cancer cells often develop various mechanisms of drug resistance during tumour progression which is the major reason for the failure of breast cancer therapy. NF-kB is constitutively active in breast cancer cells, which plays a critical role in promoting gemcitabine resistance to breast cancer (8). Hence, certain natural agents that block NF-kB activity are likely to reduce chemoresistance to gemcitabine and are possibly used in combination with gemcitabine as a novel therapeutic regimen for breast cancer patients. Thus, there is a necessity for developing novel strategies with no entity in toxicity that can sensitize breast cancer cells to chemotherapy.

Currently, phytochemicals (naturally occurring chemicals in plants) such as is flavones, gingerol, quercetin, resveratrol and curcumin have been identified to be more advantageous in treating various diseases like cancer, pancreatitis, fibrosis, etc. (10, 11). It is also known that the phytochemical, curcumin (Diferuloylmethane) has a beneficial role in the treatment of various types of cancers of the breast, pancreas, bladder and lungs (12). Curcumin is derived from turmeric (Curcuma longa), a pharmacologically safe chemical compound which has been shown to suppress NF-kB activation and its downstream regulation processes such as anti-apoptosis, proliferation, invasion and metastasis (13-15).

Hence, this investigation has focused on evaluating the beneficial role of curcumin in treatment of breast cancer in combination with gemcitabine in vitro. Curcumin has shown a positive impact on potentiating the gemcitabine anti-tumor effects as well as minimizing the side effects.

**Materials and Methods**

The human breast cancer cell lines MCF-7 or MDA MB-231 (ER-negative, HER-2-negative) obtained from the American Type Culture Collection (ATCC, Manassas, VA) were grown as a monolayer in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Grand Island, NY). The monolayer cells in the exponential growth phase were used for the experiments conducted in the present study. Gemcitabine and curcumin were obtained from Sigma, Saint Louis, USA.

**Cell Proliferation Assay**: The effect of curcumin and/or gemcitabine on MCF-7 or MDA MB-231 breast cancer cell proliferation were examined by using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) dye [16]. Briefly, the monolayer cells in growth phase were trypsinized (0.25% trypsin for 10 min) and 5,000 cells were seeded into 96-well culture plates. The cells were incubated with curcumin (10 or 20 µM) and/or gemcitabine (10 or 100 µM) in DMEM medium in a 96-well culture plates for 48 h at 37 °C, followed by 2 h incubation with 10 µl of MTT (5 mg/mL in PBS) at 37 °C. The medium was replaced with 100 µL of 99.8% dimethyl sulphoxide in each well and optical density was measured at 570 nm with reference wave length, 630 nm. All the test samples were analyzed in triplicates.

**Live / Dead Assay**: Live/Dead assay kit was used (Molecular Probes, Carlsbad, CA) to determine the curcumin and/or gemcitabine cell apoptotic effects in breast cancer cells. This method determines intracellular esterase activity and plasma membrane integrity. The assay measures the emitted fluorescence intensity by enzymatic conversion of cell permeable nonfluorescent calcein AM with ubiquitous intracellular esterase present in live cells. In
addition, dead cells were quantitated with ethidium bromide, a red fluorescent homodimer dye which can enter dead cells through damaged membranes and bind to nucleic acids (16). Briefly pre-treated MCF-7 or MDA MB-231 cells (10,000 per well) were incubated in 24-well culture plates either with curcumin (10 or 20 µM; 4 h) or gemcitabine (10 or 100 µM; 24 h). To examine the potentiating effects of curcumin, the pre-treated cultures with curcumin (20 µM; 4 h) were further treated with gemcitabine (100 µM) for 24 h at 37 ºC. After incubation, the cells were stained with Live/Dead assay reagents for 30 min at 37 ºC as per the manufacturer’s instructions. The number of live and dead cells were observed under a fluorescence microscope (Olympus, Germany), followed by counting live (green at excitation and emission wavelengths of 495 and 515 nm, respectively) and dead (red at excitation and emission wavelengths of 495 and 635 nm, respectively) cells.

**Electrophoretic Mobility Shift Assay [EMSA]**: The breast cancer MCF-7 or MDA MB-231 cells (2x10^6 cells) were homogenized in buffer A [300 mM sucrose, 60 mM KCl, 15 mM N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid (HEPES) pH 7.5 containing 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine and 14 mM mercaptoethanol, 10 mM benzamidine, 0.7 mg/ml leupeptin] after incubation with curcumin (10 or 20 µM) and/or gemcitabine in 96-well culture plates (10 or 100 µM) for 2, 4, 6 and 8 h at 37 ºC. All the steps involved in homogenization and extraction process were carried out at 4 ºC and the samples were stored at -80 ºC until the assay was performed (9). The amount of protein in nuclear extracts was determined by Bradford’s method according to the manufacturer’s instructions (Bio-Rad, Hercules, CA, USA).

Nuclear protein extracts of the breast cancer MCF-7 or MDA MB-231 cells were analyzed using an EMSA to determine NF-kB nuclear translocation (17). The EMSA was conducted by competitive binding with radiolabelled and non-radiolabelled NF-kB probe. 10 µg of nuclear protein was incubated with 0.2 µg of 32P-end-labeled double stranded oligonucleotide containing the NF-kB binding motif (Promega, Madison, WI, USA) and 1 µg of poly (dl-dC) as an inhibitor of non-specific binding in buffer B (20 mM HEPES pH 7.4 containing 60 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40 and 8% glycerol) at room temperature for 30 min. The sequence of the double-stranded oligomers used for EMSA was 5’-AGT TGA GGG GAC TTT CCC AGG C-3’. The entire reaction mixtures were run on 5% Tris-glycine EDTA gel electrophoresis, followed by autoradiography for visualizing DNA-protein complexes. The radioactive band intensity was quantitated using the Storm 820 and Image Quant software (Amersham, Piscataway, NJ).

**Statistical analysis**: All the experiments in the present study were conducted as three independent experiments and thus the obtained values were shown as Mean ± SD. In addition, the data was analyzed by one way ANOVA, followed by Duncan’s multiple comparison between control and treatment groups by using Sigma Plot 11.0. The significant differences between two sample means were compared using unpaired Student’s t-test.*p < 0.05 is considered as statistically significant.

**Results**

The aim of the present investigation was to determine the beneficial role of curcumin in potentiating anti-tumor effects of gemcitabine in breast cancer cells. To elucidate the role of curcumin, the present study was conducted on well established breast cancer cell lines, MCF-7 and MDA MB-231. The positive effects of curcumin that came to light in preventing the cancer effects, by suppressing the cell proliferation as well as NF-kB activation were examined.

**Curcumin decreased the cell proliferation in breast cancer cells in vitro**: In the present study, we have examined the role of curcumin and/or
gemcitabine on the cell viability by using MTT cell proliferation assay. Either curcumin or gemcitabine significantly ($p \leq 0.05$) decreased the MCF-7 and MDA MB-231 cancer cells in vitro in dose dependent manner (Fig. 1A). About 70 and 51% MCF-7 cancer cells survived to 48 h exposure of 10 and 20 µM curcumin, respectively in DMEM ($p \leq 0.01$). However, 10 and 100 µM gemcitabine reduced only 11 and 13.4% cells indicating that curcumin has shown major inhibition on cell proliferation ($p \leq 0.001$). In addition, 20 µM curcumin enhanced the gemcitabine anti-proliferation activity to 60.7% at higher dose (100 µM gemcitabine) as used in the present study. Similarly, MDA MB-231 cancer cells also exhibited 45% anti-proliferation activity in presence of 20 µM curcumin in combination with 100 µM gemcitabine ($p \leq 0.01$). Whereas curcumin (20 µM) or gemcitabine (100 µM) alone decreased 30% or 14.7% cell proliferation, respectively ($p \leq 0.05$).

**Curcumin enhanced the apoptosis in breast cancer cells in vitro:** Further, the potentiating effect of curcumin on gemcitabine induced apoptosis in breast cancer cells was determined by using Live/Dead assay. The assay denoted that curcumin and gemcitabine exposure to both MCF-7 and MDA MB-231 cancer cells enhanced the apoptosis (Fig. 1B). The exposure of 10 and 20 µM of curcumin exhibited 41 and 48% apoptotic cells in MCF-7 cancer cells, respectively ($p \leq 0.05$). Whereas the exposure of 10 and 20 µM of curcumin exhibited 38 and 42% apoptotic cells in MDA MB-231 cancer cells, respectively ($p \leq 0.05$). On the contrary, lesser apoptosis was observed in 10 and 100 µM gemcitabine treated cells (18 and 22% in MCF-7 cells; 14 and 20% in MDA MB-231 cells). However, apoptosis significantly decrease to 61 and 46% in MCF-7 and MDA MB-231 cells, respectively ($p \leq 0.05$) at 100 µM gemcitabine when pre-treated the cells with 20 µM curcumin. These results indicated an increase in the percentage of apoptosis in the presence of chemotherapeutic drug, gemcitabine in combination with phytochemical, curcumin. However, gemcitabine alone had a minimal effect on apoptosis in both the examined breast cancer cell lines.

**Fig.1.** Curcumin inhibits proliferation, potentiates the apoptotic effects of gemcitabine in breast cancer cells in vitro. **A.** MTT assay results showed dose dependent suppression of cell proliferation in all breast cancer cell lines tested. **B.** Live/Dead assay results indicated that curcumin potentiates gemcitabine induced cytotoxicity, percentages, proportions of apoptotic breast cancer cells. Cur: Curcumin, Gem: Gemcitabine

Mamatha Serasanambati et al
Curcumin inhibited NF-κB activation induced by gemcitabine in breast cancer cell lines:
EMSA assay was used to demonstrate whether curcumin suppresses the NF-κB activation induced by gemcitabine in breast cancer cell lines. Breast cancer cell lines were incubated with 100 µM gemcitabine with different time periods to observe maximum NF-κB activation. It has been observed that 100 µM gemcitabine induces NF-κB activation at 6 h and reaches to a maximum level at 8 h in both cell lines (Fig. 2A and Fig. 2B). MCF-7 cells have shown 5 and 5.8 fold increase of NF-κB activation at 6 h and 8 h of incubation respectively, when compared to controls; \( p < 0.001 \). Similarly, MDA-MB-231 cells have shown 5.1 and 6.9 fold increase (\( p < 0.005 \)) at 6 h and 8 h of incubation, respectively, while the 20 µM curcumin pre-treated cells exhibited 30% and 20% NF-κB suppression in MCF-7 and MDA MB-231, respectively when compared to their respective controls. Surprisingly, the curcumin in combination with gemcitabine suppressed NF-κB activation in both MCF-7 (Fig. 2C) and MDA MB-231 (Fig. 2D). The dose dependent suppression of gemcitabine induced NF-κB activation by curcumin has been observed in both cell lines. Both the cell lines when pre-treated with 10 µM curcumin for 4 h showed suppression (38%) of gemcitabine (100µM, 8h) induced NF-κB activation (\( p < 0.005 \)). In addition, the increase in curcumin concentration to 20 µM suppressed the gemcitabine (100 µM, 8 h) induced NF-κB activation up to 80% in both cell lines (\( p < 0.005 \)). The combined results enlightened the potentiating effects of curcumin in apoptosis as well as suppression of gemcitabine induced NF-κB activation.

Fig. 2. Curcumin enhances the effect of gemcitabine against expression of NF-κB in breast cancer. A. MCF-7 cells 2×10^6 were treated with 100 µM gemcitabine. B. MDA MB-231 cells 2×10^6 were treated with 10 µM gemcitabine for indicated time intervals. The cells were then analyzed for NF-κB activation using EMSA. C & D. MCF-7 cells and MDA MB-231 cells 2 ×10^6, were pretreated with 0, 10 and 20 µM curcumin for 4 h. MCF-7 cells were stimulated with 100 µM gemcitabine for 8 h, and MDA MB-231 cells were stimulated with 100µmol /L gemcitabine for same period. The cells were then analyzed for NF-κB activation using EMSA.

Antitumor effect of Gemcitabine
Discussion

The present study was conducted to elucidate the beneficial role of natural herbal compound, curcumin, to improve the therapeutic effects of gemcitabine in human breast cancer cells in vitro. It is well known that curcumin has anti-proliferation activity and it induces a high percentage of apoptosis in human breast cancer cells by regulating the expression of genes associated with programmed cell death (18), a dietary ingredient in many countries, which would be helpful in treatment of cancer (11, 12, 19, 20). In addition, it has shown to suppress NF-kB activation and NF-kB gene products in various models, including rodents and mammals (13, 14, 21). Hence, we have chosen curcumin in combination with gemcitabine that aids in potentiating the apoptosis as well as suppressing the NF-kB activity in human MCF-7 and MDA MB-231 cells. Our results reported here with the gemcitabine alone had minimal effects in anti-proliferation (up to 14%) (Fig. 1A) and apoptosis (up to 14%) (Fig. 1B) in both breast cancer cells. However, in combination with curcumin, the proliferation activity was significantly reduced to 61 and 45% in MCF-7 and MDA MB-231 cells, respectively. In addition, we have reported that the MCF-7 cells have shown more sensitivity than MDA MB-231 cells to both curcumin and gemcitabine. Previous studies have reported the potentiating effects of curcumin in combination with gemcitabine in various cancerous cells such as of human bladder 253JVB (22), KU-7, RT4V6 (14) and pancreas BxPC-3, MIA PaCa-2, Panc-1 and MPanc-96 (18). Even in vivo studies in mice revealed the potentiating beneficial role of curcumin in anticancer activity (22). Similarly, human breast cancer cells, MCF-7 and MDA MB-231 showed the significant anti-proliferation activity ($p \leq 0.05$) by combined administration of curcumin and gemcitabine. It is well known that curcumin alone has the ability to suppress apoptosis in human breast cancer cells MCF-7 and MDA MB-231 (23, 24), cholanagioma carcinoma cells, KKKU100, KKKU-M156, KKKU-M213 (25), lung cancer cells, A549 and H1299 (26) and hepatocarioma cells, HCCJ5 (27). These combined results suggest that curcumin might/does play a role in anti-proliferation activity and reduced apoptosis on breast cancer cell lines. In addition, it is well known that NF-kB has been implicated in cell survival and proliferation (5, 6). Recent reports made on NF-kB and PI3k/akt pathway activation in putative resistance mechanisms for breast cancer. However, NF-kB has been linked with chemoresistance with the therapeutic drugs such as gemcitabine that activates NF-kB in breast cancer lines such as MDA MB-231and MCF-7 as well as in tissue samples of breast carcinoma (23, 24). In the present study, EMSA denoted that the gemcitabine alone enhanced the NF-kB activation significantly ($p \leq 0.05$) in human breast cancer cells, MCF-7 (5.8 fold) and MDA MB-231 (6.9 fold). On the contrary, curcumin reduced the NF-kB activation significantly in MCF 7 and MDA MB-231 cells ($p \leq 0.005$). Some reports suggest that such an activation of NF-kB by human prostate epithelial PZ-HPV-7 cancer cells could be down regulated by curcumin sensitization by blocking phosphorylation of IkB$\alpha$ and its degradation (28), G1/S arrest in mantle cell lymphoma (29). The present investigation reports the dose dependent suppression of gemcitabine induced NF-kB activation by curcumin at 10 (38%) and 20 µM (up to 80%) in both cell lines. Similarly, previous studies using various cancer cell lines showed that curcumin is quite effective in suppressing the proliferation, NF-kB activation and promoting apoptosis, exhibiting more effectiveness when combined with gemcitabine (14,18). Hence, gemcitabine alone does not attain sufficient disease control due to intrinsic or acquired resistance of tumour cells; curcumin may help in adequate management of cancer remedy. The present study suggested that the combinational therapy with herbal compound, curcumin, may perhaps be helpful in the treatment of breast cancer in human population. Further studies of in vivo models may help in understanding the beneficial role of curcumin in breast cancer therapy.

Mamatha Serasanambati et al
References

Antitumor effect of Gemcitabine


Mamatha Serasanambati et al