

# Enhancement of Gamma Radiation-induced Cytotoxicity of Breast Cancer Cells by Curcumin

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## Abstract

Failure of cancer treatment in clinic by radio/chemotherapy is generally attributed to tumor resistance. It is therefore, important to develop strategies to increase cytotoxicity of tumor cells by radiation in combination with new tumor selective cytotoxic agents. Curcuminoids are a group of phenolic compounds isolated from the rhizome of *Curcuma longa* with various pharmacological properties. They exhibit growth inhibitory effects on a broad range of tumors and have recently been shown to act as potent radiosensitizers. Present work was designed to study the combined effects of curcumin and gamma radiation on breast cancer cells. We used MCF-7 human breast cancer cells that were treated with curcumin prior to gamma irradiation. Cell viability, induction of apoptosis, generation of ROS and loss of mitochondrial membrane potential were determined. Our results indicated that curcumin (5  $\mu$ M) exposure prior to irradiation decreased survival to 38% as compared to 52% by radiation (5 Gy) alone. It was found that treatment of cells with curcumin before radiation caused increased induction in apoptotic death as assessed by PI and annexin V dual staining and the appearance of sub G1 peak in PI cell cycle analysis. Interestingly, curcumin treatment of MCF-7 cells lead to a decrease in the generation of intracellular ROS along with a loss of the mitochondrial membrane potential. These findings, therefore, indicate that pre-treatment with curcumin caused significant enhancement of

gamma radiation-induced cell death in MCF-7 cells and this effect was potentially mediated via ROS-independent pathway.

**Keywords:** Curcumin; Gamma irradiation; MCF 7 cells; Oxidative damage; ROS generation

## Introduction

The strategy to improve cancer radiotherapy depends largely on achieving the increased tumor cytotoxicity but with least toxic effects to normal cells. This task is not straightforward to achieve in clinical settings but considerable active research has been devoted to either refining advancements on radiation treatment or to develop compounds that preferentially sensitize tumor cells to radiation.

Breast cancer is the most common malignancy in women in the World. It is estimated that one out of eight women in industrialized nations would develop breast cancer and the incidence rates in developing nations is on rise as predicted in a recent study. Approximately one-third of the women with breast cancer develops metastasis and ultimately succumbs to death from the disease. Increasing the doses of chemotherapeutic drugs and radiation fails to improve response and many of them develop resistance to therapies. An ideal strategy would be to search for anticancer drugs that trigger the process of cell death preferentially in tumor cells and spare the normal cells. Compounds occurring naturally in human diets are generally devoid of toxic effects within certain doses.

Curcumin (1, 2) is a major chemical component of turmeric (3) that is used as a spice to give a specific flavor and yellow color in Asian foods (2). Curcumin has been reported to have several pharmacological properties including anti-tumor, anti-inflammatory and antioxidant properties (2, 4). An epidemiological study has revealed that observed low incidence of bowel and prostate cancer in Indians

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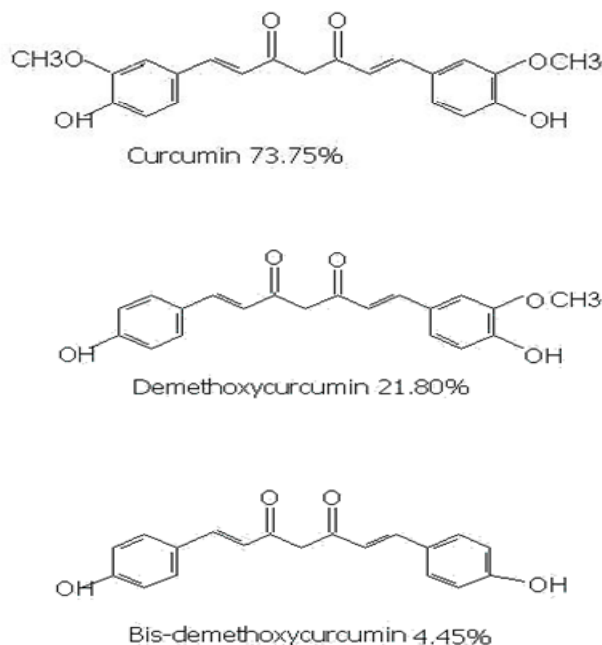
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may be due to natural additives like curcumin in their cookeries (5). Curcumin has been shown to induce apoptosis and inhibit growth in a wide variety of tumors such as mammary tumor, duodenal and colon cancer and TPA-induced skin tumors in mice (6).

Curcumin is largely a non-toxic and effective pharmacological agent that blocks the constitutive NF- $\kappa$ B activity in a broad range of cancer cells (7). The molecular mechanism of NF- $\kappa$ B inhibition by curcumin is unclear, but some studies have demonstrated inhibition of I $\kappa$ B degradation (7, 8) or direct suppression of I $\kappa$ Ba kinase (9). It has been shown that curcumin inhibits NF- $\kappa$ B activation that in turn downregulates endogenous Bcl-2 protein (10). Further, we demonstrated that curcumin radiosensitizes PC-3 prostate cancer cells by inhibiting radiation-induced pro-survival factors, such as, NF- $\kappa$ B and Bcl-2 expression (11). This is supported by a recent study that curcumin radiosensitizes head and neck cancer cells by accumulating the cells in the G2 phase which is the most radiosensitive phase of the cell cycle (12). In addition, curcumin is known to cause apoptosis in human epithelial cell lines in the G2 phase of the cell cycle (13).

Ionizing radiation and certain cytotoxic drugs are known to induce oxidative stress in cells through generation of reactive oxygen species (ROS) leading to the imbalance of the cytosolic pro-oxidant and antioxidants levels, resulting in the culmination of cell death. This ROS is implicated in PTP opening that leads to disruption of mitochondrial membrane potential and the release of cytochrome *c* in the cytosol where Apaf-1 binds to form the apoptosome complex. Most of the radiosensitizers are believed to work through upregulation of the ROS generation. The radiosensitizing effect of curcumin is a paradoxical effect for a drug that is a well-known antioxidant described as a cytoprotective agent in different models (14, 15). Curcumin has been shown to induce ROS in certain cell types like AK-5 lung carcinoma (3) but in MCF-7 and many other cell types it has been shown to scavenge ROS (16). Curcumin has been demonstrated to protect mitochondria against accumulation of ROS and lipid peroxidation of membranes on one hand and induce the PTP opening and cause loss of mitochondrial transmembrane potential on the other (17). The direct interaction of curcumin with the protein thiols of the mitochondrial membrane has been implicated for this effect (17).



**Figure 1. Structure and Composition of Curcumin C3 Complex**  
The composition of Curcumin C3 used in this study was, Curcumin 73.75%, Demethoxycurcumin 21.80%, Bis-demethoxycurcumin 4.45%.

In the present study, we chose a radio-resistant cell line MCF-7 from breast carcinoma (18, 19) as a model to study the enhancement effect of curcumin when combined with  $\gamma$  radiation. The dry rhizome of turmeric contains curcumin, the main bioactive component, demethoxycurcumin and bisdemethoxycurcumin. Studies have shown that the mixture of natural curcuminoids is more active than pure curcumin (20). Hence, for the present study, mixture of natural curcuminoids, Curcumin C3 Complex containing, Curcumin 73.75%, Demethoxycurcumin 21.80%, Bis-demethoxycurcumin 4.45%, (Figure 1) was used and this composition resembles with the commercially available curcumin.

## Material and Methods

### Materials and Cell culture

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco Co. USA, Fetal Bovine Serum was obtained from Himedia, Mumbai, India. MCF-7 cells were purchased from National Center for Cell Sciences, Pune, India. All other chemicals were obtained from Sigma-Aldrich unless mentioned otherwise. MCF-7 cells were cultured in DMEM supplemented with FBS (10%) and antibiotics

(streptomycin 200 $\mu$ g/ml and penicillin 100units /ml) at 37°C in 5% CO<sub>2</sub> environment.

### **Drug and Radiation treatment**

Curcumin C3 (Curcumin 73.75%, Demethoxycurcumin 21.80%, Bis-demethoxycurcumin 4.45%) was a kind gift from M/s Sami Laboratories, Bangalore, India. Stock solutions at 50 mM were prepared in DMSO, protected from light and stored at -20°C. After 24 of plating the cells, the medium was removed and replaced with fresh medium containing DMSO or medium containing different concentrations of curcumin. For radiation treatments, cells were either left untreated or treated with different doses of radiation alone (1-5 Gy), or different concentrations of curcumin alone or in combination of curcumin with radiation. For combination treatments, curcumin (5  $\mu$ M or 10  $\mu$ M) was added to the cultures 2 h prior to radiation treatment (5 Gy).

### **Determination of cell survival**

#### *(a) Assessment of cellular viability*

MCF-7 cells (3 x 10<sup>5</sup> cells/ml) suspended in DMEM supplemented with 10 % FBS were incubated with increasing concentrations of curcumin. Viability of the cells was determined by trypan blue dye exclusion method (final volume of the dye 0.04 % w/v). A total of four independent experiments were performed and the mean with standard deviations was calculated.

#### *(b) Determination of cytotoxicity*

The anti-proliferative effect of curcumin and radiation was investigated by MTT dye uptake method (21). Briefly, the cells (5000 per well) were incubated in quadruplicates in a 96 well plate in presence and absence of indicated concentrations of curcumin in a final volume of 0.2 ml. They were either left untreated or treated with radiation. After 96 hours of incubation, MTT dye (0.5 mg/ml) was added to samples. The plates were then further incubated for 3 hrs at 37°C. Thereafter, 0.1ml of solubilization buffer (10 % SDS in 0.01N HCl) was added. Incubation was continued overnight at 37°C and the optical density was measured at 570 nm using a microplate reader (Bio-Tek Instruments, USA) with the solubilization buffer as blank. The following formula was used for percentage cell viability, (OD of the sample/ OD of the control) x 100. A total of three independent experiments were performed and the mean with standard deviations was calculated.

### **Determination of apoptosis**

#### *(a) Annexin V and PI dual staining*

Apoptosis induction in control as well as in treated cells were determined using Annexin V-FITC and Propidium Iodide dual staining kit (Boehinger Mannheim, Germany) following the protocol provided by the manufacturer and apoptotic cells were counted using a fluorescent microscope (Optiplot 2, Nikon, Japan). To study the concentration and time dependent effect of both curcumin and radiation either alone or in combination, MCF-7 cells (3 x 10<sup>5</sup> cells/ml) were plated in 35 mm culture plates and incubated for various test conditions for 24-48 hrs under culture conditions. Cells were collected after trypsinization and were assayed for apoptosis. From the labeled cell suspension, green and red cells were counted out of 400 randomly selected cells per sample. The cells showing only green fluorescence were taken to be apoptotic while those showing both green and red were taken to be necrotic. A total of four independent experiments were performed and the mean with standard deviations was calculated.

The confirmation of Annexin-PI staining was performed by flow cytometry. Briefly, 1 x 10<sup>6</sup> cell/ml were washed in PBS and re-suspended in 100  $\mu$ l of binding buffer containing Annexin-FITC and PI (according to the kits protocol) and analyzed by Fluorescent Activated Cell Sorter (FACS) vantage flow cytometer that uses the Cell Quest acquisition and analysis program (Becton Dickerson, San Jose, CA)

#### *(b) Apoptosis by flow cytometry*

Flow cytometry by PI stained and permeabilized cells were used to quantitate the percentage of apoptotic (sub G1) cells (22). Briefly, the cells were trypsinized, washed with PBS and re-suspended in lysis solution (100 mM Tris, 154 mM NaCl, 1 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub> and 0.1% NP-40) with 50  $\mu$ g/ml of PI and 40  $\mu$ g/ml of RNase enzyme. The cells were incubated in ice for 20 min; the samples were thereafter injected into FACS Vantage flow cytometer and the apoptotic sub G1 population was sorted and analyzed using the cell quest acquisition software (Becton Dickerson, San Jose CA). A total of three independent experiments were performed and the mean with standard deviations was calculated and plotted.

### **Estimation of intracellular ROS**

The generation of intracellular ROS was measured by H<sub>2</sub>DC-FDA, (2,7,

dichlorodihydrofluorescein diacetate) fluorescent probe as described previously (23). Briefly, MCF-7 cells ( $1 \times 10^5$  cells/ml) in PBS were incubated with various concentrations of curcumin for 30 min. During the last 20 minutes of incubation  $H_2DC\text{-FDA}$  ( $10 \mu\text{M}$ ) was added to the samples. The cells were then either left untreated or treated with different doses of radiation. The measurement of fluorescent intensity ( $\lambda_{\text{ex}}=490$  and  $\lambda_{\text{em}}=520$ ) was then carried out in a fluorescent spectrophotometer (LS50B, Perkin Elmer, USA). This was repeated three times independently to obtain mean and standard deviation.

#### ***Determination of loss of mitochondrial membrane potential (MMP)***

The measurement of mitochondrial membrane potential changes was carried out using the fluorescent dye, DiOC<sub>6</sub> (Molecular Probes) as previously described (24). Cells were incubated for different treatments, at various time points the cells were trypsinised, washed and counted.  $2 \times 10^5$  cells were taken and incubated for 15 minutes with DiOC<sub>6</sub>, a fluorescent probe. The cells were then washed and fixed. After completion of the incubation period the fluorescent intensity was measured using a spectrofluorimeter ( $\lambda_{\text{ex}}=488$  and  $\lambda_{\text{em}}=525$ ). This was repeated three times independently to obtain mean and standard deviation.

#### ***Statistical Analysis***

Data was analyzed using Microsoft Excel. Statistical evaluations of cell viability, survival and percentage apoptosis was done on experimental data of three experiments done in triplicates. Data are represented as mean  $\pm$ SD (standard deviation from the mean) and compared by one or two-way ANOVA as appropriate, followed by relevant post *t* test to determine *P* values. A *p* value of  $<0.05$  was considered significant.

### **Results**

#### ***Curcumin induced dose and time dependent cytotoxicity in MCF-7 cells***

The structure and composition of curcumin C<sub>3</sub> used in the experiments is illustrated in Figure 1. We first tested the effect of curcumin on tumor cell viability. Human breast carcinoma cell line, MCF-7 was treated with different concentrations of curcumin for various time periods. After 1, 2 and 3 hrs of treatment, the cell viability was assessed by trypan blue dye exclusion. Curcumin treatment

showed both time and concentration dependent decrease in viability. It was found that  $\approx 50\%$  of the cells lost their viability after 3 hr incubation with  $100 \mu\text{M}$  curcumin (Figure 2A).

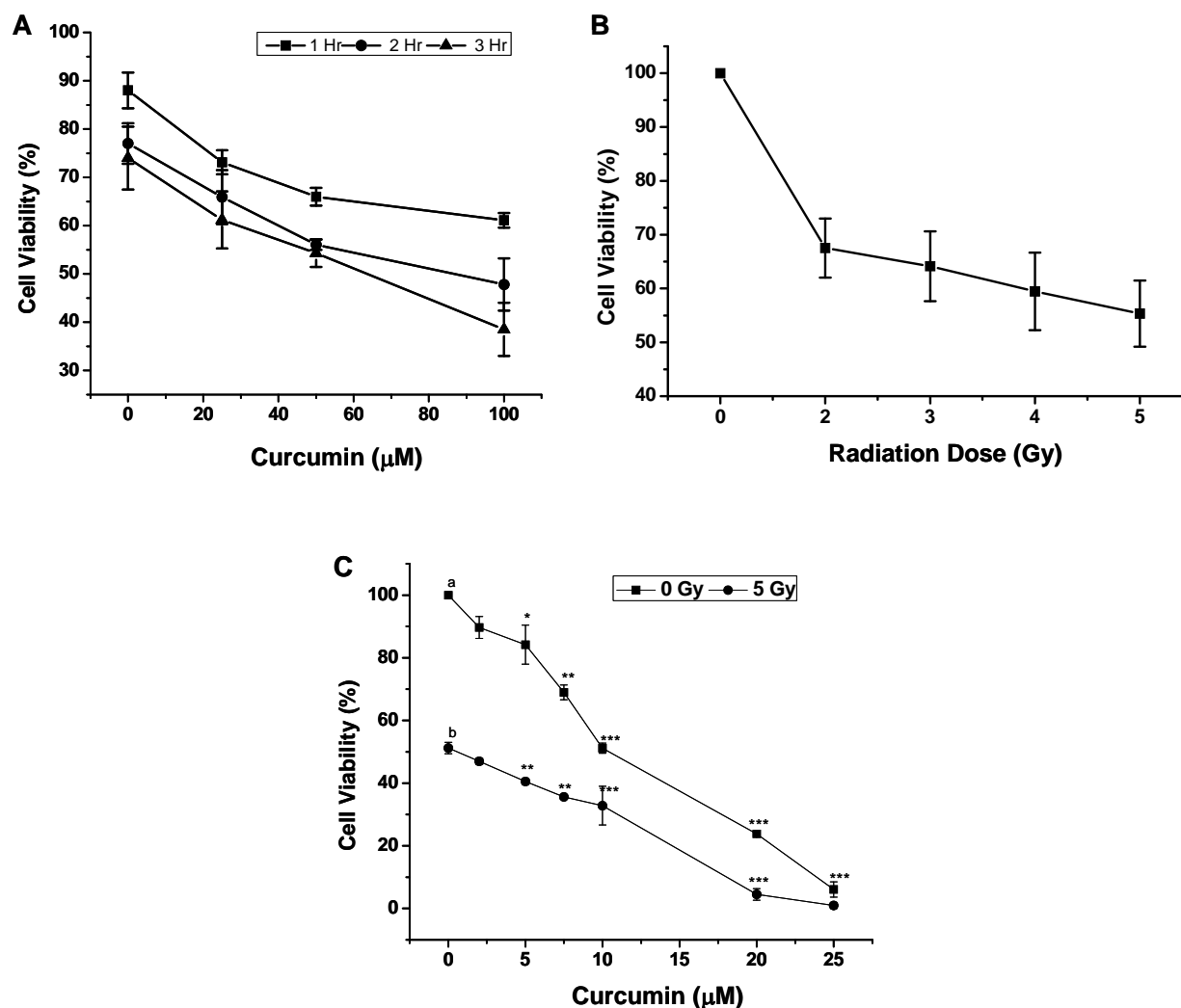
The loss of viability of MCF-7 cells in response to combined treatment of curcumin and  $\gamma$  radiation was assessed by MTT method. Treatment of cells with curcumin showed an increasing loss of cell proliferation that was directly proportional to the curcumin concentration. The IC<sub>50</sub> value of curcumin after 96 hrs of incubation was  $\sim 10 \mu\text{M}$  for MCF-7 cells, that is consistent with the previously reported data (25). Irradiation of cells by  $\gamma$  rays caused a dose dependent decrease in the proliferation of cells. Typically, the viability of cells were 67.5%, 64%, 59% and 55% after 72 hrs of radiation exposure to doses of 2, 3, 4 and 5 Gy respectively (Figure 2B).

The combination treatment of cells with curcumin and radiation showed an additive effect on the loss of cell proliferation. Typically, a dose of  $5 \mu\text{M}$  and  $10 \mu\text{M}$  of curcumin reduced the survival to 81 and 51% respectively, after 96 hrs of incubation. A dose of 5 Gy after 96 hours reduced the proliferation of MCF-7 cells to 52%. Treatment of cells with 5 and  $10 \mu\text{M}$  of curcumin followed by irradiation at 5 Gy reduced the survival of cells to 39 and 32% respectively (Figure 2C). Hence, a significant increase in cell kill by radiation was observed after pre-treatment of curcumin with an enhancement ratio of 1.4 and 1.67 for 5 and  $10 \mu\text{M}$  concentrations respectively (Table I).

#### ***Curcumin mediated radiosensitizing effect reflected in induction of apoptosis***

Effect of curcumin with and without radiation on the apoptosis of MCF-7 cells were assessed by Annexin V-PI staining after 24 and 48 hours of treatment. The cells were counted using fluorescence microscopy. Radiation caused apoptosis at 17 and 19%, whereas, cells treated with  $5 \mu\text{M}$  curcumin showed apoptosis at 18.7 and 19.2% at 24 and 48 hours respectively. The combined treatment of cells with curcumin and radiation significantly enhanced the induction of apoptosis that increased with the time of incubation. The percent apoptosis was 24% and 34% at 24 hr and 48 hr respectively (Figure 3A). The enhancement ratio for the induction of apoptosis was 1.4 and 1.8 for 5 and  $10 \mu\text{M}$  respectively.

To reconfirm the microscopy data, Annexin V-PI stained cells were subjected to flow cytometric analysis. An increase in cell death (18%) was observed with curcumin ( $5 \mu\text{M}$ ) followed by 5 Gy radiation (Figure 3B). The induction of apoptosis



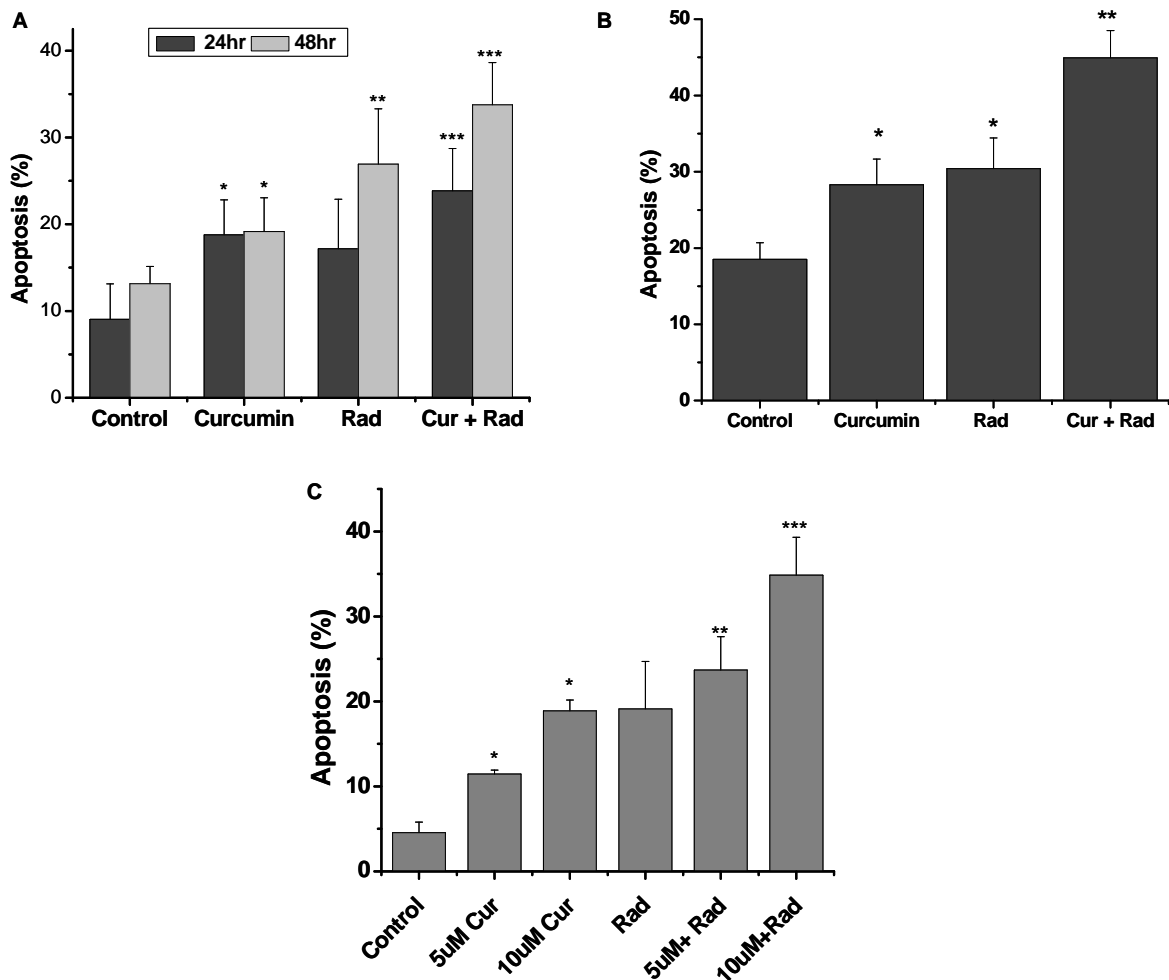
**Figure 2. (A) Curcumin-induced cytotoxicity.** MCF-7 cells,  $2 \times 10^5$  /ml, were treated with curcumin (25-100  $\mu\text{M}$ ) and viability of samples was determined after 1-3 hr of treatment by blue dye staining. Percent viability was calculated from untreated samples. Data are means  $\pm$ SD of three different experiments done in triplicates. **(B) Proliferation of MCF-7 cells treated with different doses of  $\gamma$  radiation.** Cells ( $10 \times 10^4$ ) were seeded in 96 well plates in triplicates, for 24 hrs and irradiated subsequently for a defined dose (2-5 Gy). Cell proliferation was determined after 72 hours by MTT assay. Data are means  $\pm$ SD of four different experiments done in triplicates. **(C) Effect of curcumin and  $\gamma$  radiation on the proliferation of MCF-7 cells.** Cells ( $5 \times 10^3$ ) were seeded in triplicates on a 96 well plates followed by treatment with indicated concentrations of curcumin. One hour later, plates were sham irradiated or exposed to a dose of 5 Gy. Cell proliferation was determined after 96 hrs by the MTT assay. Untreated or un-irradiated cells served as controls. Data are means  $\pm$ SD of three different experiments done in triplicates. Statistically significant difference vs the untreated control (a) and irradiated control (b) are indicated: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

was determined through cell cycle analysis by PI staining. The percentage of cells in the subG1 phase after 96 hrs of irradiation over untreated population was 19%, compared to 5 and 10  $\mu\text{M}$  curcumin alone treated cells showing 12% and 19% respectively. However, when radiation was combined with curcumin an increase in the percentage of cells in subG1 was observed and this was found to be concentration dependent (23.5 % at 5  $\mu\text{M}$  and 34 % at 10  $\mu\text{M}$ ) (Figure 3C). The apoptosis enhancement

ratios for 5 and 10  $\mu\text{M}$  were 1.2 and 1.8 respectively (Table III)

### **Curcumin downregulated radiation-induced ROS**

ROS is implicated as vital intermediate in the induction of apoptosis by radiation (26, 27). We assessed ROS generation inside the cells by using dichlorodihydrofluorescein diacetate fluorescence probe in response to these treatments. Irradiation



**Figure 3. (A) Effect of curcumin and  $\gamma$  radiation on induction of apoptosis in MCF-7 breast cancer line.** Cells were incubated with curcumin (5  $\mu$ M) and irradiated with a dose of 5 Gy after 2 hrs. Percentage apoptosis in control and treated cells was determined after ~24 hrs or 48 hrs by Annexin-V and propidium iodide dual staining by fluorescence microscopy, as described in Materials and Methods. Cells showing only green fluorescence were considered apoptotic while those showing either red or both red and green were considered as necrotic. Data are means  $\pm$  SD of three different experiments done in triplicates. Statistically significant differences vs the untreated control were determined as indicated. \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001. **(B) Determination of apoptosis induction by curcumin and  $\gamma$  radiation on MCF-7 cells.** Cells were incubated with curcumin (5  $\mu$ M and 10 $\mu$ M) and irradiated (5 Gy) after 2 hrs. Cells were collected after 24 hrs of incubation; control and treated cells were stained with FITC conjugated Annexin-V and propidium iodide as described in Materials and Methods. Data were collected using a FACS Vantage (Becton-Dickenson, USA) and analyzed by using the cell quest software. Statistically significant difference vs the untreated control were determined as indicated. \* $P$ <0.05; \*\* $P$ <0.01. **(C) Determination of Sub G1 cells after treatment with curcumin and  $\gamma$  irradiation** MCF-7 cells were incubated with different concentration of curcumin (5 and 10  $\mu$ M) for 2 hrs and subsequently irradiated with a dose of 5 Gy. Control and treated cells were stained with propidium iodide as described in Materials and Methods. Cell cycle analysis was done using the FACS Vantage (Becton-Dickenson, USA) and analyzed by using the Cell Quest software (Becton-Dickenson, USA). Cells in the sub G1 phase were taken to be apoptotic. Data are means  $\pm$  SD of three different experiments done in duplicates. Statistically significant differences vs the untreated control are indicated. \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001.

was found to cause a dose-dependent increase in generation of intracellular ROS (Figure 4A). Because curcumin acts as a free radical scavenger, it was of interest to evaluate its impact on radiation-induced generation of ROS. Curcumin alone reduced generation of ROS when compared to vehicle treated MCF-7 cells. On the other hand, treatment with 2 and 5 Gy of radiation induced a 2.2 and 4.6 fold

increase in ROS. When curcumin treated cells were irradiated, generation of ROS was inhibited and this was found to be dose dependent. Pretreatment with curcumin at concentration of 2.5, 5 and 10  $\mu$ M followed by irradiation (5 Gy) inhibited the generation of ROS by 20.9, 25.2 and 30% respectively, in MCF-7 cells. Indeed incubation with a 20 $\mu$ M concentration of curcumin 30 minutes prior

**Table 1. Enhancement ratio in survival fraction of MCF-7 cells treated with curcumin and radiation**

Treatment	Survival Fraction	Enhancement Ratio
Radiation alone (5 Gy)	55.13	-
Curcumin 5 $\mu$ M	78.7	-
Curcumin 10 $\mu$ M	51.465	-
Curcumin 5 $\mu$ M + 5 Gy	39.353	1.400
Curcumin 10 $\mu$ M + 5 Gy	32.85	1.678

to irradiation dose of 2 Gy reduced ROS to the levels comparable with the vehicle treated control cells (Figure 4B), demonstrating curcumin's ability to act as a free radical scavenger.

### **Curcumin treatment leads to mitochondrial transmembrane potential change**

There is increasing evidence that altered mitochondrial function plays a role in process of cell apoptosis and a decreasing mitochondrial transmembrane potential ( $\psi_m$ ) (MMP) is associated with mitochondrial dysfunction. Curcumin treatment is known to cause a loss in the MMP (3, 17, 28, 29). In this study, we assessed the change in MMP,  $\Delta\psi_m$  using the fluorescent probe, DiOC<sub>6</sub> employing spectrofluorimetric method. As shown in Figure 5A, MCF-7 cells exposed to 2.5, 5, and 10  $\mu$ M of curcumin for 30 minutes showed a marked decrease in DiOC<sub>6</sub> fluorescence, with the mean values of 40, 18.2 and 8.5 indicating that curcumin induced alterations in membrane potential. The fluorescence intensity of the control was taken to be 100. Further, we measured  $\psi_m$  24 hrs after irradiation (5 Gy) of MCF-7 cells. Cells treated with curcumin (10  $\mu$ M) followed by irradiation (5 Gy) showed a decrease of 35 % in the fluorescence of DiOC<sub>6</sub> as compared to vehicle control (Figure 5B). These findings demonstrate that curcumin can cause a dynamic change in MMP that can be further altered in presence of radiation.

### **Discussion**

Extensive evidence has been documented in the literature to suggest that both curcumin and ionizing radiation cause induction of apoptosis in tumor cell. It is generally accepted that radiation induced apoptosis is mediated through the generation of reactive oxygen species (ROS) in tumor cells causing oxidative damage to vital molecules (26,

27). Whereas as curcumin is a well-known antioxidant that has the ability to scavenge free radicals and is reported to offer protection to normal cells against the ionizing radiation. In fact oral administration of curcumin (200  $\mu$ mol/kg) was reported to significantly reduce lung toxicity in rats treated with whole-body irradiation (10 Gy in five fractions) by inhibiting liver and serum lipid peroxidation (29). In addition, curcumin was shown to reduce radiation-induced genotoxicity by preventing DNA damage (29, 30) and late effects of radiation (31). But on the other hand there have been reports of curcumin acting as a radiosensitizer to tumor cells. Our group was the first to report that curcumin enhanced the inhibitory effects of X-ray irradiation in prostate cancer cell line PC-3 (11) and others have demonstrated the same effect in head and neck cancer cell lines (12). In another study, it was demonstrated that curcumin induced a dual effect on irradiated cells; in one situation curcumin caused an anti-oxidant effect (31), whereas, in certain other conditions, it can cause pro-oxidant effect leading to increased DNA damage (1).

Our studies were aimed to investigate the effect of curcumin on  $\gamma$  radiation treated MCF-7 cell line. It has recently been demonstrated that mitochondria might be the target for curcumin-induced apoptosis in tumor cells, which involves alterations in mitochondrial membrane potential (24). Curcumin is reported to downregulate ROS in MCF-7 cells (16) and at the same time cause the loss of mitochondrial transmembrane potential and induce apoptosis (25, 32). It was of interest to examine the role of curcumin on the modification of these factors in irradiated cells. Our results demonstrate that curcumin treated tumor cells exhibited enhanced potentiation of radiation induced inhibition of viability as assessed by MTT assay (Figure 2B). Reduced survival in response to combined treatments can be owed to increased apoptosis

**Table 2. Enhancement ratio for apoptosis induction in MCF-7 cells after treatment with curcumin and radiation as calculated by annexin-V and PI dual staining after 24 and 48 hrs**

Treatment	Percentage Apoptosis	Enhancement ratio
Radiation alone (5 Gy) 24 hr	17.15	-
Curcumin 5 $\mu$ M 24 hr	18.76	-
Curcumin 5 $\mu$ M + 5 Gy 24 hr	23.80	1.39
Radiation alone (5 Gy) 48 hr	19.10	-
Curcumin 5 $\mu$ M 48 hr	26.90	-
Curcumin 5 $\mu$ M + 5 Gy 48hr	33.75	1.767

Table 3. Enhancement ratio for apoptosis induction in MCF-7 cells following exposure to curcumin and radiation as calculated by the percentage of cells in the sub G1 phase of cell cycle

Treatment	Percentage apoptosis	Enhancement ratio
Radiation alone (5 Gy)	19.1	-
Curcumin 5 $\mu$ M	11.43	-
Curcumin 10 $\mu$ M	18.885	-
Curcumin 5 $\mu$ M+ 5 Gy	23.68	1.24
Curcumin 10 $\mu$ M+5 Gy	34.855	1.824

observed (Figure. 3). Further, we found that radiation induced ROS generation was reduced by curcumin probably by scavenging process which is in line with the reported effects in a number of studies (16) as shown in (Figure 4B). In contrast, curcumin was found to cause a significant loss in MMP of tumor cells post treatment after 30 minutes. Apparently these observations are paradoxical because upregulation of ROS and loss of MMP have been widely accepted as a mechanism of induced apoptosis by several agents/ drugs.

It is known that modulation of PTP activity involves multiple regulatory steps including the role of the redox phenomenon caused due to an enhanced induction of ROS, and specifically the oxidation and cross-linkage of mitochondrial membrane protein thiol groups. Morin et al showed that curcumin significantly inhibited the generation of ROS associated with an increase in membrane permeability of rat liver mitochondria resulting in swelling, loss of membrane potential and inhibition of ATP synthesis (17, 33). These effects were suggested to be mediated by the opening of the permeability transition pore (PTP) leading to cell apoptosis. It was shown that curcumin protected the mitochondria against the accumulation of ROS as well as lipid peroxidation of membranes due to its free radical scavenging activity yet caused an opening of the PTP. The major pathway for curcumin mediated pore induction was shown to involve the oxidation of membrane protein thiol function and not necessarily through ROS pathway.

Hence, the findings of this study might strongly suggest that curcumin induced membrane protein thiol oxidation might cause opening of permeability transition pore and it simultaneously competes to abrogate the ROS generation, The balance between its protective antioxidant effect and its ability to induce PTP (a direct cause of apoptosis), probably dictate the cell to undergo survival or death. Based on the findings reported here and as well as by others, it is strongly indicated that the

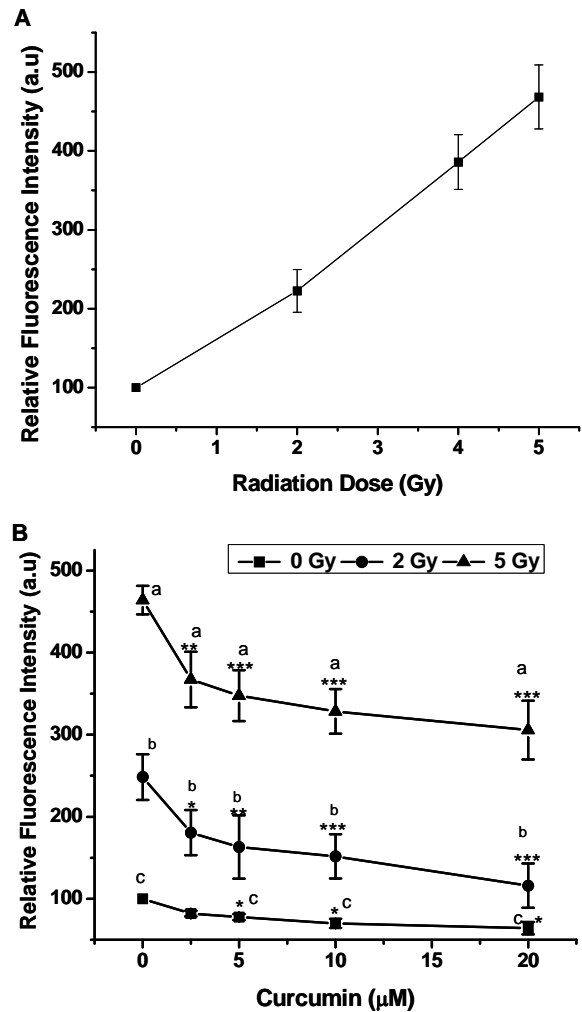
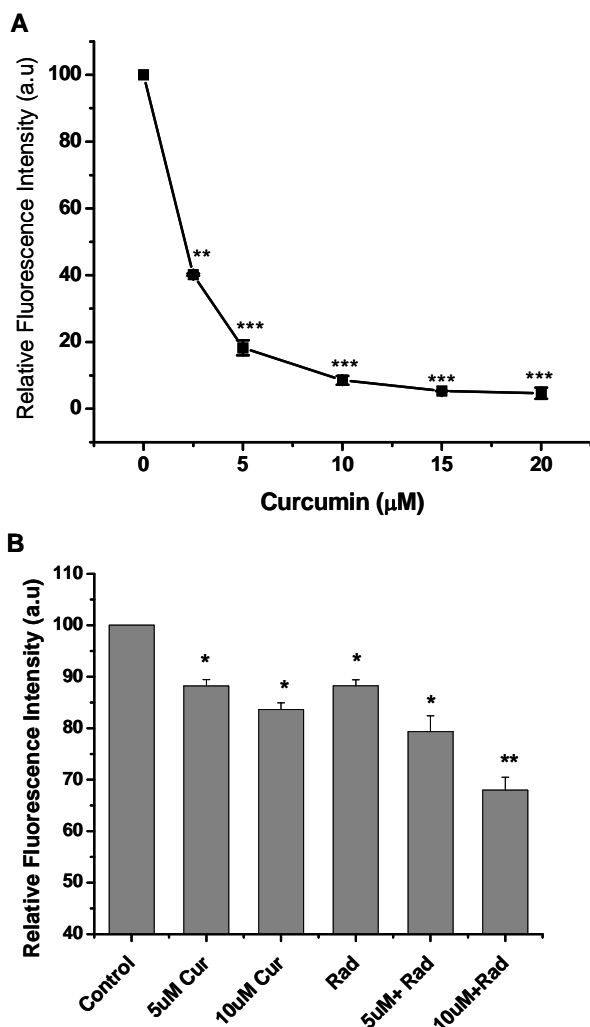


Figure 4. (A) Generation of ROS as a function of radiation dose. MCF-7 cells were labeled with fluorescent probe  $H_2DCFDA$  for 20 minutes and then irradiated (2-5 Gy). Fluorescent intensity ( $\lambda_{ex} = 490$  nm and  $\lambda_{em} = 520$  nm) was thereafter measured in a quartz cuvette using Fluorescence Spectrometer (LS50B, Perkin Elmer). Data are means  $\pm$  SD of three different experiments done in triplicates. (B) Effect of curcumin treatment on gamma radiation induced ROS. MCF-7 cells were incubated with various concentrations of curcumin (2.5-20  $\mu$ M) for 30 minutes. During the last 20 minutes of this incubation, fluorescent probe  $H_2DCFDA$  (10  $\mu$ M/ml) was added. Cells were subsequently sham irradiated or treated with a dose of 2 Gy or 5Gy. The fluorescent intensity ( $\lambda_{ex} = 490$  and  $\lambda_{em} = 520$ ) was thereafter measured. ROS levels were compared to samples which were not submitted to any treatment i.e. 0 Gy (a) or to respective irradiated controls, 2 Gy (b) and 5 Gy (c). Data are means  $\pm$  SD of three different experiments done in triplicates. Statistically significant difference vs the untreated control (a) and irradiated control (b, c) are indicated. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

enhancement effect of curcumin on gamma radiation-induced cell death effect is via a pathway not chiefly dependent on the ROS generation.

We earlier reported that curcumin caused enhanced radio-sensitization of PC-3 a p53 null





**Figure 5. (A) Estimation of loss of mitochondrial membrane potential by curcumin.** MCF-7 cells were incubated with various concentrations of curcumin (2.5-20 µM) for 30 minutes and subsequently irradiated (5 Gy). DiOC<sub>6</sub> (40 nM), fluorescence probe was added and cells were incubated for another 15 minutes. Thereafter the fluorescent intensity was measured at  $\lambda_{ex} = 488$  and  $\lambda_{em} = 525$ , using a fluorescence spectrometer. Data are means  $\pm$  SD of three different experiments done in duplicates. Statistically significant differences vs untreated control are indicated. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001. **(B) Evaluation of MMP of MCF-7 cells after 24 hrs of treatment with curcumin and  $\gamma$  radiation.** Cells were treated with different concentrations of curcumin (5 and 10µM), and irradiated with a radiation dose of 5 Gy. Following incubation for 24 hrs, cells were trypsinised and counted and  $1 \times 10^5$  cells were loaded with DiOC<sub>6</sub> dye and incubated at 37°C for 30 minutes. The fluorescent intensity of the cells was then measured at  $\lambda_{ex} = 488$  and  $\lambda_{em} = 525$ .

prostate cancer cell line (11) and of the underlying mechanism was due to inhibition of radiation-induced NFκB activity. Altogether, this effect was mediated through down regulation of radiation-

induced pro-survival factors and enhancing radiation-induced pro-apoptotic signaling. This may possibly be one of the mechanisms for the radiosensitizing effect of curcumin on MCF-7 cells. An, another investigation by Khafif et al (12) reported the radiosensitizing effect of curcumin in head and neck cancer cell lines. They suggested that the observed effect was due to the fact that curcumin abrogated the G2/M block caused by radiation. In our study we did not observe abrogation of radiation-induced G2/M block (data not shown). In this study, we demonstrated that curcumin is able to radio-sensitize a classical radio-resistant cell line MCF-7 that harbor wild type p53 with caspase-3 negative background. Altogether, the findings of this study conclude that mitochondria might be an important target through which curcumin mediates its radio-sensitizing effects and this may not necessarily involve a ROS dependent pathway.

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### Conflicts of Interest

No potential conflicts of interest to disclose.

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