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Curcumin in Prostate Cancer

PRINCIPAL INVESTIGATOR: Damodaran Chendil, Ph.D.

CONTRACTING ORGANIZATION: Kentucky University Research Foundation
Lexington, Kentucky 40506-0057

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13. ABSTRACT (Maximum 200 Words) In prostate cancer, constitutive activation of NFkB and AP1, non-functional p53 and defective androgen receptor signaling together may play a role in rendering intrinsic resistance to radiation in prostate cancer. Many reports in literature have showed that Curcumin (diferulylmethane is a major chemical component of a curry spice, turmeric) is a potent inhibitor of prostate cancer cell growth. It was found that Curcumin inhibits TNF- α mediated activation of NFkB and down-regulates Bcl-2 expression. From this reported observation, we hypothesized that Curcumin will abrogated the upregulation of pro-survival genes by radiation. Our preliminary data demonstrated that Curcumin significantly inhibited the growth of androgen-independent prostate cancer cell line PC-3. In addition, Curcumin conferred significant enhancement of radiation-induced clonogenic inhibition and apoptosis in prostate cancer cell line PC-3. Interestingly, Curcumin also inhibited the radiation induced prosurvival factors such as NFkB activity and Bcl-2 expression. These results underscore the need to formally study the functional relevance of Curcumin in synergizing the effects of radiation in prostate cancer. Since the proposal was funded for two years and the PI was asked to drop aim 3 and request to resubmit the aims. Hence, the first aim is completed in the proposed time.				
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Introduction

Inhibition of radiation-induced pro-survival genes by Curcumin in prostate cancer.

PI: Damodaran Chendil., PhD

BACKGROUND: In prostate cancer, constitutive activation of NF κ B and AP1, non-functional p53, defective androgen receptor signaling, and high constitutive levels of Cyclooxygenase (COX-2) enzyme and inactivation of tumor suppressor gene PTEN/MMAC together may play a role in rendering intrinsic resistance to radiation in prostate cancer. In wild-type p53 background, radiation often induces p53 that culminates the upregulation of Bax with a concomitant decrease in the level of Bcl-2 leading to cell death. On the other hand, in p53 mutant background, radiation causes induction of bcl-2 through activation of NF κ B. Many reports in literature have showed that Curcumin (diferulylmethane is a major chemical component of a curry spice, turmeric) is a potent inhibitor of prostate cancer cell growth. It was found that Curcumin inhibits TNF- α mediated activation of NF κ B and down-regulates Bcl-2 expression. From this reported observation, we hypothesized that Curcumin will abrogated the upregulation of pro-survival genes by radiation. Our preliminary data demonstrated that Curcumin significantly inhibited the growth of androgen-independent prostate cancer cell line PC-3. In addition, Curcumin conferred significant enhancement of radiation-induced clonogenic inhibition and apoptosis in prostate cancer cell line PC-3. Interestingly, Curcumin also inhibited the radiation induced pro-survival factors such as NF κ B activity and Bcl-2 expression. These results underscore the need to formally study the functional relevance of Curcumin in synergizing the effects of radiation in prostate cancer.

HYPOTHESIS: Based on these factorial observations mediated above, it is hypothesized that in prostate cancer cells that lack functional p53, radiation will upregulate NF κ B activity and Bcl-2 expression and confer an "induced radiation resistant phenotype". Curcumin will inhibit the radiation-induced NF κ B and Bcl-2 expression and will allow radiation to enhance the apoptosis. Thus, we hypothesize that addition of Curcumin will abrogate the "induced radiation resistance" phenotype and enhanced pro-apoptotic action of radiation in prostate cancer. **SPECIFIC AIMS:** To test the above hypotheses, two specific aims are proposed:

(1) Effect of Curcumin with or without radiation on the clonogenic inhibition, cell cycle phase and apoptosis of prostate cancer cell lines.

(2) To determine the mechanism of action of Curcumin mediated radiosensitizing effect by analyzing the pro-survival transcription factor activity (such as AP-1, NF κ B and AR activation) and its target genes.

STUDY DESIGN: Specific aims 1 and 2 will be studied using PC-3, DU-145, 22Rv -1 and LNCaP cells. In aim 1, we will study the effect of Curcumin with or without radiation on clonogenic inhibition and cell cycle phases and correlate with apoptosis. In the second aim, we will study the kinetics of pro-survival transcription factor activity such as AP1, AR and NF κ B in response to Curcumin in combination with and without radiation. This will be analyzed by protein-DNA array and confirmed gel shift analysis and reporter assays.

RELEVANCE: Curcumin is a natural dietary compound that is not consumed in USA. On the contrary, everyday use of Curcumin as a dietary agent in India may have led to reduce incidence of prostate cancer. In addition to chemopreventive effects of Curcumin on prostate cancer, it kills prostate cancer cells and is a potent chemo-adjuvant for enhancing the effects of radiation. Thus, if our *in-vitro* studies demonstrate that Curcumin plays pivotal role in enhancing the effects of radiation, then we will plan studies to test this concept in animal models and patients with prostate cancer.

Final report.

BODY

In this section a detailed description of the research accomplishment will be described as per outlined in the approved states of work. The following tasks pertain to the first year of the grant period.

TASK-1. Effect of curcumin in PC-3, DU-145, 22Rv1 and LNCaP cells, months 1-12

Dose response curves for curcumin and curcumin plus radiation will be generated for all the four prostate cancer cell lines. In response to these treatments, cell death will be assayed by TUNEL staining and flow cytometry (Propidium Iodide staining). Cell cycle changes will be assessed by flow cytometry.

The response data on clonogenic inhibition and apoptosis will be compared with cell cycle changes. Months 1-12.

Aim 1: Effect of Curcumin with or without radiation on the clonogenic inhibition, cell cycle assays and apoptosis of androgen dependent and androgen independent prostate cancer cell lines.

Purpose: The present study was taken to determine that curcumin is a potent sensitizer of radiation-induced pro-apoptotic action in prostate cancer cells. This was accomplished by partially by aim 1.

Results: Curcumin (diferuloylmethane) is a major chemical component of turmeric (*Curcuma longa*) and is used as a spice to give a specific flavor and yellow color in Asian food. Curcumin exhibits growth inhibitory effects in a broad range of tumors as well TPA-induced skin tumors in mice. This study was undertaken to investigate the radio sensitizing effects of curcumin in prostate cancer cell lines. Results of PC-3 results are given in abstract form and these results are published in peer reviewed journal (Oncogene) and a copy of published article is enclosed herewith in Appendix II along with the figures.

PC-3

Curcumin [Diferuloylmethane] is a major chemical component of turmeric [*Curcuma longa*] and is used as a spice to give a specific flavor and yellow color in Asian food. Curcumin exhibits growth inhibitory effects in a broad range of tumors as well as in TPA-induced skin tumors in mice. This study was undertaken to investigate the radiosensitizing effects of curcumin in p53 mutant prostate cancer cell line PC-3. Compared to cells that were treated with curcumin alone (fig-1 and table-1) or irradiated alone ($SF_2=0.635$; $D_0=231\text{cGy}$) (Fig-2), curcumin at 2 and 4 μM concentrations in combination with radiation showed significant enhancement of radiation induced clonogenic inhibition ($SF_2=0.224$; $D_0=97\text{cGy}$ and $SF_2=0.080$; $D_0=38\text{cGy}$) (Table 1) and apoptosis (fig 3A & 3B). Curcumin caused a strong G_2/M block which is an important phase sensitive to radiation. Curcumin treated cells showed 32.55% of G_2/M block at 12 hours, 48.10 % at 24 hours, 42.75% at 48 hours and 35.89% at 72 hours. (Table-2) It has been reported that curcumin inhibits $\text{TNF-}\alpha$ induced $\text{NF}\kappa\text{B}$ activity that is essential for Bcl-2 protein induction. In PC-3 cells, radiation up-regulated $\text{TNF-}\alpha$ protein leading to an increase in $\text{NF}\kappa\text{B}$ activity resulting in the induction of Bcl-2 protein. However, curcumin in combination with radiation treatment had shown inhibition of $\text{TNF-}\alpha$ mediated $\text{NF}\kappa\text{B}$ activity resulting in bcl-2 protein down regulation. Bax protein levels remained constant in these cells after radiation or curcumin plus radiation treatments. However, the down regulation of Bcl-2 and no changes in Bax protein levels in curcumin plus radiation treated PC-3 cells, together, altered the Bcl2:Bax ratio and this caused the enhanced radiosensitization effect. In addition, significant activation of cytochrome-c, caspase-9 and

caspase-3 were observed in curcumin plus radiation treatments. Together, these mechanisms strongly suggest that the natural compound curcumin is a potent radiosensitizer, and it acts by overcoming the effects of radiation induced pro-survival gene expression in prostate cancer.

DU-145

Different concentrations of curcumin (2 μ M, 4 μ M and 5 μ M) enhance the radio sensitization effect on DU-145 cells (Table 1) compared to curcumin alone (fig-4) or radiation alone (fig-5). In combination with radiation, curcumin induced a significant induction of apoptosis (31% at 24 h and 69% at 48 h) compared to radiation alone 4.1% at 24 h and 10.8% at 48 h) or curcumin alone (12% at 24 h and 23.08% at 48 h) (fig-6). There were no significant changes observed in cell cycle assays either treatment with curcumin alone or radiation alone or in combination. (Table-3)

22Rv1

As shown in table one, 22Rv1 cells are very sensitive to curcumin ($IC_{50}=1.97 \mu$ M)(fig-7) and radiation ($SF_2 =0.29$ and $D_{010}= 1.86$ Gy) (fig-8). In clonogenic inhibition assays radiation and curcumin in combination, these cells even in lower concentrations showed more sensitivity compare to other four cell lines (PC-3, DU-145, LN3 and LNCaP). In addition curcumin enhances radiation induced apoptosis in 22Rv1 cells (fig-9 & fig-10). Interestingly, curcumin abrogates radiation induced G_2/M block in 22Rv-1 cells (table-4).

LNCaP

As we mentioned earlier, LNCaP cells were failed to form colonies, hence we used MTT assay to detect the cytotoxic effect of curcumin in this cell line. Curcumin (Fig-11) or radiation alone (fig-12) induced cytotoxicity in LNCaP cells, however in combination no significant cytotoxicity effects were observed (Fig-12). Similarly apoptotic assays demonstrated that curcumin or radiation alone induced significant amount of apoptosis compared in combination with curcumin and radiation. (Fig-13 & fig 14) There were no changes in cell cycle pattern in LNCaP cells treated either with radiation or curcumin or in combination. (Table-5)

Second year:

TASK-2. The functional role of pro survival transcription factors on the mechanism of action of Curcumin with and without radiation on prostate cancer cell lines, Months 1-24:

An array of 54 -transcription factor activity will be screened using Protein-DNA array kit purchased from Panomics Inc. Results obtained from array will be confirmed by gel shift and reporter assays.

In particular, AP1, NF κ B and AR activity will be tested using gel-shift and reporter assays in cells exposed to Curcumin with or without radiation.

Aim-2 the functional role of pro survival transcription factors on the mechanism of action of Curcumin with or without radiation on prostate cancer cell lines.

Rationale: Based on the reasoning aforementioned in the background, it is hypothesized that in prostate cancer cells that lacks functional p53, radiation may up regulate pro-survival factors such as NF κ B activity and Bcl-2 expression and confer an "induced radiation resistant phenotype Curcumin inhibit radiation-induced pro-survival factors such as AP-1, NF κ B and Bcl-2 expression that will lead to enhanced radiation-induced apoptosis.

In this aim we used Protein-DNA array kit to find out whether curcumin regulates any transcriptional factors resulting curcumin mediated radiosensitization effect on prostate cancer cells. For this study we used PC-3 and LNCaP cell lines, and results revealed no additional transcriptional factors in these cell lines. Hence, support from University of Kentucky, we used RNA based microarray studies, to find out new genes which is regulated by curcumin. We used four groups (untreated, curcumin alone, radiation alone and in combination with curcumin and radiation) (Fig-15 and table-6) and these results confirmed that NFkB and AP1 are significantly down regulated in AR independent prostate cancer cell lines (PC-3 and DU-145) (Fig 16 and 17) than AR dependent prostate cancer cell lines (LNCap and 22RV1) (Fig 18 and 19). AR dependent prostate cancer cell lines demonstrate very low or no detectable basal levels of NFkB and AP-1 were seen.

PC-3 and DU-145:

In both cell lines the endogenous NFkB and AP-1 levels are high and curcumin down regulated the basal levels in both cell lines and radiation upregulated both the pro-survival factors (NFkB and AP-1) and combination with radiation and curcumin down regulated both NFkB and AP-1 activation in both the cell lines (figure-16 and 17). Further, DHT induced AR activation was abolished in combination with curcumin.

LNCap and CWR22RV-1: As stated about in both cell lines no significant induction of radiation induced NFkB and AP-1 activation were not seen. (figures-18 and 19) Also there is no significant activation AR activation by DHT were seen.

Key Research Accomplishments:

1. Curcumin sensitizes prostate cancer cells and induced apoptosis in all the proposed prostate cancer cell lines. In 22Rv1 and PC-3 cells curcumin inhibits G₂/M block that is an important phase of cell cycle. Curcumin enhanced radiosensitized prostate cancer cell lines (except LNCaP).
2. Curcumin down regulated NFkB, AP-1 and AR activation resulting radiation induced resistance were completely abolished.
3. No significant changes of other pro-survival factors were not seen curcumin treatment in prostate cancer cell lines by DNA-protein arrays.
4. Curcumin specifically target prostate cancer cells without causing any toxicity to normal prostate epithelial cells.

Reportable outcomes

1. Presentations were made in the National meetings:

1. Chendil.D, Rama.S, David.M, Sathishkumar.S, Inayat.S.M, Dey.S, Mohiuddin.M, and Ahmed.M.M. **Inhibition of radiation-induced pro-survival genes by Curcumin in prostate cancer.** Presented at 94th annual conference of American Society for Cancer Research (2003), Washington.
2. R.S Ranga and D.Chendil. **Molecular effects of curcumin on prostate cancer.** Presented at University of Kentucky International student symposium (2003)

2. Publications.

Damodaran Chendil, Rama S Ranga, David Meigooni, Sabapathi Sathishkumar and Mansoor M Ahmed. Curcumin confers radio-sensitizing effect in prostate cancer cell line PC-3. **Oncogene (2004 23, 1599-1707.**

Conclusions

Our results suggest that curcumin specifically targets prostate cancer cells, without causing any toxicity to normal prostate epithelial cells. In addition, curcumin act as potent radiosensitizer by abrogating radiation induced G2/M block of cell cycle and other hand it down regulated NFkB, AP1 and AR activation in prostate cancer cell lines.

Appendix-1

Figure-1 Curcumin induces clonogenic inhibition in PC-3 cells:

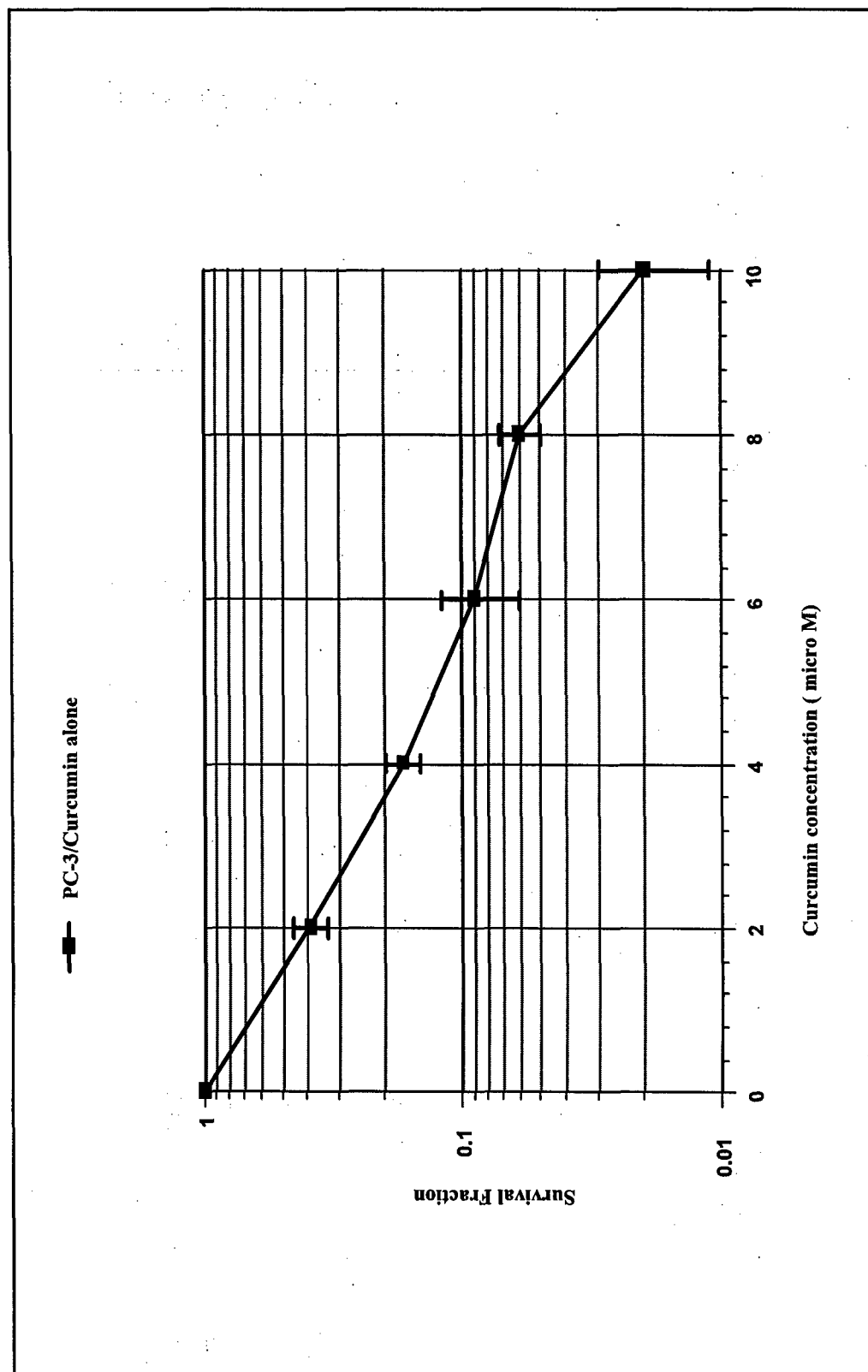


Figure-2 Curcumin enhances radiation induced clonogenic inhibition in PC-3 cells

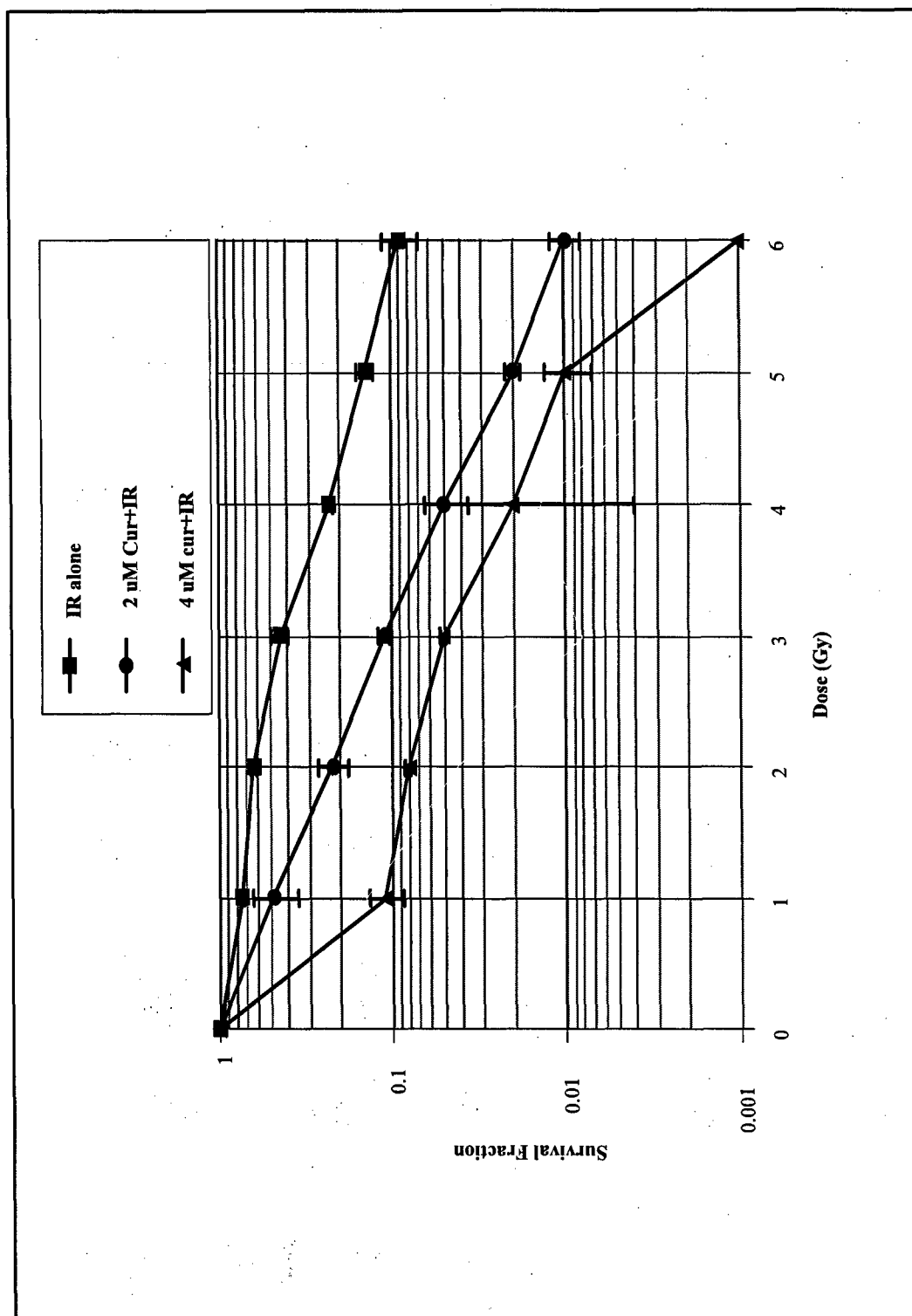
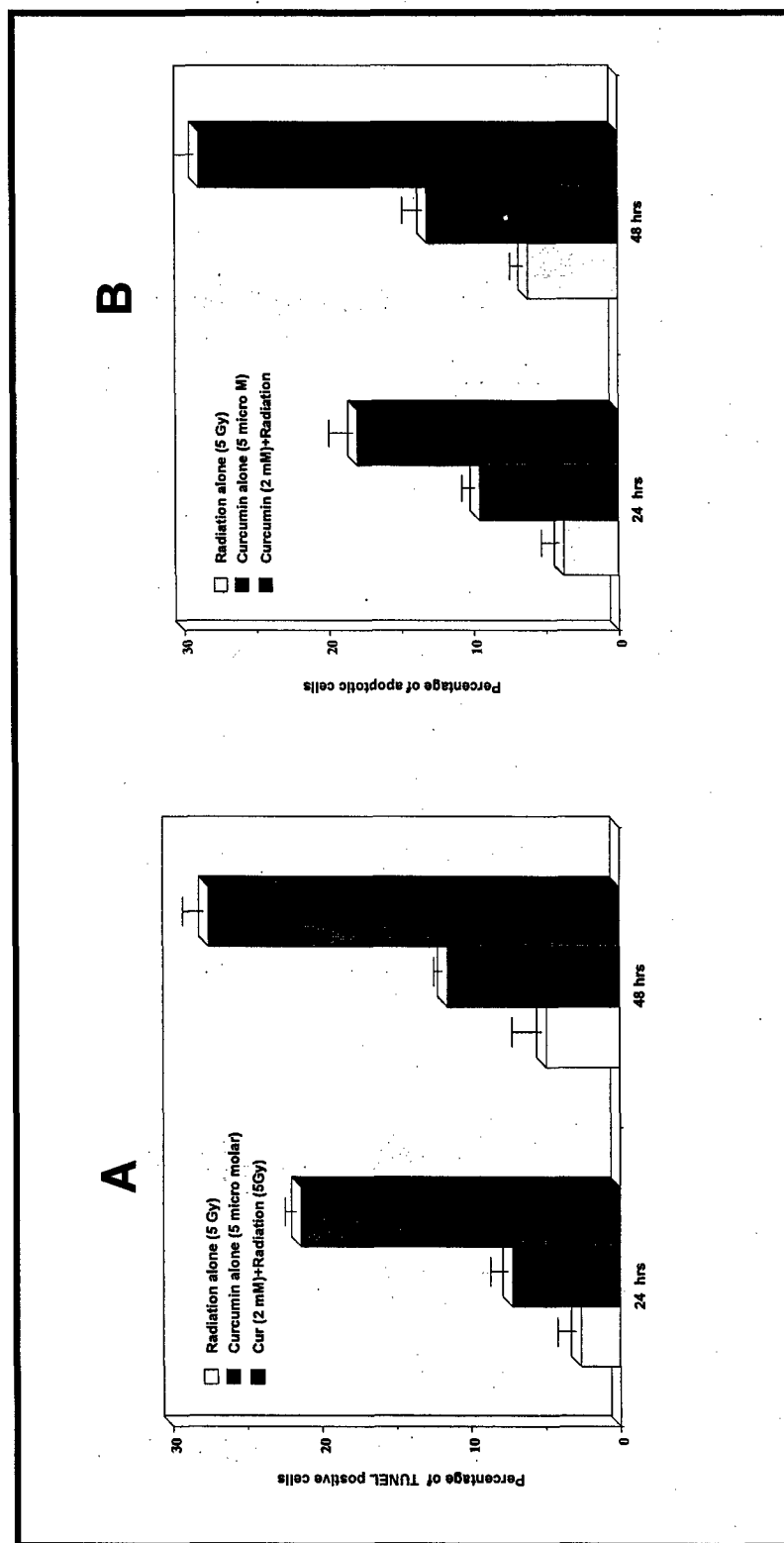


Table-1 SF₂,D₀ values of curcumin, radiation and incombination treatments on prostate cancer cells.

Cell line	Treatment	SF ₂ (Gy)	SF ₂ ER	D ₀	D ₀ ER	ER
PC-3	IR alone	0.635	-	2.31Gy	-	-
	IR+2 μ MCur	0.224	2.83	0.97Gy	2.38	5.21
	IR+4 μ MCur	0.080	7.93	0.38Gy	6.07	14.0
DU145	IR alone	0.51	-	2.74Gy	-	-
	IR(2Gy)+2 μ MCur	0.33	1.545	-	-	1.545
	IR(2Gy)+4 μ MCur	0.20	2.55	-	-	2.55
	IR(2Gy)+5 μ MCur	0.09	5.66	-	-	5.66
	Curcumin alone	-	-	2.97 μ M	-	-
LN3	Cur + IR(2Gy)	-	-	2.52 μ M	1.18	1.18
	IR alone	0.40	-	2.07Gy	-	-
	Curcumin alone	-	-	1.61 μ M	-	-
22rv1	IR +0.25 μ MCur	0.27	1.48	1.08Gy	1.90	3.38
	IR alone	0.29	-	1.86Gy	-	-
	Curcumin alone	-	-	1.97 μ M	-	-

Figure 3 Curcumin enhance radiation induced apoptosis in PC-3 cells:



**Table-2 Effects of curcumin or Ionizing radiation or in combination
on cell cycle distribution in PC-3 cells**

Cell cycle	Treatments	12 hours	24 hours	48 hours
G0/G1	Untreated	55.45 $\pm$ 2.21	56.53 $\pm$ 4.21	53.23 $\pm$ 4.28
	curcumin alone	28.45 $\pm$ 3.43	18.65 $\pm$ 3.76	20.05 $\pm$ 1.92
	radiation alone	15.62 $\pm$ 2.34	21.61 $\pm$ 1.72	36.11 $\pm$ 2.69
	IR+Cur	24.21 $\pm$ 3.99	25.22 $\pm$ 2.24	20.85 $\pm$ 1.65
S	Untreated	24.21 $\pm$ 3.05	21.32 $\pm$ 5.11	28.39 $\pm$ 3.76
	curcumin alone	39.00 $\pm$ 1.98	33.25 $\pm$ 1.07	37.20 $\pm$ 4.56
	radiation alone	35.47 $\pm$ 2.98	13.25 $\pm$ 1.09	22.86 $\pm$ 3.30
	IR+Cur	48.32 $\pm$ 3.12	44.20 $\pm$ 5.12	54.65 $\pm$ 5.74
G2-M	Untreated	20.34 $\pm$ 3.67	22.15 $\pm$ 1.98	18.38 $\pm$ 2.01
	curcumin alone	32.55 $\pm$ 3.10	48.10 $\pm$ 2.16	42.75 $\pm$ 3.21
	radiation alone	48.82 $\pm$ 2.73	65.14 $\pm$ 5.92	41.03 $\pm$ 4.19
	IR+Cur	27.48 $\pm$ 2.12	30.58 $\pm$ 4.21	24.50 $\pm$ 1.99

Figure-4 Curcumin induces clonogenic inhibition in DU-145 cells

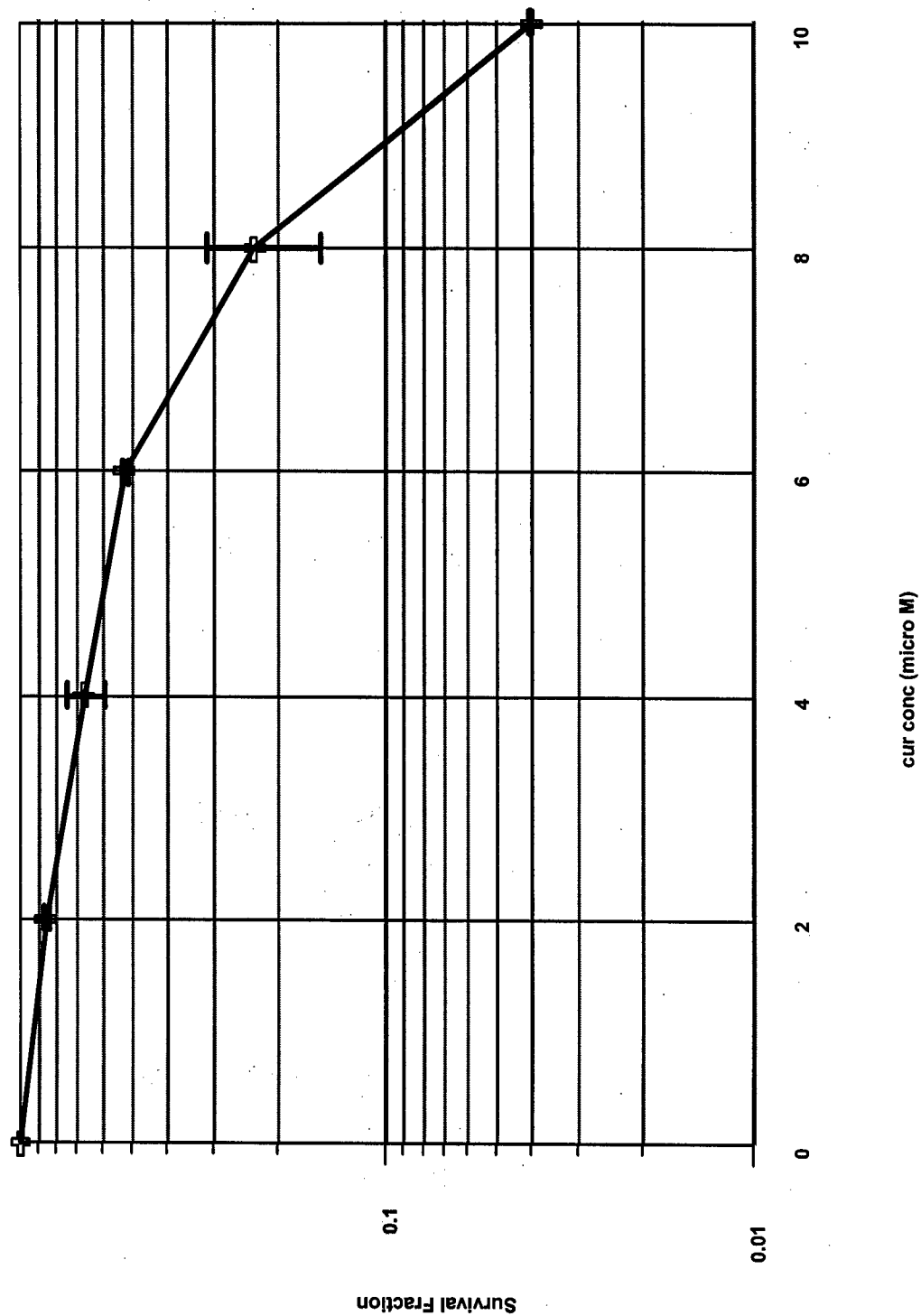


Figure- 5 Curcumin enhances radiation induced clonogenic inhibition in DU-145 cells

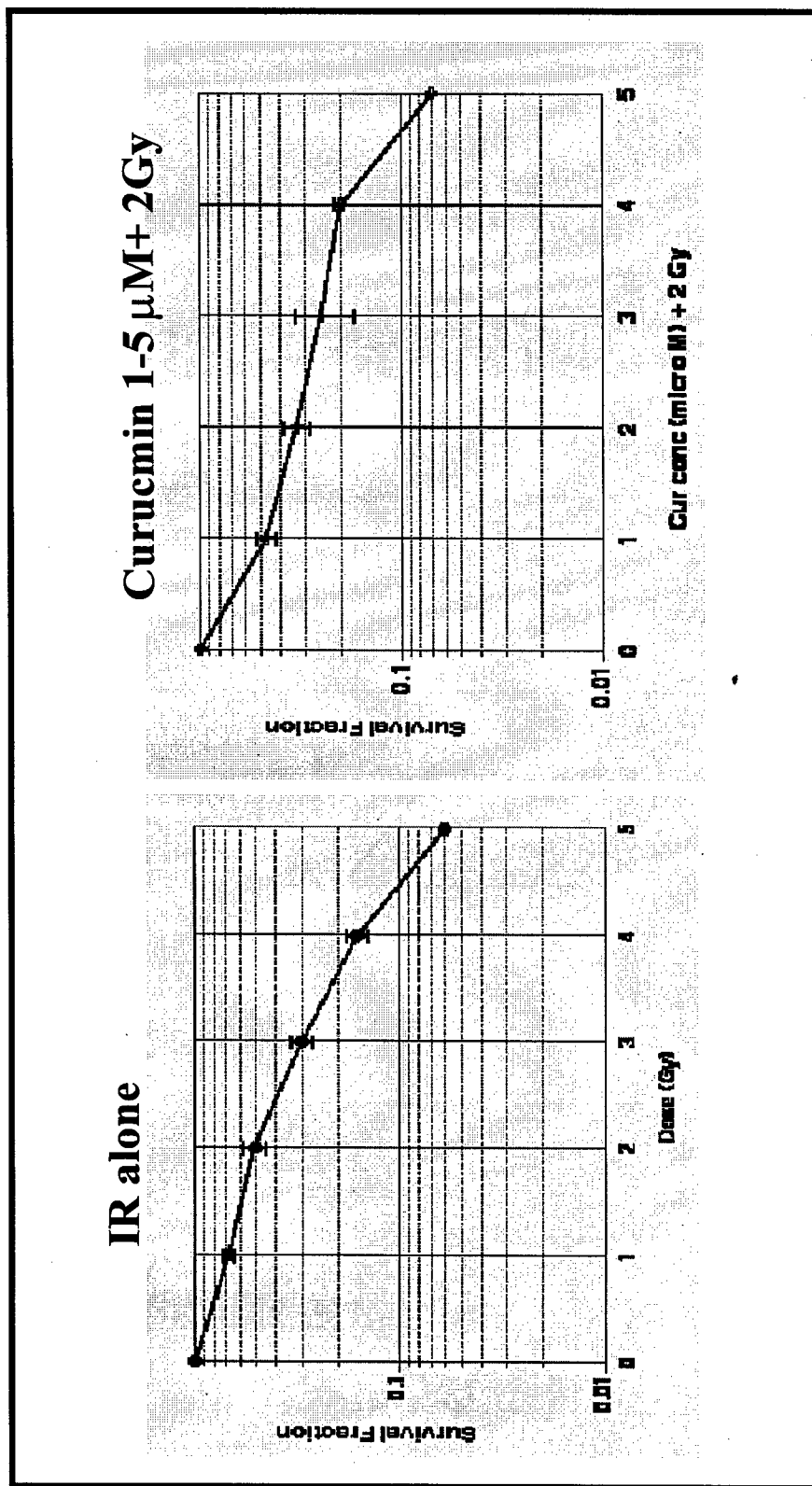


Figure 6 Curcumin enhance radiation induced apoptosis in DU-145 cells

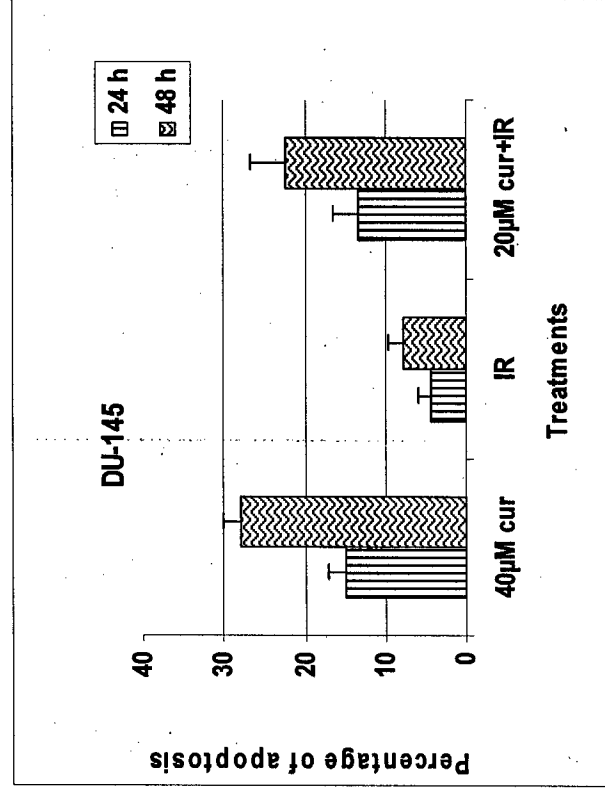
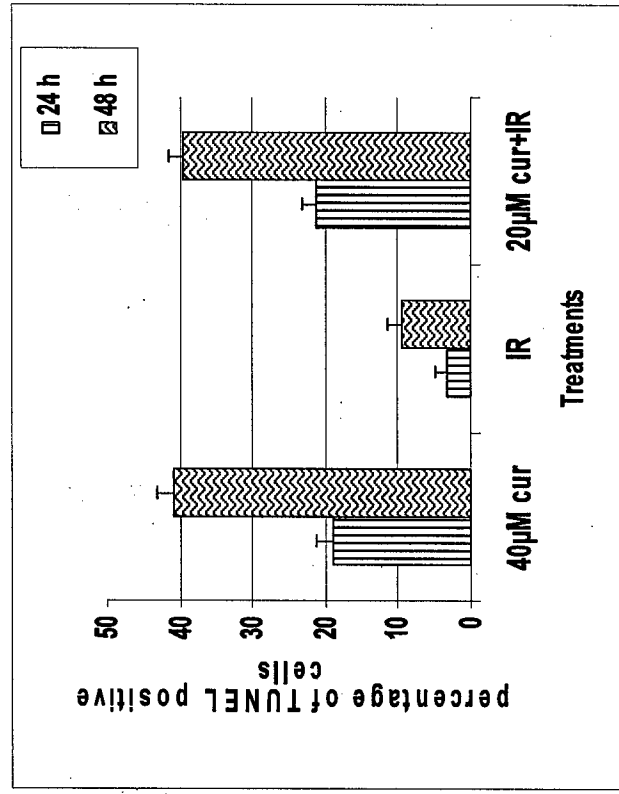


Table-3 Effects of curcumin or ionizing radiation or in combination on cell cycle distribution in DU-145 cells

Treatment		6hrs	12hrs	24hrs	48 hrs	72hrs
G0/G1	UT	31.2±2.0	34.1±1.9	36.1±1.4	57.7±3.0	53.3±2.9
	Cur 10 µM	37.8±3.2	35.1±1.7	24.2±2.2	28.7±3.7	43.3±5.0
	15Gy	26.4±2.7	15.5±2.6	30.2±3.7	32.7±1.8	39.4±2.6
	Cur 10 µM+ 5 Gy	31.9±2.8	19.8±1.0	12.1±4.6	20.6±1.1	31.0±4.8
S	UT	22.9±3.7	26.0±4.2	27.3±4.2	11.1±5.0	9.7±.7
	Cur 10 µM	24.3±1.6	25.2±2.4	28.1±2.0	46.3±2.9	9.9±1.0
	5Gy	31.1±1.2	31.7±3.7	16.6±3.5	33.6±4.2	12.6±.8
	Cur 10 µM+ 5Gy	25.97±2.9	30.9±4.0	32.5±1.0	32.7±1.5	03.1±.0
G2/M	UT	45.9±2.7	40.1±1.8	36.5±2.3	31.2±2.4	36.9±1.6
	Cur 10 µM	37.9±1.4	39.7±1.5	47.6±3.1	25.0±1.3	46.7±5.4
	5Gy	42.6±2.1	53.1±3.2	53.2±1.4	33.6±3.8	47.9±2.6
	Cur 10 µM+ 5Gy	42.1±1.6	49.3±2.7	55.3±2.7	46.6±3.6	65.8±4.6

Figure-7 Curcumin induces clonogenic inhibition in 22Rv-1 cells

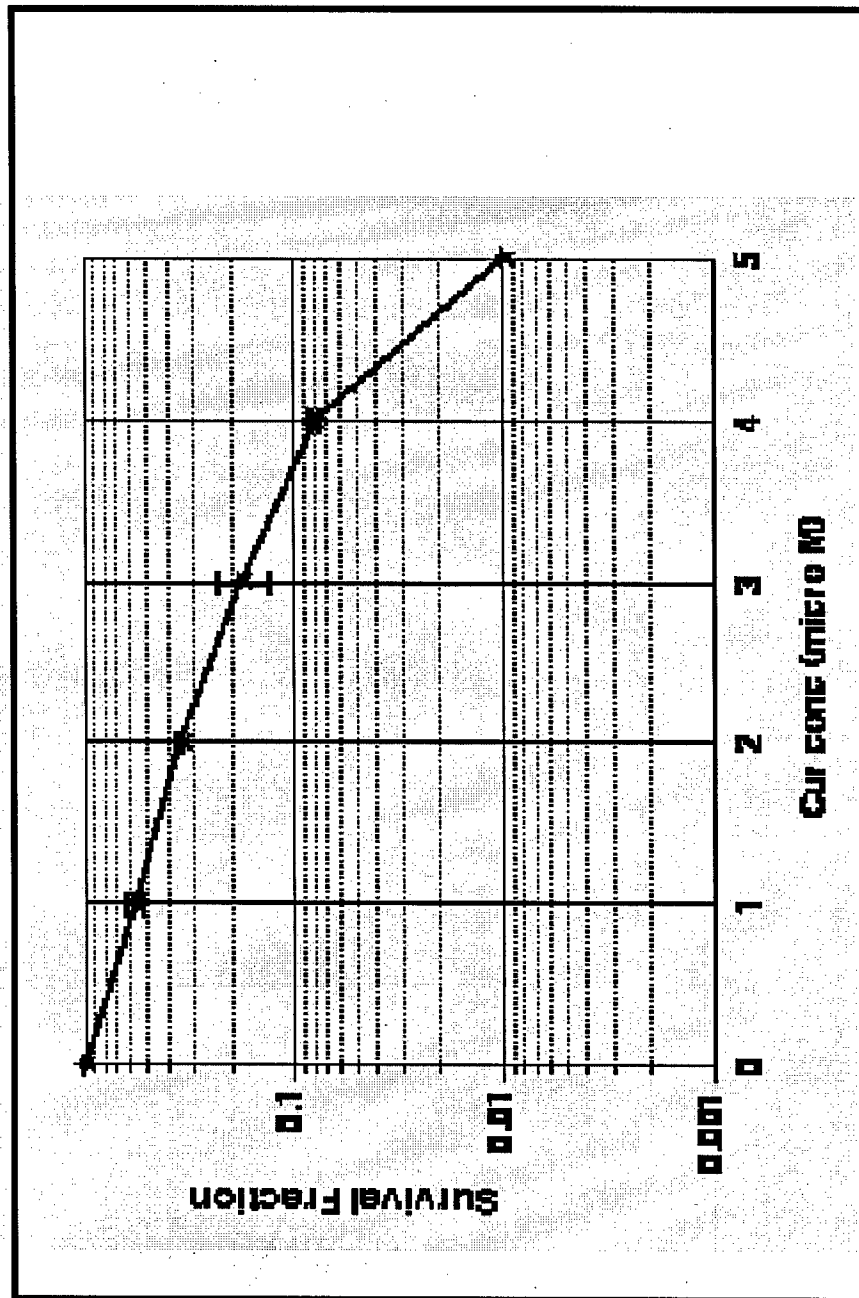
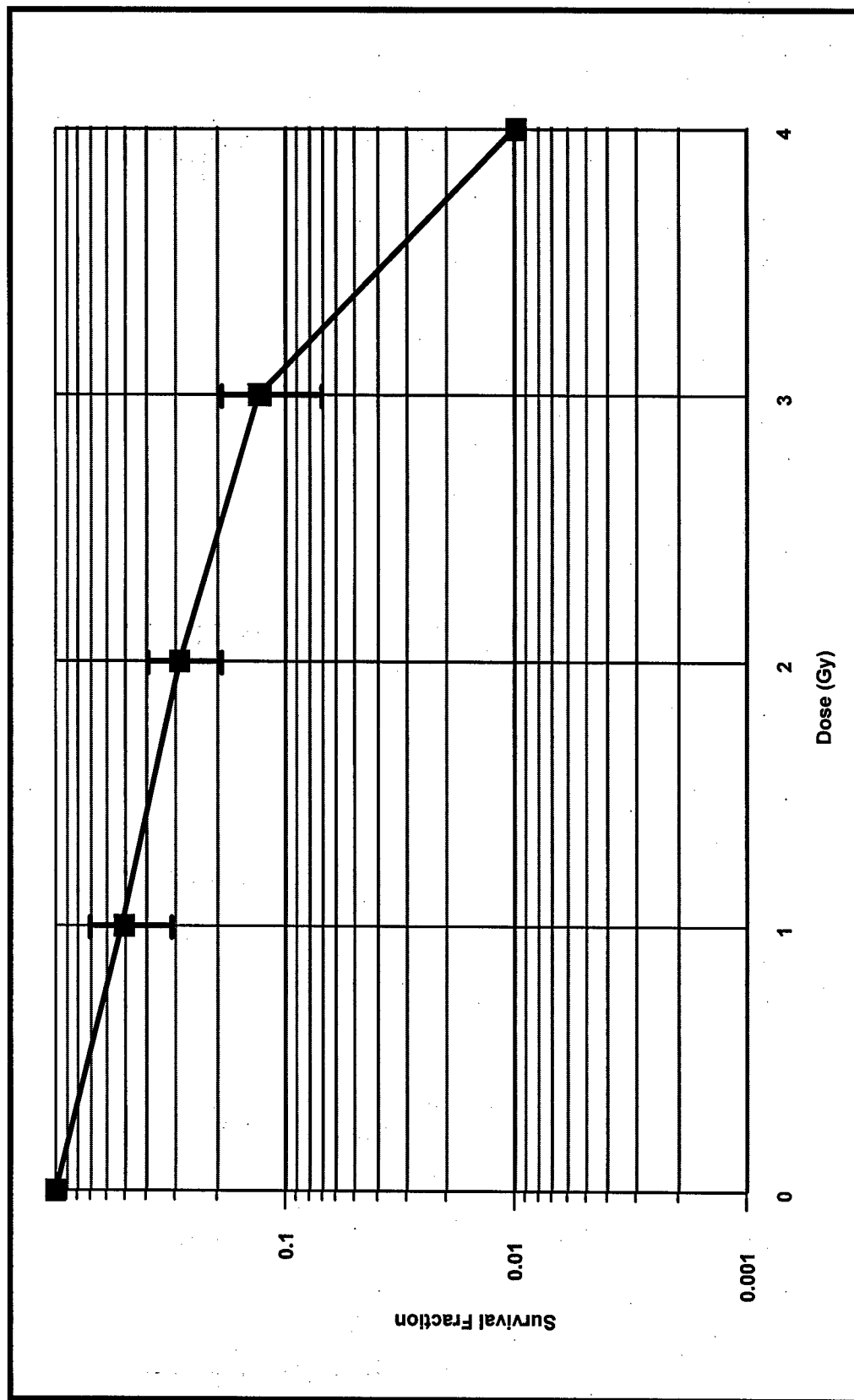


Figure-8 radiation induced clonogenic inhibition in 22Rv-1 cells



**Figure 9 Curcumin enhances radiation induced apoptosis in 22Rv-1 cells
By TUNEL**

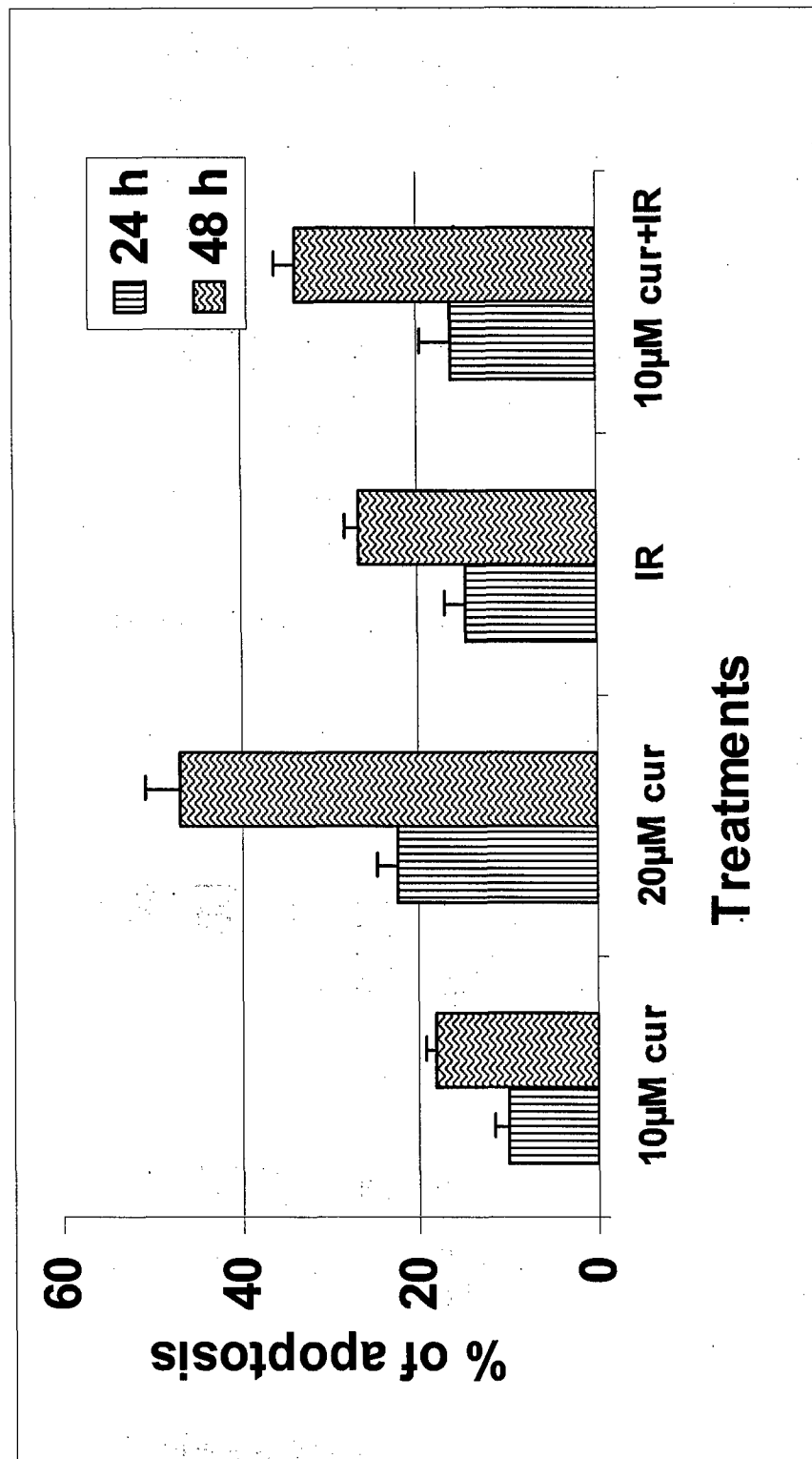


Figure 10 Curcumin enhances radiation induced apoptosis in 22Rv-1 cells
By Annexin V-FITC

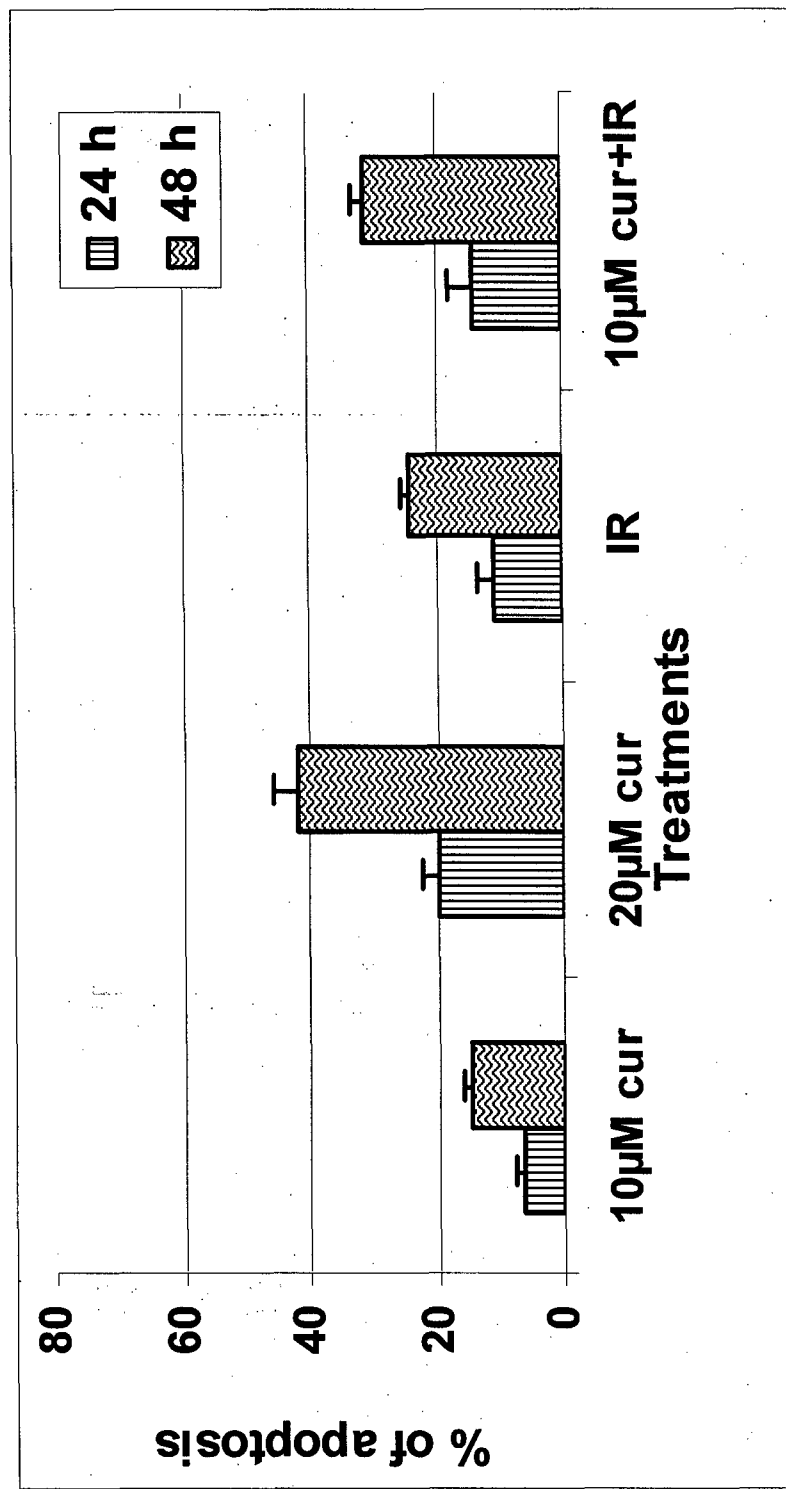


Table-4 Effects of curcumin or Ionizing radiation or in combination on cell cycle distribution in 22Rv-1 cells

Treatment/		6hrs	12hrs	24hrs	48hrs	72hrs
G0/G1	UT	46.0 $\pm$ 2.9	43.3 $\pm$ 4.2	38.5 $\pm$ 3.7	36.6 $\pm$ 1.8	59.7 $\pm$ 1.0
	Cur 20 μ M	45.3 $\pm$ 3.7	57.1 $\pm$ 3.1	37.4 $\pm$ 2.1	51.1 $\pm$ 2.7	39.5 $\pm$ 1.5
	5Gy	35.1 $\pm$ 4.0	24.1 $\pm$ 2.8	38.9 $\pm$ 0.8	37.2 $\pm$ 2.1	67.7 $\pm$ 3.0
	Cur 20 μ M+ 5Gy	43.2 $\pm$ 2.1	31.8 $\pm$ 1.6	29.6 $\pm$ 2.5	45 $\pm$ 1.8	51.2 $\pm$ 2.6
S	UT	44.9 $\pm$ 4.0	46.8 $\pm$ 3	49.9 $\pm$ 2.6	49.1 $\pm$ 4.5	28.9 $\pm$ 2.8
	Cur 20 μ M	38.7 $\pm$ 3.7	30.3 $\pm$ 2.3	48.4 $\pm$ 1.2	38 $\pm$ 3.2	45.5 $\pm$ 3.6
	5Gy	40.8 $\pm$ 3.5	37.3 $\pm$ 4.6	26.1 $\pm$ 4.2	32.8 $\pm$ 1.6	11.5 $\pm$ 2.6
	Cur 20 μ M+ 5Gy	36.1 $\pm$ 1.9	48.2 $\pm$ 5.1	39.5 $\pm$ 5.4	33.8 $\pm$ 1.0	30.9 $\pm$ 1.5
G2/M	UT	09.1 $\pm$ 2.6	10.1 $\pm$ 1.9	11.6 $\pm$ 1.9	14.3 $\pm$ 2.4	11.2 $\pm$ 1.8
	Cur 20 μ M	16.2 $\pm$ 2.9	11.7 $\pm$ 2.1	14.2 $\pm$ 3.2	10.0 $\pm$ 3.1	14.8 $\pm$ 1.9
	5Gy	24.3 $\pm$ 1.3	38.2 $\pm$ 6.7	35.0 $\pm$ 2.8	32.1 $\pm$ 1.7	20.7 $\pm$ 4.0
	Cur 20 μ M+ 5Gy	20.8 $\pm$ 3.2	19.9 $\pm$ 4.6	30.9 $\pm$ 3.1	21.2 $\pm$ 1.1	17.8 $\pm$ 3.7

Figure-11 Curcumin induced cytotoxicity on LNCaP cells

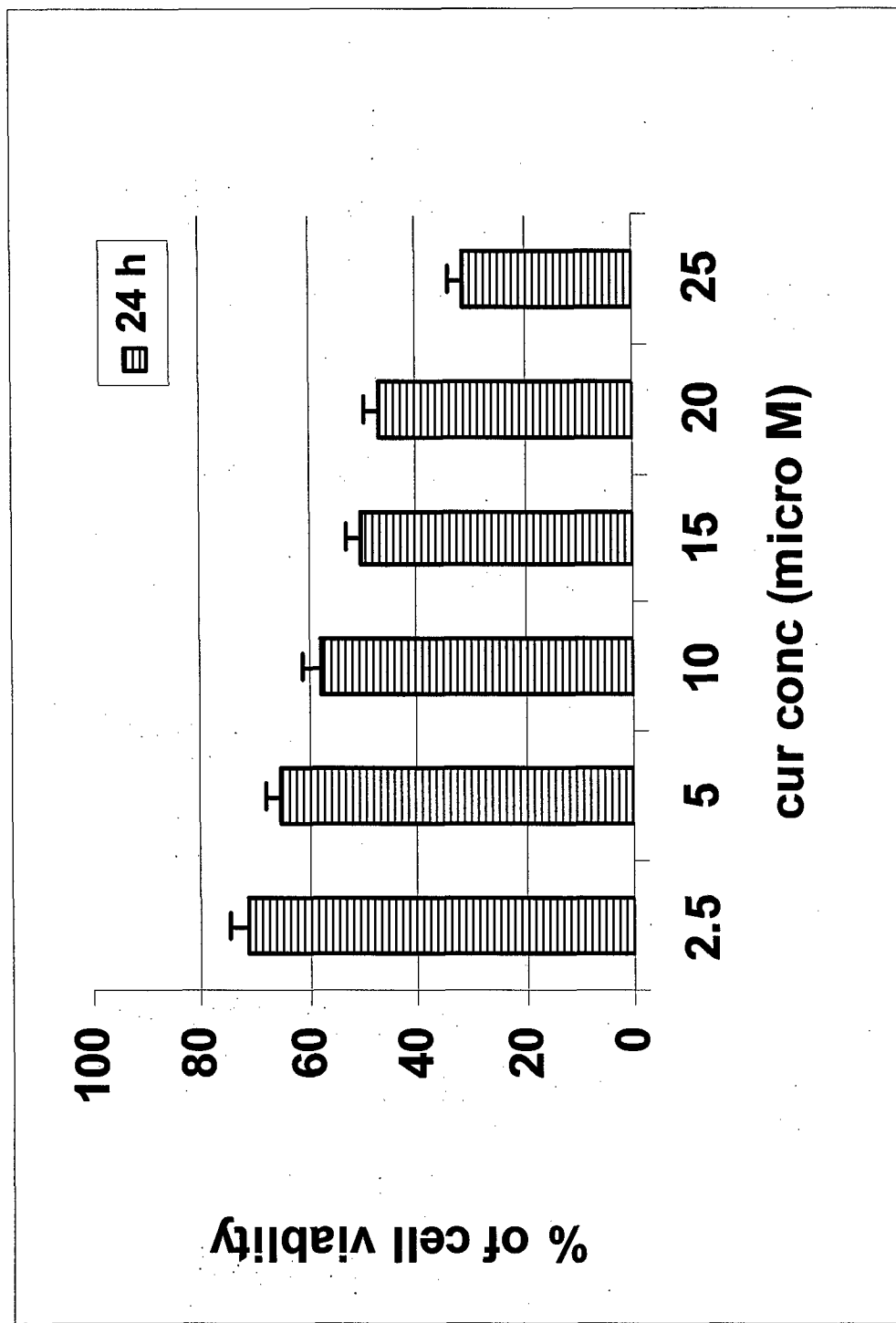
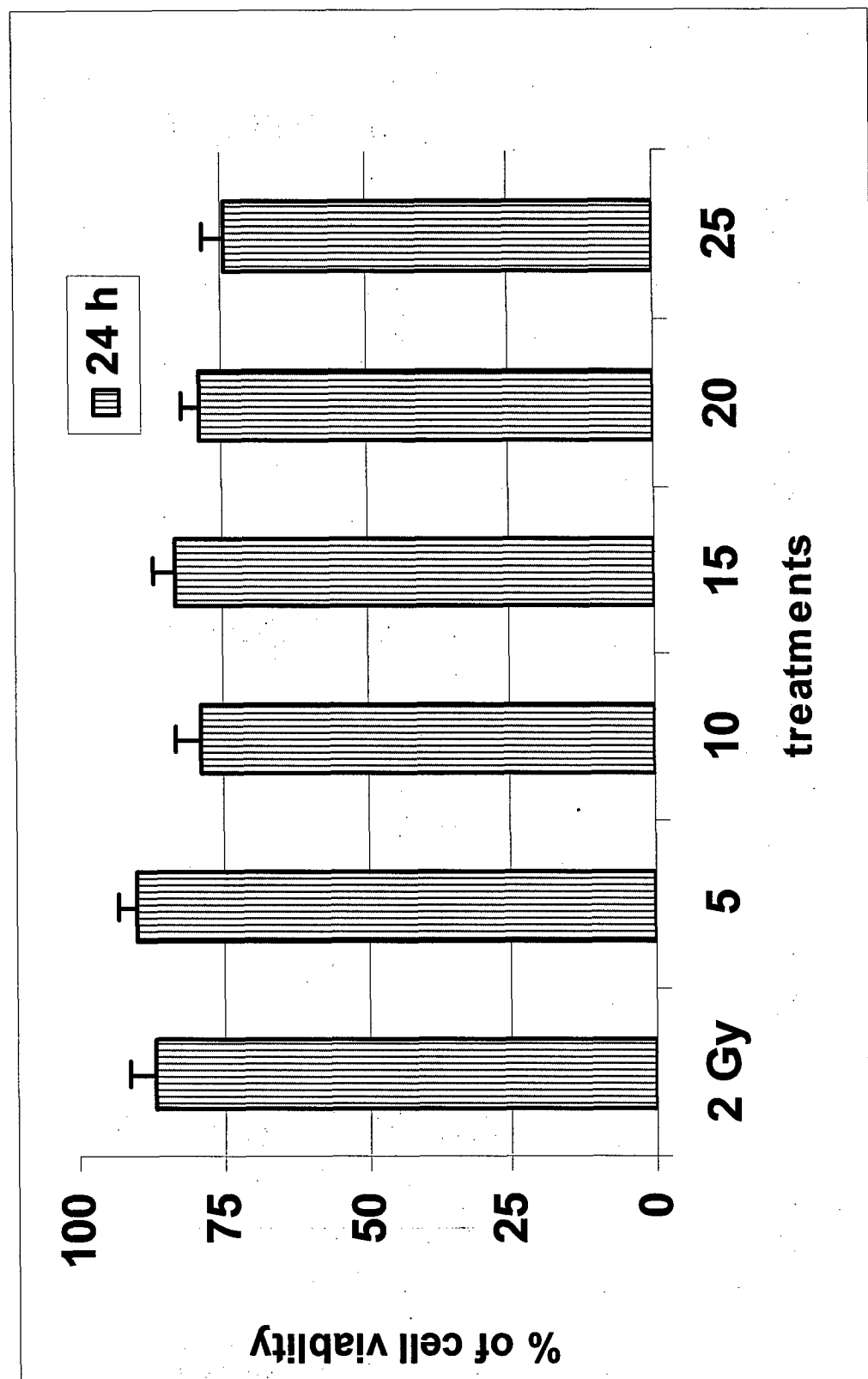


Figure-12 Cytotoxicity effects of curcumin and radiation on LNCaP cells.



**Figure 13 Curcumin and radiation induced apoptosis in LNCaP cells
BY Annexin V-FITC**

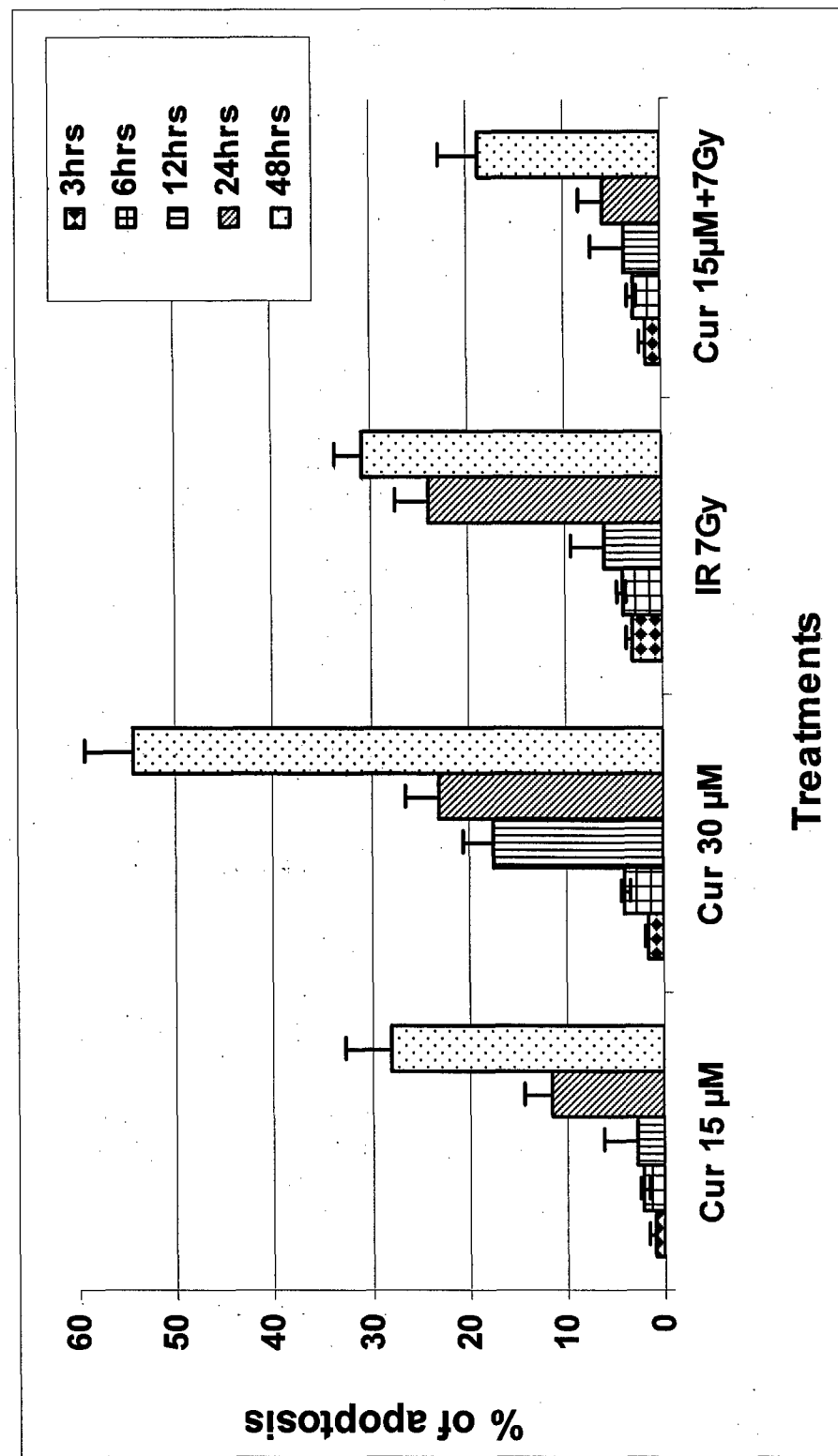


Figure-14 Curcumin and radiation induced apoptosis in LNCaP cells by TUNEL.

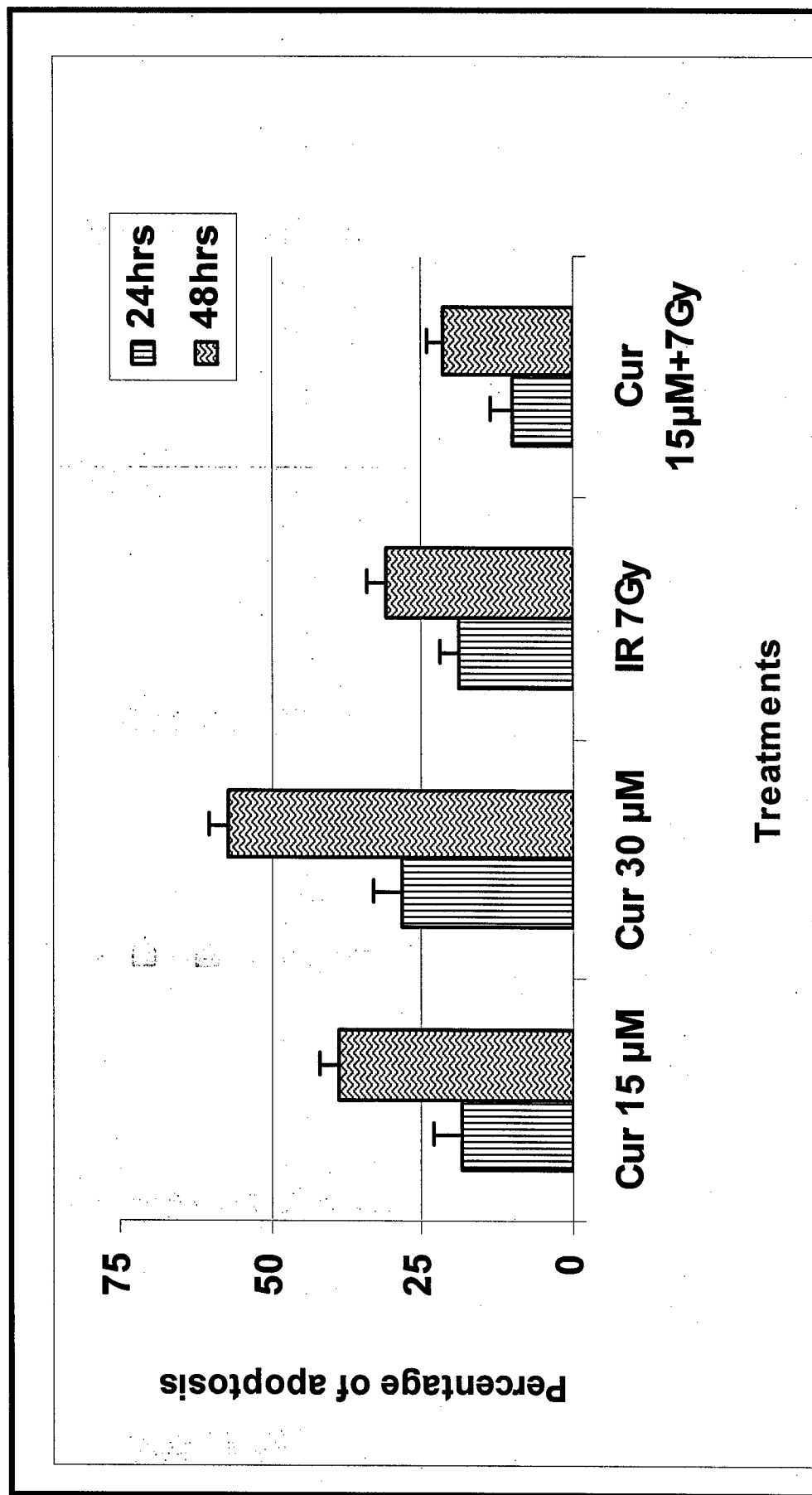
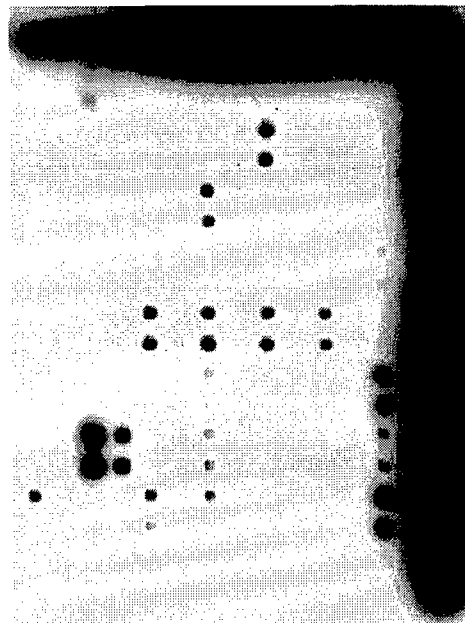


Table-5 Effects of curcumin or Ionizing radiation or in combination on cell cycle distribution in LNCaP cells.

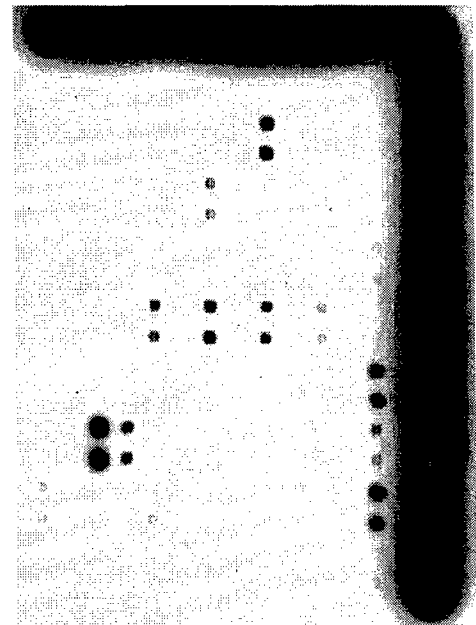
Treatments		Time			
		6hrs	12hrs	24hrs	48hrs
G0/G1	UT	63.77± 3.78	58.39 ± 4.64	66.35 + 7.01	63.51± 9.37
	Cur 15 µM	64.73± 4.21	65.79 ± 7.01	62.97 ± 6.76	73.51± 6.89
	IR 7Gy	57.13± 2.59	55.51± 6.23	73+ 7.24	87.95 ± 6.99
	Cur 10µM+7Gy	61.34± 1.09	56.93 ± 3.90	61.62+3.25	56.45 ± 6.12
S	UT	31.12 ± 1.27	36.52 + 5.03	31.55+2.19	29.52 ± 3.90
	IR 7Gy	41.2 ± 5.01	35.33+ 4.76	12.92 ± 1.09	11.35 ± 30
	Cur 15 µM	30.6 ± 2.08	28.99+1.99	36.82 ± 3.0	26.49 + 2.18
	Cur 10µM+7Gy	32.4± 3.09	38.46+ 4.65	34.98 ± 3.01	37.56 ± 3.01
G2/M	UT	5.12 ± 2.10	5.09 ± 1.98	2.1 ± 1.5	6.98 ± 2.67
	Cur 15 µM	4.67 ± 0.85	5.23± 2.67	0.21± 0.01	0
	IR 7Gy	1.67 ± 1	9.16± 2.54	14.08 ± 2.19	0.7
	Cur 10µM+7Gy	6.26 ± 2.34	4.61± 1.37	3.4 ± 1.02	5.99 ± 1.23

Fig 15. Du145 DNA/Protein micro-array

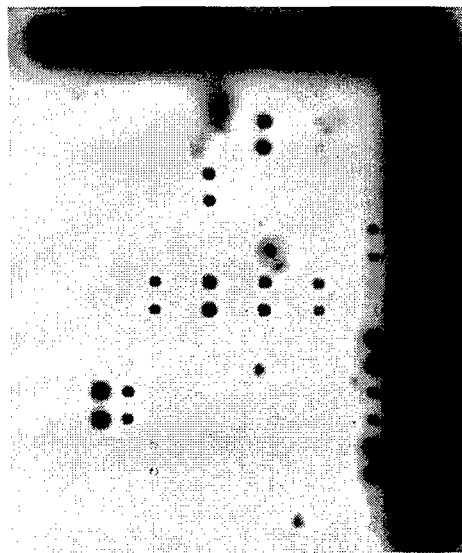
CURCUMIN



CURCUMIN+IR



UT



IR

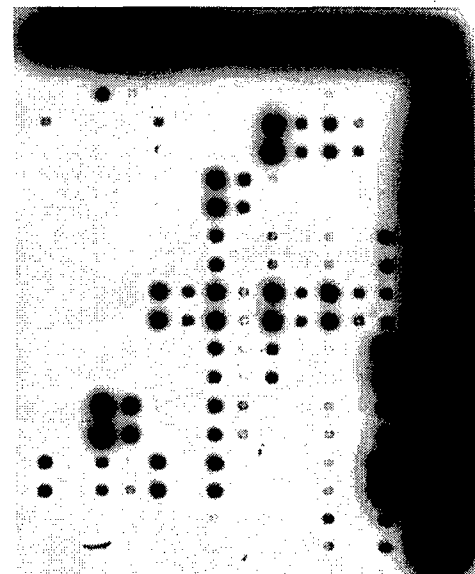


Table-6 Schematic Diagram of the TransSignal Protein/DNA Array I

LEGEND

	1	2
A	API	API
B	API	API

NOZAL

OUTLET TO

Figure 3. Schematic diagram of the TransSignal™ Protein/DNA Array 1. The genes on the array are spotted in duplicate; the first row is DNA spotted normally, the second row is DNA spotted in duplicate. The dark grey columns along the right and bottom sides of the array indicate where biotinylated DNA has been spotted. These spots are intended for alignment. Note that the notch is at the top, right-hand corner.)

Fig.16 Curcumin down regulates radiation induced NF κ B AP 1 in PC-3 cells

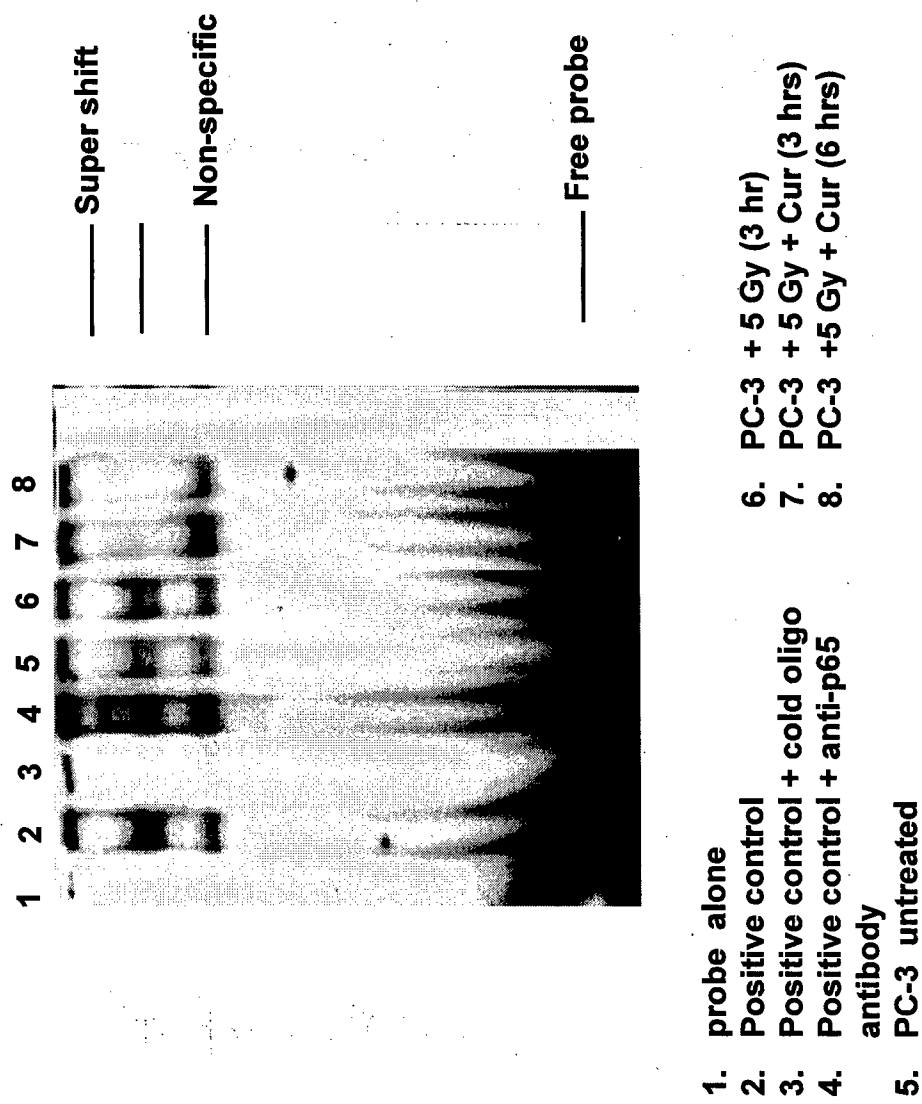


Fig.17 Curcumin down regulates radiation induced NF κ B AP 1 in DU-145 cells

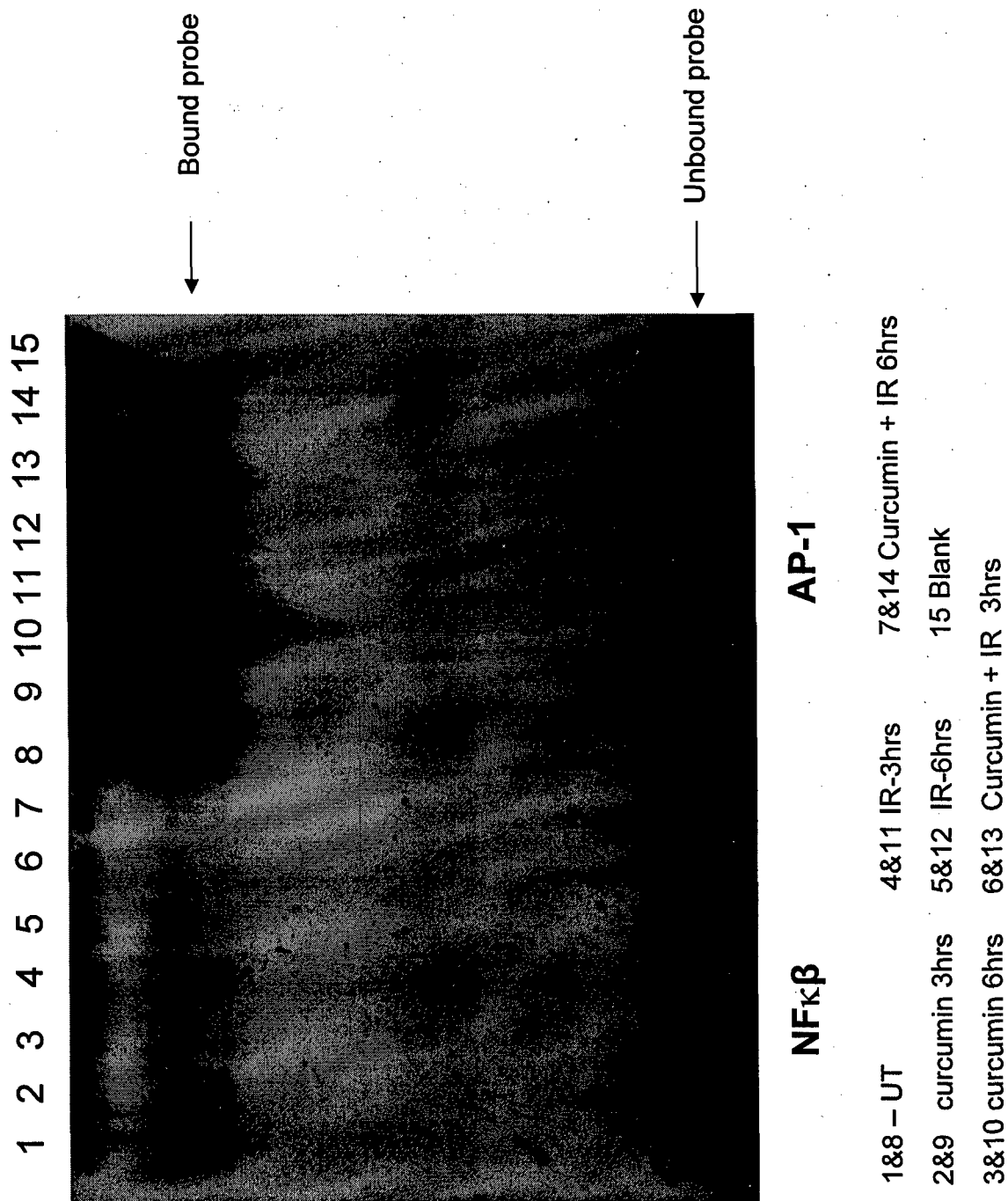


Fig-18. Effect of curcumin on NF κ B & AP-1 in 22Rv1

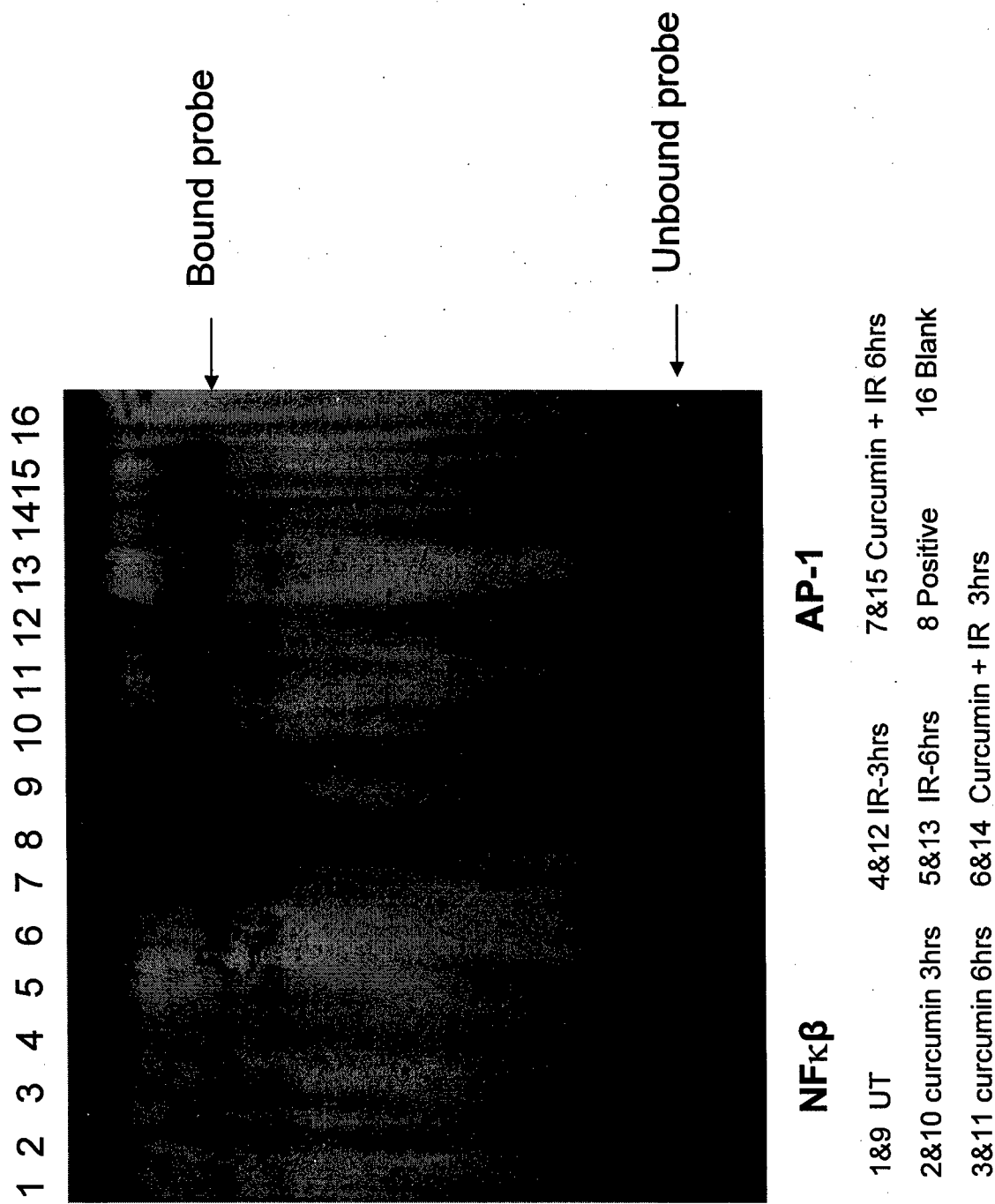
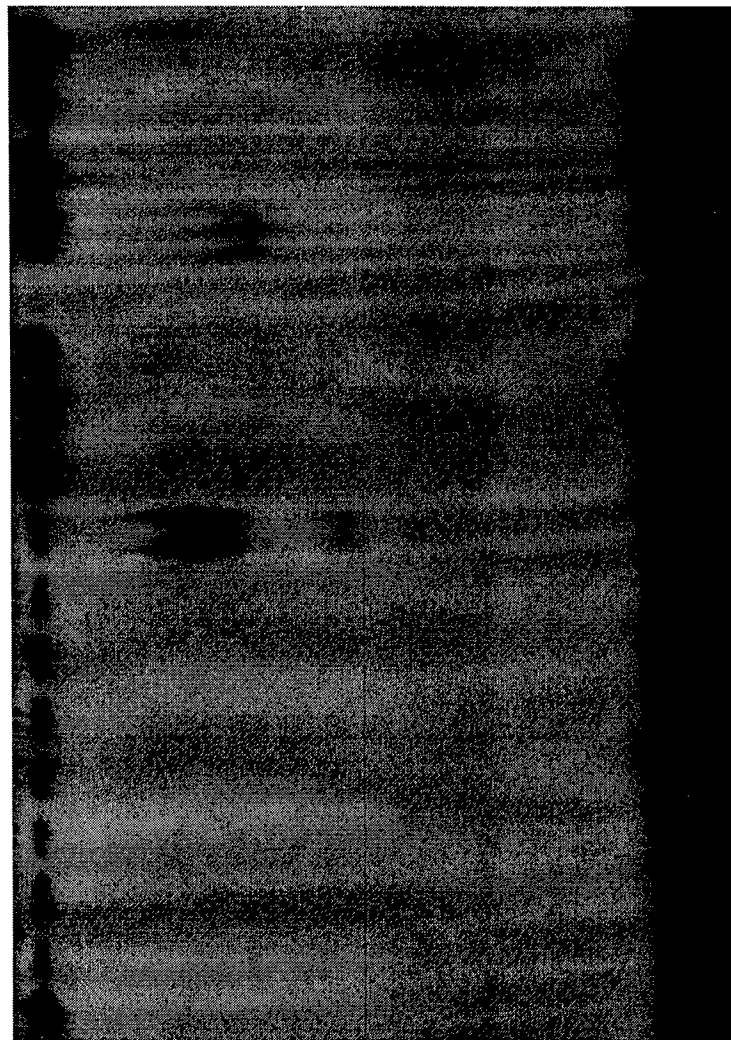


Fig 19. Effect of curcumin on NF κ B & AP-1 in LNcaP

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



AP-1

NF κ B

1 Blank	9 Positive	4&12 Curcumin-6hrs	7&15 Curcumin + IR 3hrs
2&10 UT		5&13 IR-3hrs	8&16 Curcumin + IR 6hrs
3&11 curcumin 3hrs		6&14 IR 6hrs	

Curcumin confers radiosensitizing effect in prostate cancer cell line PC-3

Damodaran Chendil^{*1}, Rama S Ranga¹, David Meigooni¹, Sabapathi Sathishkumar² and Mansoor M Ahmed²

¹Department of Clinical Science, University of Kentucky, Lexington, KY 40536, USA; ²Department of Radiation Medicine, University of Kentucky, Lexington, KY 40536, USA

Curcumin (Diferuloylmethane) is a major chemical component of turmeric (*curcuma longa*) and is used as a spice to give a specific flavor and yellow color in Asian food. Curcumin exhibits growth inhibitory effects in a broad range of tumors as well as in TPA-induced skin tumors in mice. This study was undertaken to investigate the radiosensitizing effects of curcumin in p53 mutant prostate cancer cell line PC-3. Compared to cells that were irradiated alone ($SF_2 = 0.635$; $D_0 = 231$ cGy), curcumin at 2 and 4 μ M concentrations in combination with radiation showed significant enhancement to radiation-induced clonogenic inhibition ($SF_2 = 0.224$; $D_0 = 97$ cGy and $SF_2 = 0.080$; $D_0 = 38$ cGy) and apoptosis. It has been reported that curcumin inhibits TNF- α -induced NF κ B activity that is essential for Bcl-2 protein induction. In PC-3 cells, radiation upregulated TNF- α protein leading to an increase in NF κ B activity resulting in the induction of Bcl-2 protein. However, curcumin in combination with radiation treated showed inhibition of TNF- α -mediated NF κ B activity resulting in bcl-2 protein downregulation. Bax protein levels remained constant in these cells after radiation or curcumin plus radiation treatments. However, the downregulation of Bcl-2 and no changes in Bax protein levels in curcumin plus radiation-treated PC-3 cells, together, altered the Bcl2:Bax ratio and this caused the enhanced radiosensitization effect. In addition, significant activation of cytochrome *c* and caspase-9 and -3 were observed in curcumin plus radiation treatments. Together, these mechanisms strongly suggest that the natural compound curcumin is a potent radiosensitizer, and it acts by overcoming the effects of radiation-induced prosurvival gene expression in prostate cancer.

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Keywords: prostate; curcumin; radiation; TNF- α ; NF κ B; Bcl-2; apoptosis

Introduction

Prostate cancer is the cancer of second largest incidence among the male populations in the US, and the incidence has been increasing rapidly in the recent years (Greenlee *et al.*, 2000). According to a WHO report, 36% of the prostate cancer patients of the world, as in 2000, belong to US population (Wilkinson *et al.*, 2002). Prostate cancer cells are only modestly responsive or even unresponsive to the cytotoxic effects of chemotherapeutic agents or radiotherapy. Increased concentrations of cytotoxic drugs and higher dosages of irradiation fail to improve the response to therapy and it leads to resistance to apoptosis in prostate cancer cells. Thus, it is imperative to identify anticancer agents that are nontoxic and highly effective to induce cell death preferentially in tumor cells. Compounds occurring naturally in the human diet may be devoid of toxicity. Curcumin (Singh *et al.*, 1996) is a major chemical component of turmeric (Bhaumik *et al.*, 1999) and is used as a spice to give a specific flavor and yellow color in Asian food (Sharma, 1976). It is also used as a cosmetic as well as in some medical preparation. Curcumin has been reported to have several pharmacological effects including antitumor, anti-inflammatory and antioxidant properties (Sharma, 1976; Huang *et al.*, 1991). An epidemiological study revealed that low incidence of bowel cancer in Indians can be part in attributed to the presence of natural additives like curcumin in Indian cookery (Mohandas and Desai, 1999). Curcumin is inhibitory to a broad range of tumors such as mammary tumor, duodenal and colon cancer and TPA-induced skin tumors in mice (Huang *et al.*, 1998).

Curcumin has a potent role in inhibiting cellular migration and curcumin treatment of DU-145 cells suppressed the constitutive activation of both NF- κ B and AP-1 (Mukhopadhyay *et al.*, 2001). The molecular mechanism of NF κ B inhibition by curcumin is unclear, but involved inhibition of I κ B degradation (Jobin *et al.*, 1999; Mukhopadhyay *et al.*, 2001). In addition, the antiproliferative activity of curcumin may also relate to its ability to block activation of RAS protein by inhibiting farnesyl protein transferase (Jiang *et al.*, 1996). Curcumin significantly inhibits prostate cancer growth (Dorai *et al.*, 2001) and has the potential to prevent the progression of this cancer to its

*Correspondence: D Chendil, Division of Clinical Health Sciences, Room # 209D, College of Health Sciences, University of Kentucky, 900 South Limestone street, Lexington, KY 40536-0200, USA; E-mail: dchen2@uky.edu

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hormone-refractory state (Huang *et al.*, 1998). Curcumin induced apoptosis in both androgen-dependent and androgen-independent prostate cancer cells, by interfering with the signal transduction pathways and prevent the progression of the tumor to the hormone-refractory state (Singh and Aggarwal, 1995).

It has been shown that curcumin inhibits NF κ B activation that in turn downregulates endogenous bcl-2 and bax_L protein (Mukhopadhyay *et al.*, 2001). These observations lead us to hypothesize that curcumin can potentially inhibit radiation-induced prosurvival factors such as NF κ B activation and Bcl-2 expression. To ascertain this hypothesis, we performed the experiments using androgen-independent, p53-null PC-3 cells. Our results show that curcumin in combination with radiation inhibits TNF- α -mediated NF κ B activity, resulting in Bcl-2 protein downregulation in PC-3 cells. Also, curcumin enhanced radiation-induced apoptosis by releasing cytochrome *c* and activated caspases in combinations with radiation in PC-3 cells.

Results

Radiation-induced resistance through upregulation of prosurvival factors in prostate cancer cells

Resistance to radiation or chemotherapy may be due to interference of apoptotic pathways in cancer treatment. NF κ B activation is thought to exert antiapoptotic effects in most cancer cells. In some cell types, the antiapoptotic effects of TNF- α appears to be mediated by the upregulation of NF κ B activity (Beg and Baltimore, 1996; Wang *et al.*, 1996), resulting in induction of bcl-2 gene expression that causes resistance to treatments in cancer cells (Chendil *et al.*, 2002; Inayat *et al.*, 2002). Radiation caused an induction of TNF- α protein expression (Figure 1a), NF κ B activity (Figure 1b) and Bcl-2 upregulation (Figure 1c) in PC-3 cells. The peak induction of TNF- α protein expression was observed at 48 h, and these results show that radiation induced prosurvival factors in p53-null prostate cancer cells.

Anti-TNF- α neutralizing antibody inhibits radiation-induced NF κ B activity leading to repression of Bcl-2 protein

The protective responses of cells against radiation are DNA repair, activation of prosurvival transcription

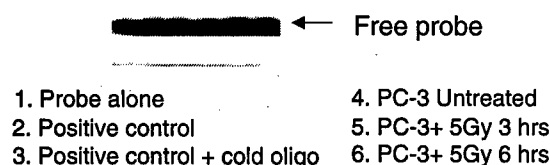
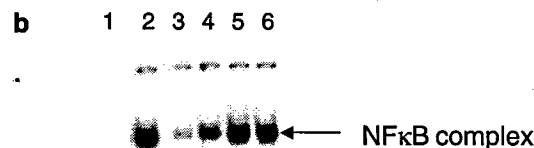
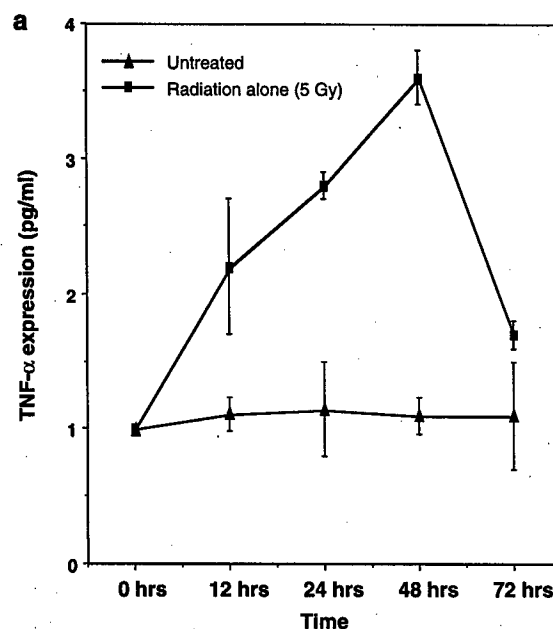


Figure 1 Ionizing radiation induces TNF- α protein, NF κ B activity and Bcl-2 protein in PC-3 cells. (a) TNF- α protein estimation in a medium of untreated and irradiated PC-3 cells by enzyme-linked immunosorbent assay (ELISA). Error bars represent s.e.m. of two separate experiments performed in triplicate. (b) The effect of radiation on DNA binding activity of NF κ B in PC-3 cells. EMSAs of NF κ B binding complexes from PC-3 cells with 5 Gy of radiation. Lanes 2–5, positive control nuclear was used for appropriate experimental controls. Arrows indicate the position of super shift, NF κ B complex band shift nonspecific binding and free probe. (c) Western blot analysis of Bcl-2 protein induction in PC-3 cells. Whole cell protein extracts were prepared from PC-3 cells that were left untreated (UT) or exposed to a 5 Gy dose and incubated for the indicated time interval. The blot was probed with an antibody for Bcl-2 or β -actin

factors and induction of antiapoptotic genes. One of the prosurvival transcription factor, NF κ B, is activated by ionizing radiation (Brach *et al.*, 1991). NF κ B activates several downstream target genes such as Bcl-2 (Dixon *et al.*, 1997; Tamatani *et al.*, 1999), which is responsible for the protection of cells against radiation-induced apoptosis. The activation of NF κ B by radiation also depends on radiation-induced TNF- α protein (Baldwin, 1996; Bierhaus *et al.*, 1997). Moreover, NF κ B is known to be a cell survival and antiapoptotic molecule (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996). To test whether TNF- α induction by radiation is necessary for NF κ B activation and Bcl-2 upregulation, we performed Western blot analysis of cells either left untreated or treated with anti-TNF- α neutralizing antibody plus radiation. As shown in Figure 2a, DNA binding activity of NF κ B increased approximately sixfolds in these cells treated with recombinant TNF- α protein. Whereas, the radiation-induced NF κ B was inhibited when PC-3 cells were exposed to anti-TNF- α neutralizing antibody (Figure 2a). Similarly, recombinant TNF- α protein induced Bcl-2 expression in these cells while anti-TNF- α neutralizing antibody repressed the radiation-induced Bcl-2 expression (Figure 2b). These results suggest that radiation-induced NF κ B activity depends on radiation-induced expression of TNF- α . Altogether, these observations indicate that radiation induces TNF- α that in

turn triggers NF κ B activation and Bcl-2 induction in PC-3 cells.

Curcumin inhibits radiation-induced prosurvival factors in PC-3 cells

Previously, it has been shown that curcumin inhibits TNF- α -mediated NF κ B activation. In addition, curcumin downregulates endogenous bcl-2 and bax_{XL} protein level (Mukhopadhyay *et al.*, 2001). In order to inhibit the radiation-induced antiapoptotic function in cells, we treated PC-3 cells either with curcumin or radiation alone or in combination. Interestingly, curcumin inhibited radiation-induced TNF- α protein expression (Figure 3a) and resulted in downregulation of NF κ B activation (Figure 3b) and Bcl-2 protein expression (Figure 3c). Together, these data strongly suggest that curcumin is a potent inhibitor of radiation-induced prosurvival factors in PC-3 cells.

Curcumin enhance radiation-induced clonogenic inhibition in PC-3 cells

PC-3 cells conferred enhanced resistance to radiation since curcumin inhibits radiation-induced prosurvival factors such as NF κ B and Bcl-2. Effects of curcumin either alone or in combination with radiation on cell survival studied with colony-forming assay. Curcumin enhanced significantly the radiation-induced clonogenic inhibition ($SF_2=0.224$; $D_0=97$ cGy and $SF_2=0.080$; $D_0=38$ cGy at 2 and 4 μ M concentrations) compared to cells treated with curcumin alone (Figure 4a) or radiation alone ($SF_2=0.635$; $D_0=231$ cGy) (Figure 4b). Interestingly, a significant enhancement in the radiosensitizing effect of curcumin was observed at 2 and 4 μ M concentrations. These results indicate that the natural compound curcumin inhibited the growth of PC-3 cells and significantly enhanced the effect of radiation (Table 1).

Curcumin enhances radiation-induced apoptosis in PC-3 cells

Terminal transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) staining was performed with or without curcumin/radiation after 24 and 48 h of treatment to determine the induction of apoptosis. By TUNEL assay, the incidence of apoptosis after 24 and 48 h of radiation over the untreated population was 2.61 and 4.88% compared to curcumin alone treated cells (7.23 and 11.56%, respectively). The combination of curcumin and radiation significantly enhanced induction of apoptosis in these cells after 24 h (21.39%) and 48 h (27.57%) of treatment (Figure 5a). By flow cytometry assay, using MC-540 and Hoechst 342 staining, the incidence of apoptosis after 24 and 48 h of radiation over the untreated population was 3.81 and 6.25%, compared to curcumin alone treated cells (9.6 and 13.22%, respectively). However, with combination of radiation and curcumin the incidence of apoptosis after 24 and 48 h was 18.31 and 29.90%, respectively

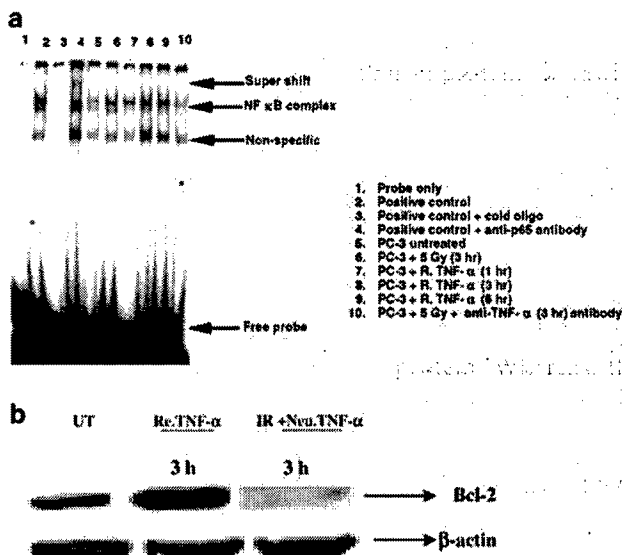


Figure 2 Neutralizing antibody to TNF- α or AD5-I κ B super-repressor inhibits radiation-induced NF κ B activity leading to repression of Bcl-2 protein in PC-3 cells. (a) EMSAs of NF κ B binding complexes from PC-3 cells were left untreated or treated with recombinant TNF- α and/or 5 Gy of radiation with neutralizing anti-TNF- α antibody. Nuclear cell extracts (5 μ g) from untreated or irradiated cells were incubated with 32p-labeled NF κ B DNA probe, followed by analysis of DNA binding activities. Arrows indicated the position of NF κ B complex bindings. (b) Whole cell protein extracts were prepared from PC-3 cells that were left untreated (UT) or treated with recombinant TNF- α and/or irradiated at 5 Gy and incubated for the indicated time interval. The blot was probed with an antibody for Bcl-2 or β -actin

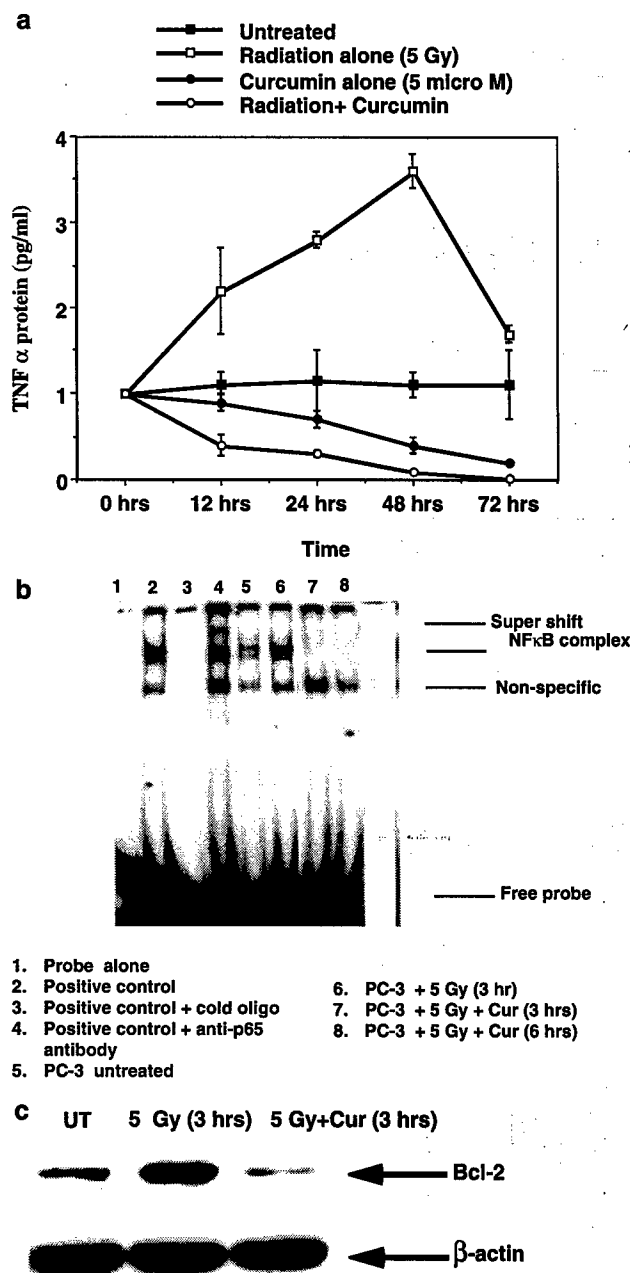


Figure 3 Curcumin downregulates radiation-induced NF κ B activity and Bcl-2 protein expression. (a) TNF- α protein estimation in a medium of untreated/rtreated with curcumin (5 μ M) or radiation (5 Gy) or in combination (curcumin 2 μ M plus radiation (5 Gy)) of PC-3 cells by ELISA. Error bars represent s.e.m. of two separate experiments performed in triplicate. (b) The effect of Curcumin on radiation-induced NF κ B activity in PC-3 cells. Arrows indicated the position of super shift, NF κ B complex, nonspecific binding and free probes. (c) Western blot analysis of Bcl-2 expression on PC-3 treated with curcumin with or without radiation. Whole cell protein extracts were prepared from PC-3 cells that were left untreated (UT) or treated with or without curcumin or exposed to a 5 Gy dose. The blot was probed with antibodies for Bcl-2, or β -actin

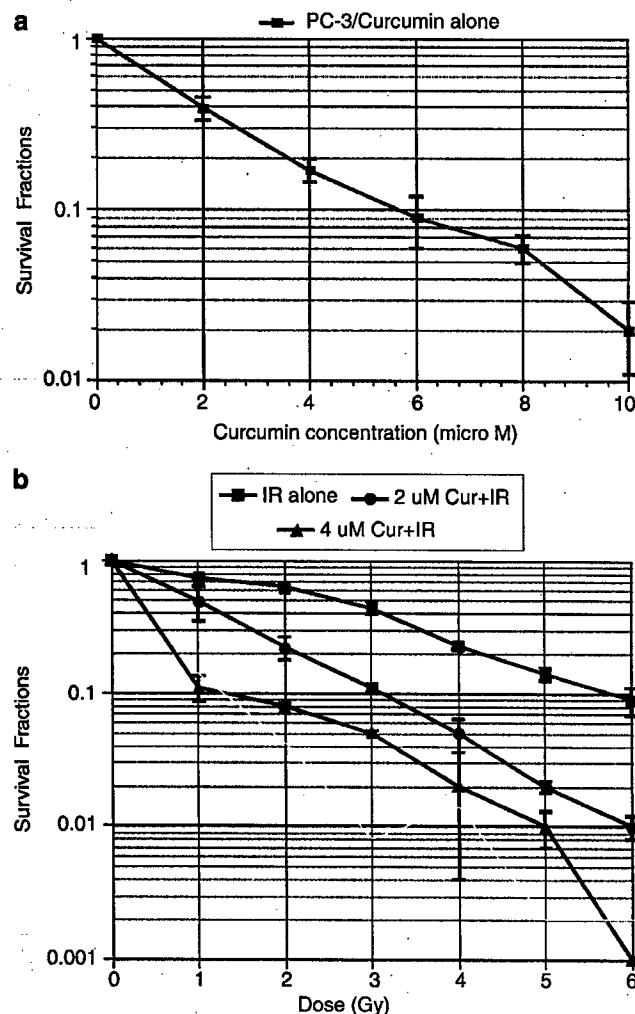


Figure 4 Curcumin enhance radiation-induced clonogenic inhibition in PC-3 cells: (a) Curcumin alone or (b) radiation or in combination with 2 or 4 μ M concentration of curcumin induced clonogenic inhibition of PC-3 cells. Cell survival curve of PC-3 cells with treatments as assayed by colony-forming ability and analysed by Single Hit Multi-Target (SHMT) model curve fit. The data shown are representative of the combined mean of two independent experiments

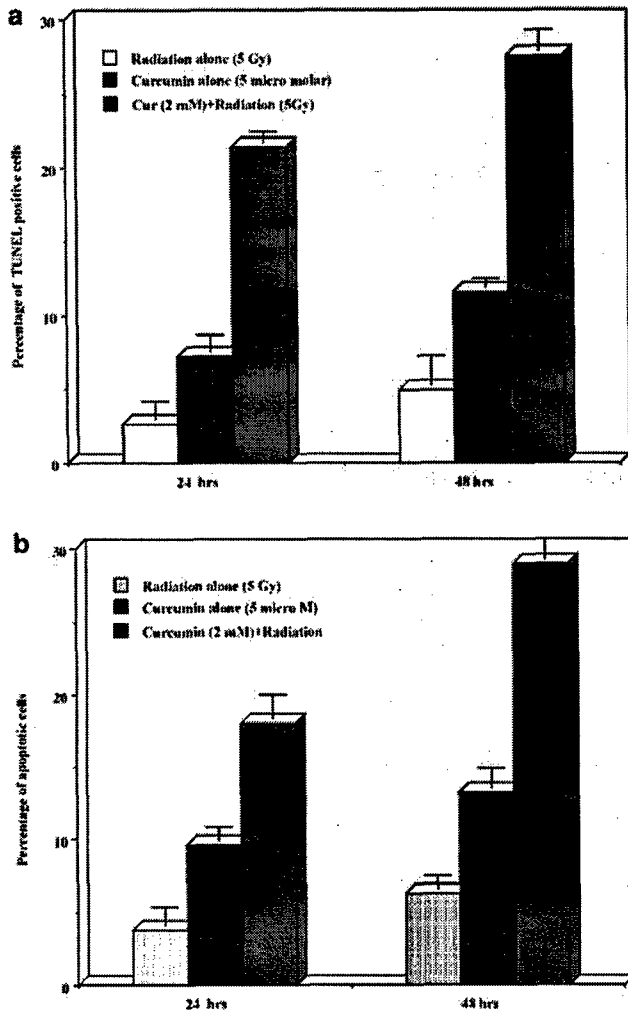
(Figure 5b). Thus, these results demonstrate that curcumin significantly enhanced the radiation-induced apoptosis in PC-3 cells.

Curcumin downregulates radiation-induced Bcl-2 protein expression in PC-3 cells

After confirming that curcumin inhibits radiation-induced prosurvival factors in PC-3 cells, we performed Western blot analysis for Bcl2 and Bax protein expression after treating PC-3 cells with curcumin or radiation or in combination. Bax and Bcl-2 are two discrete members of the gene family involved in the regulation of cellular apoptosis. Interestingly, no change in the level of bax protein was observed after the combined treatment (Figure 6). Since curcumin downregulates radiation-induced Bcl-2 and no changes in the

Table 1 Radiation inactivation estimates of PC-3 cells treated with curcumin or radiation alone or in combination

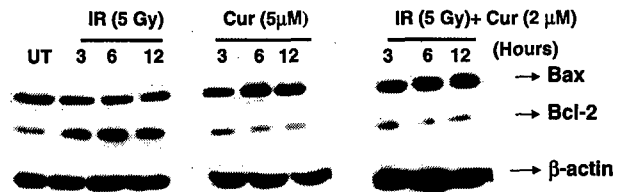
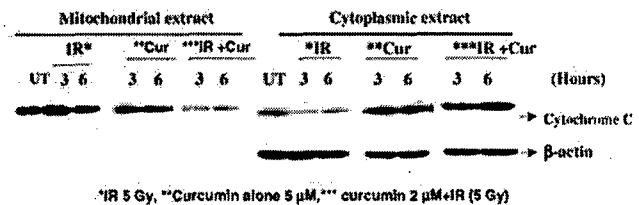
Treatment	SF ₂	SF ₂ ER	D ₀ (Gy)	D ₀ ER	ER
Radiation alone	0.635	—	231 cGy	—	—
IR + 2 μ M curcumin	0.224	2.83	97 cGy	2.38	5.21
IR + 4 μ M curcumin	0.080	7.93	38 cGy	6.07	14

**Figure 5** Curcumin enhances radiation-induced apoptosis in PC-3 cells: (a) Quantification of apoptosis by TUNEL method. (b) Quantification of apoptosis in by Hoechst 33342 (Ho342) and merocyanin 540 (MC540) staining. The error bar represents s.e. and the data shown are representative of the combined mean from two independent experiments

bax protein levels were observed, Bcl2:Bax ratio changed and it may have caused the induction of apoptosis in PC-3 cells.

Curcumin induced cytochrome c release and caspases activation in PC-3 cells

To confirm the involvement of the mitochondrial pathway of apoptosis, we analysed the activation of

**Figure 6** Curcumin downregulated radiation-induced Bcl-2 protein in PC-3 cells: Whole cell protein extracts were prepared from PC-3 cells that were left untreated or treated with radiation or curcumin or in combination and incubated for the indicated time interval. The blot was probed with antibodies for Bcl-2, Bax and β -actin**Figure 7** Curcumin induced cytochrome c release in PC-3 cells: Cytochrome c was assessed in untreated, treated with radiation or curcumin or in combination after 3 and 6 h in PC-3 cells, and cytosol and mitochondria extracts were extracted. Western blot analysis was used to determine the cytochrome c expression in both cytosol and mitochondria extracts. Loading control β -actin antibody was used for cytosol extracts

cytochrome c release from the mitochondria by Western blot analysis. It is known that cytochrome c releases from mitochondria into the cytosol and binds to the apoptotic protease activating factor (Apaf) complex and triggers the activation of procaspase-9 to the active caspase-9 (Reed, 1997). As shown in Figure 7, a marked fraction of the cytochrome c was released from the mitochondria, of curcumin and in combination with radiation-treated cells at 3 h, and the release was more pronounced at 6 h.

Caspases play a pivotal role in the execution of programmed cell death (Janicke *et al.*, 1998; Juo *et al.*, 1998; Kuida *et al.*, 1998; Earnshaw *et al.*, 1999), and in particular, we evaluated caspase-9 activity because it represents the apical caspase of the mitochondrial (intrinsic) pathway (Kuida *et al.*, 1998). Caspase-3 activation has been shown to be one of the most important cell executioners for apoptosis (Janicke *et al.*, 1998). A marked time-dependent increase in the activities of caspase-9 and -3 was observed in cells treated with 5 μ M curcumin or in combination of curcumin (2 μ M) with radiation. However, the sequential pattern of activation of these caspases was markedly different. There was significant activation of caspase-9 as early as 3 h of treatment, and the activity continued to increase till 24 h of the assay (Figure 8a). On the other hand, significant activation of caspase-3 activity was seen at 12 h after treatment (Figure 8b). The increase in the release of cytochrome c was in agreement with the data showing the continued increase in caspase-9 activity after 3 h treatment. The close association of the release of cytochrome c from mitochondria with the

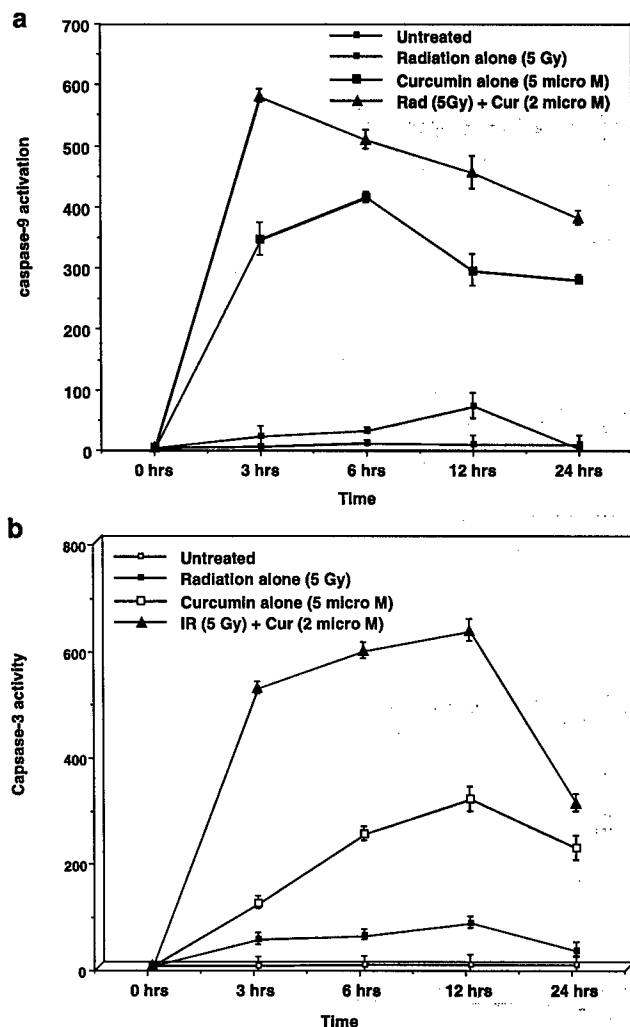


Figure 8 Curcumin induce caspase-3 and caspase-9 activation in PC-3 cells: PC-3 cells were left untreated or treated with radiation or curcumin or in combination and cell lysates prepared for time interval indicated. The enzymatic activity of cell lysates towards tetrapeptide chromogenic substrates, (a) DEVD-pNA (for caspase 3-like) and (b) LEHD-pNA (for caspase 9) was determined. Caspase activities are expressed as fold change of control and presented as mean $\pm$ SE of two samples

concurrent increase in caspase-9 provide evidence that curcumin induces apoptosis in PC-3 cells through the mitochondrial pathway.

Curcumin radiosensitizes PC-3 cells by inducing G₂/M block of cell cycle

To understand the mechanism that curcumin causes enhanced radiation-induced clonogenic inhibition, we performed flow cytometry to analyse the cell cycle changes induced by radiation in PC-3 cells. Curcumin caused a strong G₂/M block, which is an important phase sensitive to radiation. Curcumin-treated cells showed 32.55% of G₂/M block at 12 h, 48.10% at 24 h, 42.75% at 48 h and 35.89% at 72 h (Table 2). Thus, the G₂ block in curcumin-treated cells when combined with radiation caused enhanced radiosensitization, whereas radiation- or curcumin-treated cells showed no significant G₂/M block.

Discussion

Our studies showed that radiation induces prosurvival factors such as increased NF κ B activity and Bcl-2 upregulation in PC-3 cells. Ionizing radiation induce NF κ B activation (Hallahan *et al.*, 1989; Van Antwerp *et al.*, 1996) and it plays an important role in inhibiting TNF- α or chemotherapy-induced apoptosis (Wang *et al.*, 1996; Plummer *et al.*, 1999). TNF- α is also a potent inducer of NF κ B activity (Hallahan *et al.*, 1989; Van Antwerp *et al.*, 1996). The multiplicity of mechanisms of NF κ B activation and its role in inhibition of antiapoptotic function is more complex. The antiapoptotic target genes for NF κ B includes Bcl-2 (Tamatani *et al.*, 1999), Bcl-xL (Dixon *et al.*, 1997; Tamatani *et al.*, 1999) and Bcl-2 homologue A1/Bfl-1 (Wang *et al.*, 1999). We reported that ectopic overexpression of Bcl-2 in prostate cancer cells showed enhanced radiation resistance and inhibition of apoptosis in prostate cancer cells and other tumor cell types (Hockenbery *et al.*, 1990; Sentman *et al.*, 1991). Induction of prosurvival and antiapoptotic genes

Table 2 Effects of curcumin or ionizing radiation or in combination with curcumin and radiation on cell cycle distribution

Cell cycle	Treatments	12 h	24 h	48 h
G ₀ /G ₁	Untreated	55.45 $\pm$ 2.21	56.53 $\pm$ 4.21	53.23 $\pm$ 4.28
	Curcumin alone	28.45 $\pm$ 3.43	18.65 $\pm$ 3.76	20.05 $\pm$ 1.92
	Radiation alone	15.62 $\pm$ 2.34	21.61 $\pm$ 1.72	36.11 $\pm$ 2.69
	IR + Cur	24.21 $\pm$ 3.99	25.22 $\pm$ 2.24	20.85 $\pm$ 1.65
S	Untreated	24.21 $\pm$ 3.05	21.32 $\pm$ 5.11	28.39 $\pm$ 3.76
	Curcumin alone	39.00 $\pm$ 1.98	33.25 $\pm$ 1.07	37.20 $\pm$ 4.56
	Radiation alone	35.47 $\pm$ 2.98	13.25 $\pm$ 1.09	22.86 $\pm$ 3.30
	IR + Cur	48.32 $\pm$ 3.12	44.20 $\pm$ 5.12	54.65 $\pm$ 5.74
G ₂ -M	Untreated	20.34 $\pm$ 3.67	22.15 $\pm$ 1.98	18.38 $\pm$ 2.01
	Curcumin alone	32.55 $\pm$ 3.10	48.10 $\pm$ 2.16	42.75 $\pm$ 3.21
	Radiation alone	48.82 $\pm$ 2.73	65.14 $\pm$ 5.92	41.03 $\pm$ 4.19
	IR + Cur	27.48 $\pm$ 2.12	30.58 $\pm$ 4.21	24.50 $\pm$ 1.99

strongly suggests that PC-3 cells harbor a tight regulatory loop that inhibits the cell killing effects of ionizing radiation.

Curcumin has been reported to be a potent anti-proliferative agent for many tumor types (Rao *et al.*, 1995; Sikora *et al.*, 1997) and it acts as a proapoptotic agent in a variety of cancer cell lines (Kuo *et al.*, 1996; Khar *et al.*, 1999). Exposure of PC-3 cells to curcumin inhibited radiation-induced Bcl-2 expression, indicating that radiation-induced TNF- α is necessary to activate NF κ B, which is required for the induction of Bcl-2 protein. This is the first report showing that curcumin inhibits endogenous TNF- α as well as radiation-induced TNF- α protein expression in PC-3 cells. Inhibitory effects of curcumin on NF κ B activation have been documented in prostate cancer cells (Mukhopadhyay *et al.*, 2001), mouse fibroblast cells (Huang *et al.*, 1991), human leukemia cells (Singh and Aggarwal, 1995) and human colon epithelial cells (Plummer *et al.*, 1999). Curcumin inhibits NF κ B activation by inhibiting I κ B α phosphorylation that is necessary to export NF κ B from cytosol to nucleus and to activate its target genes. However, this is the first documented report demonstrating that curcumin is a potent radiosensitizer in prostate cancer cells and this sensitization is conferred by the inhibition of radiation-induced prosurvival factors such as NF κ B and Bcl-2. Hence, the down-regulation of endogenous and radiation-induced bcl-2 protein expressions in PC-3 cells will have significant therapeutic benefits in a majority of prostate cancer patients, since bcl-2 protein is overexpressed in these patients. Curcumin also inhibits cell proliferation induced by growth factors. Correlation between inhibition of cell proliferation and different phases of cell cycle by curcumin has been reported in the literature (Chen and Huang, 1998; Chen *et al.*, 1999). Curcumin induces cell cycle arrest in G2/M phase in breast cancer cells. These findings well correlated with our results since curcumin-treated cells showed G2/M arrest of cell cycle, which is sensitive to radiation and therefore this leads to enhanced radiosensitization.

Activated protein (AP-1) is a transcription factor activated by UV radiation, phorbol ester and asbestosis (Shaulian and Karin, 2002). AP-1 promotes several cellular genes that are responsible for cell proliferation and also transformation of preneoplastic to neoplastic state (Dong *et al.*, 1995). Several reports show that curcumin suppresses AP-1 activation; in our study also curcumin inhibits endogenous and radiation-induced AP-1 in PC-3 cells (data not shown).

We found that curcumin induced apoptosis by the activation of the downstream caspase-9, which has been shown to play an important role in apoptosis induced by several conditions (Ohta *et al.*, 1997; Mow *et al.*, 2001). In this study, caspase-9 activation was preceded by the activation of caspase-3, the apical caspase of the intrinsic mitochondrial pathway of apoptosis. Similarly, curcumin induced the release of cytochrome *c* into the cytosol after 3 h, and this release markedly increased after 6 h of treatment. Our results show curcumin downregulates radiation-induced bcl-2 protein expres-

sion that suggests that this protein is involved in the release of cytochrome *c* from mitochondria.

In recent clinical trials, curcumin was given a dosage of 8000 mg/day, and the peak serum concentration of $1.77 \pm 1.87 \mu\text{M}$ after 2 h of intake of curcumin has been reported (Cheng *et al.*, 2001). In our results, a significant enhancement of radiosensitizing effect was observed at 2 and 4 μM concentrations of curcumin by colony-forming assays. Hence, it is possible to achieve 2 μM concentration of curcumin in the serum concentration by consumption of 8000 mg/day of curcumin and this dose of curcumin in the serum will enhance the radiation effect in prostate cancer patients.

Apoptotic assays indicate that radiation caused significantly enhanced apoptosis in curcumin-treated cells. These results indicate that the natural compound, curcumin, at nontoxic doses inhibited the growth and induced apoptosis in PC3 cells and significantly enhanced the effect of radiation. It has been shown that curcumin induce apoptosis either by mitochondrial-dependent or mitochondrial-independent mechanism depending on the cell types. Curcumin-induced mitochondrial-independent apoptosis has been shown in breast cancer cell lines (Mehta *et al.*, 1997), basal cell carcinomas (Jee *et al.*, 1998) and T-Jurkat cells (Piwocka *et al.*, 1999).

In conclusion, curcumin, a major active component of turmeric, has been reported to induce growth inhibition and induce apoptosis in many cancer cell types. In this study we, for the first time, report that curcumin is a potent radiosensitizer that inhibits growth of human prostate PC-3 cancer cells and downregulates radiation-induced prosurvival factors and enhance radiation-induced sensitivity in PC-3 cells.

Materials and methods

Cell line

Human prostate cancer cells (PC-3 cells) were obtained from the American Type Culture Collection and maintained as adherent monolayer cultures in RPMI-1640 medium supplemented with 10% fetal bovine serum.

Curcumin

Curcumin (99% purity) (E,E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) was purchased from Sigma Chemical Co. (St Louis, MO, USA) and stored as 100 mM stock solution in DMSO, protected from light at -20°C . At 24 h after plating the cells, the medium was removed and replaced with fresh medium containing DMSO or medium containing different concentrations of curcumin in RPMI medium. For treatments, cells were left untreated or treated with radiation (5 Gy) alone or curcumin (5 μM) alone or in combination of curcumin with radiation. For combination treatment, curcumin (2 μM) was added to the cultures 2 h prior to radiation (5 Gy).

Irradiation

A 100 kV industrial X-ray machine (Phillips, Netherlands) was used to irradiate the cultures at room temperature. The dose

rate with a 2 mm Al plus 1 mm Be filter was ~ 2.64 Gy/min at a focus-surface distance of 10 cm.

Western blot analysis

Total protein extracts from untreated cells or cells treated with curcumin alone or irradiated alone or in combination (curcumin plus radiation) at various time intervals were subjected to Western blot analysis as described (Chendil *et al.*, 2002) using Bcl-2 monoclonal antibody (sc-509) (Santa Cruz, CA, USA), Bax monoclonal antibody (sc-493) (Santa Cruz, CA, USA), cytochrome *c* monoclonal antibody (Biovision, Inc., CA, USA), or for loading control the β -actin antibody (Sigma Chemical Co, St Louis, MO, USA) were detected using the chemiluminescence method.

Colony-forming assay

For clonogenic cell survival studies, two different cell concentrations in quadruplet sets were used for each treatment. Cell lines were left untreated or exposed to 0.5–6 Gy dose of radiation or treated with various concentration of curcumin or for combination treatment curcumin (2 μ M or 4 μ M) was added to the cultures 2 h prior to radiation (5 Gy). After incubation for 10 or more days, each flask was stained with crystal violet and the colonies containing more than 50 cells were counted. The surviving fraction (SF) was calculated as a ratio of the number of colonies formed and the product of the number of cells plated and the plating efficiency. The curve was plotted using *X-Y* log scatter (Delta Graph[®]4.0), and by using the formula of the SHMT model, the D_0 was calculated. D_0 is the dose required for reducing the fraction of cells to 37%, indicative of single-event killing. SF_2 is the survival fraction of exponentially growing cells that were irradiated at the clinically relevant dose of 2 Gy.

Flow cytometry

Flow cytometry was performed as described earlier (Chendil *et al.*, 2002). Untreated and treated cells (1×10^6) were washed in phosphate-buffered saline (PBS) and fixed in ice-cold ethanol. Fixed cells were pelleted and resuspended in 500 μ l of PBS. RNA was eliminated by treating cells with RNase A (Sigma, St. Louis, MO, USA). Then, the cells were stained by propidium iodide in PBS and analysed for cell cycle phases by flow cytometry, a FACStar calibur (Becton Dickinson) cell sorter.

Quantification of apoptosis

Apoptosis was quantified by TUNEL staining and flow cytometry. The ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD, USA), which detects DNA strand breaks by TUNEL, was used as described (Ahmed *et al.*, 1996). Briefly, cells were seeded in chamber slides and the next day the cells were left untreated or treated with curcumin or radiation or in combination. After 24 and 48 h, the DNA was tailed with digoxigenin-dUTP and conjugated with an anti-digoxigenin fluorescein. The specimen was counterstained with propidium iodide and antifade. The stained specimen was

observed in triple band-pass filter using Nikon-microphot epifluorescence microscope. To determine the percentage of cells showing apoptosis, four experiments in total were performed, and approximately 1000 cells were counted in each experiment. For flow cytometry, cells were lifted by using nonenzymatic cell dissociation medium (Sigma) and washed with PBS and stained with Hoechst (Ho342) and merocyanine (MC540) and analysed by flow cytometry using a FACStar Plus cell sorter as described (Ahmed *et al.*, 1996).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from untreated and treated cells and EMSA was performed as described previously (Chendil *et al.*, 2002) with some modification. Briefly, cells were scraped and washed with cold PBS and repelleted. The pellet was suspended in 1 ml of Icecold buffer A (10 mM HEPES pH 7.8, 2 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 10 μ g/ml of aprotinin, 0.5 μ g/ml leupeptin, 3 mM PMSF and 3 mM dithiothreitol) for 5 min on ice. The crude nuclei were pelleted by centrifugation for 5 min. The crude nuclei pellet was suspended in 50 μ l of buffer B (10 mM HEPES, pH 7.8, 2 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 10 μ g/ml of aprotinin, 0.5 μ g/ml leupeptin, 3 mM PMSF, 3 mM dithiothreitol and 10% NP-40). After 20 min of incubation on ice, the suspension was centrifuged, and supernatant was collected. Equal volume of cold buffer C (50 mM HEPES, pH 7.4, 300 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 3 mM PMSF, 3 mM DTT and 10% (v/v) glycerol) was added to the supernatant and incubated on ice for 5 min with intermittent vortexing. The extracts were then centrifuged for 10 min and the supernatant was divided into aliquots and frozen at -80°C .

Analysis of DNA binding by EMSA was performed using 2 mg of poly(dI-dC) (Sigma Chemical Co, St Louis, MI, USA) as nonspecific competitor DNA. The binding reactions contained 10 000 c.p.m. of ^{32}P -labeled double-stranded oligonucleotide probe with a high affinity for NF κ B binding (Promega, Madison, WI, USA). For supershift experiments, anti-p65 antibody was incubated with binding buffer and nuclear extract for 1 h prior to adding the oligo probe. Binding reactions were electrophoresed on a 4% PAGE in 0.5 $\times$ TBE buffer to separate the bound and unbound probe.

Caspase activity

To measure the activity of caspases-3 and -9 in PC-3 cells, a fluorimetric assay was used according to the instruction of the manufacturer (Biovision, CA, USA). Briefly, cells were left untreated and treated either with curcumin or radiation or in combination. Cells were collected and resuspended in cold lysis buffer and incubated for 10 min on ice. In all, 50 μ l of 2 $\times$ reaction buffer was added and incubated for 2 h at 37°C with fluorogenic substrates, DEVD-AFC (caspase-3) and LEHD-AFC (caspase-9) in a reaction buffer. The release of fluorochrome AFC was measured at 400 nm excitation and 505 nm emissions using a fluorescence spectrophotometer.

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