Chapter 4

Doxycycline potentiates anticancer effect of cyclophosphamide
Summary

Cyclophosphamide (CPA) is a widely used chemotherapeutic drug in neoplasias. It is a DNA and protein alkylating agent that has a broad spectrum of activity against variety of neoplasms including breast cancer. The therapeutic effectiveness of CPA is limited by the high dose hematopoietic, renal and cardiac toxicity that accompanies the systemic distribution of liver-derived activated drug metabolites. The present study examines the potential of combining well-tolerated antibiotic doxycycline (DOX) with CPA and understanding the mechanism of cell killing. DOX potentiates MCF-7 cell killing by CPA in vitro. Interestingly, we also found that DOX significantly enhances the tumor regression activity of CPA on xenograft mice model bearing MCF-7 cells. In presence of DOX (3 μg/ml) the IC$_{50}$ value of CPA decreased significantly from 10 mM to 2.5 mM. Additional analyses indicate that the tumor suppressor p53 and p53-regulated proapoptotic Bax were up-regulated in vivo and in vitro following CPA treatment in combination with DOX, suggesting that upregulation of p53 may contribute to enhancement of antitumor effect of CPA by DOX. Furthermore, down regulation of antiapoptotic Bcl-2 was observed in animals treated with CPA and CPA plus DOX treated animals. Also, the enhancement of therapeutic efficiency of this combination therapy has also been attributed to increase in reactive oxygen species following drug treatments. Our results raise the possibility that this combination chemotherapeutic regimen may lead to additional improvements in the treatment of breast cancer.
4.1 Introduction

The concept of combining chemotherapeutic agents to increase cytotoxic efficacy has evolved greatly over past several years. The underlying rationale is the realization that except for Burkitt’s lymphoma and Choriocarcinoma, individual chemotherapeutic agents for the majority of tumors have not increased cure rates in the treatment of cancer (Shah and Schwartz, 2001). However with the advancements in our understanding for the effects of chemotherapeutic drugs on cancer cells, it is becoming apparent that combination therapy involving two or more drugs may have greater beneficial effects on cancer treatment and management.

CPA is a cell cycle-dependent DNA and protein alkylating agent that has a broad spectrum of activity against variety of neoplasms, and is widely used in the clinical management of human malignancies including breast cancer (Sladek, 1988; Moore, 1991). In conventional chemotherapy, CPA is one of the most commonly employed drugs and is used in high dose regimen to treat metastatic breast cancer (Legha et al., 1979; Tormey et al., 1982). In this setting the therapeutic efficacy of this drug is limited by host toxicity as a result of the systemic distribution of activated drug metabolites that have significant cytotoxic effects including cardiac and renal toxicity (Chen et al., 1996). Therefore drugs that could potentiate CPA antineoplastic effect would be of significant importance and will lessen the toxic effects associated with high dose CPA treatment. Potentiation of antitumor effect of CPA by AQ4N and tirapazamine on mammary carcinoma (Friery et al., 2000), and by bis-indole alkaloid on breast cancer cells has been reported (Leung et al., 2000). Very recently it has been demonstrated that coadministration of thalidomide and CPA gave markedly greater activity against Colon 38 tumors compared with either drug alone (Ding et al., 2002).

Doxycycline (DOX), a commonly used antibiotic has antitumor activity against several malignancies (Fife et al., 1998; Rubins et al., 2001). Recently it has been reported that DOX has potential treatment value in bone metastasis of breast cancer cells (Duivenvoorden et al., 2002). DOX inhibits these effects by inhibiting matrix metalloproteinases (MMPs), not only in breast cancer cells but also in human endothelial, prostate cancer, osteocarcinoma cells of patients (Fife et al., 1997;
Hanemaaijar et al., 1998). Moreover, it also inhibits cell proliferation and induces apoptosis in various cancer cells (Rubins et al., 2001). All these studies demonstrate that this well tolerated antibiotic may be effective in treatment of various human cancers, either alone or in combination therapy. In our quest to identify anticancer drugs other than CPA in which combination with DOX may enhance therapeutic efficiencies, we also assayed cytotoxicity of commonly used anticancer drugs such as 5-fluorouracil (5-FU), carboplatin (Carb) or cycloheximide (CHX) alone as well as with DOX on breast cancer cells. However, only in combination with CPA, DOX enhanced its therapeutic efficacy on MCF-7 breast cancer cells both in vitro and in vivo.

The ultimate vulnerability of cells to diverse apoptotic stimuli such as anticancer drugs is determined by the relative ratio of various proapoptotic and antiapoptotic members of the Bcl-2 family (Oltvai et al., 1993; Yang and Korsmeyer, 1996) and tumor suppressor p53. The Bcl-2 family includes antiapoptotic Bcl-2 and its homologous protein, Bax, which competes with Bcl-2. In all, this report suggests a coordinate performance of these molecules is crucial for controlling life and death of a cell (Basu and Haidar, 1998). Furthermore, there are several reports suggesting that free radicals are powerful inducers of p53 activity and that they play a role in the execution of p53-dependent apoptosis (Martinez et al., 1997). Nonetheless, there are investigations reporting ROS as one of the downstream mediators of p53-dependent apoptosis (Johnson et al., 1996). Additionally, it has been reported that CPA-induced genotoxicity is also due to the generation of reactive oxygen molecules during its bioactivation (Stankiewicz et al., 2002). Thus, we also examined whether ROS generation is additive in the enhanced cytotoxic effect of this combination therapy.

DOX is potentially beneficial in bone metastasis of breast cancer cells and CPA is an important component of chemotherapeutic regimen for treatment of breast cancers. Therefore we postulated that this combination treatment might enhance antitumor effect of CPA on breast cancer cells in vivo. We found that DOX significantly enhances the tumor regression activity of CPA on xenograft mice model of MCF-7 cells. We have explored the molecular basis of synergistic effect of
CPA and DOX, and investigated mechanism of action both in vitro and in vivo with emphasis on Bax-Bel-2 ratio and ROS generation. Our results raise the possibility that this combination chemotherapeutic regimen may lead to additional improvements in the treatment of breast cancer.
4.2 Materials and Methods

4.2.1 Reagents and cell cultures

Drugs such as 5-Fluorouracil (5-FU), Carboplatin (Carb), Cycloheximide (CHX), Cyclophosphamide (CPA), Doxycycline (DOX) and methyl-thiazolyl tetrazolium (MTT) dye were purchased from Sigma St. Louis, USA and dissolved in sterile water to prepare a stock of 50 mM for drugs, 5 mg/ml for DOX and 1 mg/ml for MTT. Human breast cancer line MCF-7 (ATCC HTB 22) was obtained from ATCC, Virginia, USA and maintained in our in-house National Cell repository. MCF-7 cells were routinely cultured in MEM, supplemented with 10% heat inactivated fetal bovine serum (HyClone, UT, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen Life Technologies, MD, USA), at 37°C with 5% CO₂. Wherever tetracycline inducible system (Tet system) has been used, MEM was supplemented with Tet system approved fetal bovine serum (Clontech, CA, USA).

4.2.2 MTT cell proliferation assay

To assay the cell viability after drug treatment, cells were seeded at a density of 7500 cells per well into 96 well plates and allowed to adhere for 24 h at 37°C. Cells were treated with 5-FU and Carb in range of 1 μM to 1000 μM, and with CHX and CPA in range of 10 μM to 10 mM, with or without DOX (1 and 3 μg/ml) for 24 h in triplicates and further incubated for additional 48 h in absence of drugs. Thereafter, media was decanted and 50 μl of MTT (1 mg/ml) in MEM (without phenol red) was added to each well and incubated for 4 h at 37°C. Formazan crystals were solubilized in 50 μl of iso-propanol by incubating with shaking at room temperature for 10 min. Absorbance was measured at 570 nm using 630 nm as reference filter. Absorbance given by untreated cells was taken as 100% cell growth.

4.2.3 RNA extraction, cDNA synthesis and RT-PCR

Total cellular RNA from treated and untreated cells was extracted using TRIzol™ reagent (Invitrogen Life Technologies, MD, USA), according to the manufacturer’s instructions. Five micrograms of total RNA and oligo (dT)₁₂₋₁₈ primers were taken in diethyl pyrocarbonate (DEPC)-treated water. cDNA synthesis was initiated using 200 units of M-MLV reverse transcriptase (Invitrogen Life
Technologies, MD, USA), under conditions recommended by manufacturer and the reaction was allowed to proceed at 37°C for 50 min. Reaction was terminated by heating at 70°C for 15 min. Each RT-PCR contained 10% of cDNA, 20 pmoles of each primer in 20 mM Tris-HCl (pH 8.4) containing 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP mix, and 1 unit of platinum Taq DNA polymerase (Invitrogen Life Technologies, MD, USA) in a final volume of 20 μl. After an initial denaturation for 1 min at 95°C, 30 or 35 cycles of denaturation (94°C for 1 min), annealing (for 1 min), and extension (72°C for 1 min) were performed on a DNA thermal cycler (Techne, Cambridge, UK) with a final extension for 10 min at 72°C.

The primer pairs and their respective annealing temperatures as well as product size are tabulated below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temp</th>
<th>Product size (bp)</th>
</tr>
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<tbody>
<tr>
<td>p53</td>
<td>(F) 5'-CTG AGG TTG GCT CTG ACT GTC CCA CCA TTC-3' (R) 5'-CTC ATT CAG CTC TCG GAA CAT CTG GAA GCG-3'</td>
<td>55°C</td>
<td>371</td>
</tr>
<tr>
<td>p21</td>
<td>(F) 5'-GAC ACC ACT GGA GGG TGA CT-3' (R) 5'-GGC GTT TGG AGT GGA AA-3'</td>
<td>59°C</td>
<td>299</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>(F) 5'-TTC GGG CGA GAT GTC CGC C-3' (R) 5'-TCA CTT GTG GCC CAG ATA GG-3'</td>
<td>61°C</td>
<td>373</td>
</tr>
<tr>
<td>Bax</td>
<td>(F) 5'-ATT ATG GAC GGG TCC GGG GA-3' (R) 5'-TGT CCA GCC CAT GAT GGT TCT-3'</td>
<td>59°C</td>
<td>419</td>
</tr>
<tr>
<td>β-actin</td>
<td>(F) 5'-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3' (R) 5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3'</td>
<td>55°C</td>
<td>838</td>
</tr>
</tbody>
</table>

4.2.4 Immunoblotting

Following 36 h treatment with CPA in the presence or absence of DOX, cells were washed thrice with ice cold PBS, and lysed in 100 μl of lysis buffer (20 mM HEPES pH 7.4 containing 1% triton X-100, 2 mM EGTA, 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 mM TPCK, 2 mM NaN₃VO₄ and protease inhibitor cocktail tablet from Boehringer Mannheim, Germany) per 1x10⁶ cells on ice. An equal amount of protein samples (75 μg) were resolved on 10-12% SDS-polyacrylamide gel and then transferred onto PVDF membrane (Amersham, Aylesbury, UK). The membranes were probed with antibodies against p53, Bax and β-actin (Santa Cruz Biotechnology, CA, USA). The immunoblots were
detected by enhanced chemiluminescence (ECL) reagent (New England Biolabs, MA, USA).

4.2.5 Transfection and chloramphenicol acetyl transferase (CAT) reporter assay

The p53 CAT reporter construct PG_{13}CAT, which contains 13 repeats of p53 binding site inserted 5' to polyomavirus basal promoter linked to CAT reporter gene (kind gift of Dr. Bert Vogelstein, John Hopkins, Baltimore, USA) along with a β-galactosidase expression vector (pCH110), was transiently transfected MCF-7 cells by lipofectamine method (Invitrogen Life Technologies, MD, USA). Almost 80% confluent cells in 35 mm culture plate were transfected with 4 μg of DNA including 1 μg pCH110 plasmid as an internal control to assess the transfection efficiency. Vector plasmids were used as carrier DNA to makeup the final DNA concentration to 4 μg. 1 h before transfection, 1 ml of fresh medium was added to each plate. For each plate to be transfected, 4 μg of DNA was diluted 250 μl of Opti-MEM (Gibco). For each plate, 4 μl of LF2000 reagent was diluted into 250 μl of Opti-MEM (Gibco) and incubated for 5 min at room temperature. Diluted DNA was mixed with diluted LF2000 reagent and incubated at room temperature for 40-45 min to allow LF2000-DNA complex formation. 500 μl of LF2000-DNA complex was added drop wise to the plate and mixed gently by rocking the plate. Cells were incubated at 37 °C for 24 h. After transfection, cells were washed and fresh media with or without drugs was added to the culture plates for an additional 24 h. Cells were then collected and washed thrice with PBS and resuspended in 0.25 M Tris-Cl (pH 7.5) buffer. Cells were lysed by four cycles of rapid freeze–thaw. CAT assay was performed by taking equal amounts of lysate protein in presence of 1 μCi C^{14}-chloramphenicol (NEN, Boston, MA, USA) and 100 μg of acetyl CoA (Amersham, Aylesbury, UK) in 250 mM Tris-Cl (pH 7.5) in a total reaction volume of 100 μl. Reaction mixture was incubated at 37°C for 5 h and terminated by adding ethyl acetate to the sample tubes. Products were resolved by thin layer chromatography (TLC), using mixture of chloroform and methanol (19:1 ratio). TLC plates were analyzed by autoradiography and scanning on a phosphorimager (Bio-Rad, CA, USA). The specific CAT activity was calculated by determining the fraction of
chloramphenicol that had been acetylated during the reaction. pCH110, (Clontech, CA, USA) was also used as internal control for normalization of transfection efficiencies by quantifying activity of β-galactosidase in the transfected cells. The activity of β-galactosidase was assayed in pCH110 transfected cells by using CPRG (chlorophenolred-β-D-galactopyranoside) as substrate. Principally, CPRG is cleaved by β-galactosidase to yield chlorophenol red and absorbance of this red colour can be measured at 570 nm. 20 µg of cell lysates from each sample was taken in the wells of flat bottom 96-well plate and diluted to 100 µl with PBS to estimate the β-galactosidase activity in the cells. 1 mM of CPRG was added to each well and incubated at 37 °C for 6 h. Absorbance was taken in microplate reader (Multiskan Ascent, Labsystems) at 570 nm.

4.2.6 Transient expression of antisense p53 in MCF-7 cells

p53 dependent cell death in MCF-7 cells treated with drug combinations was assessed by transiently co-transfecting cells with pTet-On (Clontech, CA, USA) and pTRErevp53 (vector for antisense p53 expression). In pTRErevp53 plasmid, p53 was sub-cloned for antisense orientation in parental pTRE vector (Clontech, CA, USA), which has tetracycline responsive element (TRE) specifically responsive to minimal doses of DOX. Briefly, 1800 bp fragment of wild type p53 was excised with Bam H1 (New England Biolabs, MA, USA) restriction digestion from pC53-SN3 vector (kind gift of Dr. Bert Vogelstein, John Hopkins, Baltimore, USA) and sub cloned in antisense orientation in multiple cloning sites of pTRE vector. The correctness of pTRErevp53 was confirmed previously by antisense p53 dependent functional assays of chapter 3 of this thesis. This system has got inherent advantage as it utilizes DOX as an inducer. Transfection was performed by lipofectamine method (Invitrogen Life Technologies, MD, USA) as described in CAT reporter assay method. After transfection, cells were washed and fresh media with or without respective drug was added to the cell in culture plates for an additional 48 h. Cells were then harvested for PI staining as well as for immunoblotting. Transfection efficiency was determined simultaneously by transfecting green fluorescent protein expressing plasmid pEGFPN1 (Clontech, CA, USA). GFP intensity was directly measured from the cell lysates of pEGFP-N1 transfected cells by fluorometer to confirm equal transfection efficiency as well to normalize the reporter activity.
Equal amount (50 μg) of cell lysates from pEGFP-N1 transfected cells were taken in the wells of 96-black-well plates. The fluorescence intensity of GFP was recorded on plate reading fluorometer (Fluoroskan Ascent FL, Labsystems) with filter set at excitation 485 nm and emission 510 nm.

4.2.7 Transient expression of sense Bcl-2 in MCF-7 cells

Cell survival effect of Bcl-2 was assessed by transient overexpression of human Bcl-2 construct in MCF-7 cells. The Bcl-2 expression vector pSSVBcl-2 was provided by Dr. Silvia Soddu, Regina Elena Cancer Institute, Rome, Italy. Cells were seeded at a density of 7500 cells per well into 96 well plates and allowed to adhere for 24 h at 37°C. Transfection was performed by lipofectamine method (Invitrogen Life Technologies, MD, USA). Cells were seeded at the density of 10^4 per well of 96-well culture plate and transfected with 0.5 μg of DNA including 0.1 μg pEGFP-N1 plasmid as an internal control to assess the transfection efficiency. Vector plasmids were used as carrier DNA to makeup the final DNA concentration to 0.5 μg. 1 h before transfection, 100 μl of fresh medium was added to each well. For each well to be transfected, 0.5 μg of DNA was diluted 25 μl of Opti-MEM (Gibco). For each plate, 0.2 μl of LF2000 reagent was diluted into 25 μl of Opti-MEM (Gibco) and incubated for 5 min at room temperature. Diluted DNA was mixed with diluted LF2000 reagent and incubated at room temperature for 40-45 min to allow LF2000-DNA complex formation. 50 μl of LF2000-DNA complex was added drop wise to the plate and mixed gently by rocking the plate. Cells were incubated at 37 °C for 24 h. After transfection, cells were washed and fresh media with or without a drug was added to the cell in culture plates for an additional 48 h. Cells were then subjected to MTT cytotoxicity assay as given in section 4.2.2. Transfection efficiency was determined simultaneously by transfecting green fluorescent protein expressing plasmid pEGFPN1 (Clontech, CA, USA) as described in section 4.2.6.

4.2.8 Flowcytometry for cell cycle analysis

Cells were plated at a density of approximately 8 × 10^5 cells in 60 mm tissue culture plates and allowed to grow for 24 h. Ten millimolar CPA and 3 μg/ml DOX...
were added to the cells for 48 hours. Cells were harvested by trypsinization and subsequently processed for flow cytometric analysis. In brief, cells were washed twice in chilled PBS and fixed in 70% ethanol on ice. After RNase A (200 μg/ml) treatment for 30 min at 37°C, 50 μg/ml propidium iodide (PI) was added to cell pellet and incubated in the dark for 30 minutes on ice. The fluorescence of PI was collected through a 585 nm filter in FACScan flowcytometer (Becton Dickinson GmbH, Heidelberg, Germany). The data were analyzed using the Cell Quest Software, for 10^6 cells. The percent of cells in sub G0-G1 fraction was obtained from the data.

4.2.9 Total cell death determination by flowcytometric analysis

For total cell death (necrotic and late apoptotic) determination by flowcytometric assay, MCF-7 cells were treated with various concentrations of DOX (0.3 to 6 μg/ml) in presence of CPA (10mM) as described in MTT cell proliferation assay. The cells were stained with PI (50 μg/ml) and analyzed by the method of Nicoletti et al., 1991 and Wolbers et al., 2004. In brief, cells were washed twice in chilled PBS. After RNase A (200 μg/ml) treatment for 30 min at 37°C, 50 μg/ml PI was added to cell pellet and incubated in the dark for 30 minutes on ice. This method does not rely on ethanol fixing of the cells and offers advantage for being quantitative for calculation of total cell death indices. The fluorescence of PI was collected through a 585 nm filter in FACScan flowcytometer (Becton Dickinson GmbH, Heidelberg, Germany). Both dot plot and histogram analyses were performed using the Cell Quest Software (Becton Dickinson GmbH, Heidelberg, Germany) for 10^6 cells.

4.2.10 Enhanced reactive oxygen species (ROS) generation in CPA treated cells after DOX addition

Confocal microscopy for assessing levels of ROS in drug treated MCF-7 cells was performed using 6-Carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA) from Sigma. When applied to intact cells the non-ionic, non-polar DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to non-fluorescent DCFH. In presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF). Therefore the intracellular DCF fluorescence can be
used as an index to quantify overall oxidative stress in cells. The MCF-7 cells were seeded on multiwell microslides (ICN Pharmaceuticals, CA, USA) and allowed to adhere for at least 24h. The cells were then treated with DOX and CPA at known concentrations for another 24h. The cells were briefly washed with PBS and loaded with 10 mM DCFH-DA for 30 min at 37°C. After washing with cold PBS, fluorescent intensity was measured under Laser Confocal Microscope (Zeiss, LSM-510) with excitation at 485 nm and emission at 530nm. The results are represented as fold increase in fluorescence intensity performed in triplicates. Representation of intensity in form of 2.5D plot was also obtained.

4.2.11 Animals, animal-keeping conditions and tumor growth regression model

Eight weeks old female homozygous (nu+/nu+) nude athymic NIH mice (NIV, Pune, India), 18-22 g, were used as human cancer xenograft models. The animals were housed in specific pathogen-free isolators (Harlan Isotec, USA) in an environmentally controlled Experimental Animal Facility of our institute. They were caged (6 per cage) in polypropylene boxes and had free access to sterilized pelleted laboratory rodent chow and water. All animal experiments have been performed following the requirement of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and after permission of the Institute’s Animal Care and Use Committee (IACUC). Five consecutive subcutaneous (sc) estradiol valerate (30 μg/day) injections were administered in each mouse prior to sc injection of MCF-7 tumor cells in the exponential growth phase (2 x 10^7 cells in 0.1 ml), at right flank of each mouse. Drug treatments were initiated 2 weeks after palpable tumors developed. The mice were randomized into four groups having six mice per group (n=6) to receive vehicle alone (control), DOX (5 mg/kg/day), CPA (100 mg/kg/day), or CPA plus DOX by intraperitonial (ip) injection for four consecutive days. CPA and DOX were dissolved in PBS. Four days following last injection mice were sacrificed and tumors were surgically excised. Tumor growth was determined by weight measurements and host toxicity was monitored by body weight measurements. Representative tumor tissues (200 mg from each animal of a group) were processed and samples were pooled for n=3 per treatment group for reverse transcriptase polymerase chain reaction (RT-PCR) analysis as mentioned in section 4.2.3.
4.2.12 Statistical Analysis

Data are expressed as the mean of three independent results. Statistical comparisons are made using student's $t$ test and $P$ value $< 0.05$ was considered as significant.
4.3 RESULTS

4.3.1 Effect of various anticancer drugs on MCF-7 breast cancer cells in presence or absence of doxycycline as evaluated by MTT cytotoxicity assay

A dose response curve of the MCF-7 cells to various concentrations of commonly used anticancer drugs such as 5-FU, Carb, CHX and CPA, with and without DOX was generated. As shown in Fig. 1A and 1B, the treatments with increasing concentration of 5-FU and Carb illustrated the dose dependent cell death in MCF-7 cells. However addition of DOX (1 and 3 μg/ml) did not potentiate the toxicity of 5-FU and Carb.

Fig.1. In vitro chemosensitivity assays on MCF-7 breast cancer cell. MTT assay was performed as documented in materials and methods. MCF-7 cells were incubated in the presence of various chemotherapeutic drugs 5-FU (A) and Carb (B), with and without Doxycycline (DOX) for 24 h, and after 48 h of post drug incubation the dose dependent cell survival was determined by MTT metabolism. Cell survival (mean±standard deviation, n=5) is expressed as a fraction of untreated control cells.
Fig. 2. In vitro chemosensitivity assays on MCF-7 breast cancer cell. MTT assay was performed as documented in materials and methods. MCF-7 cells were incubated in the presence of various chemotherapeutic drugs CHX (A) and CPA (B), with and without Doxycycline for 24 h, and after 48 h of post drug incubation the dose dependent cell survival was determined by MTT metabolism. Cell survival (mean±standard deviation, n=5) is expressed as a fraction of untreated control cells.

A dose response curve of the MCF-7 cells to various concentrations of CHX and CPA, with and without DOX was generated. As shown in Fig. 2A and 2B, the treatments with increasing concentration of CHX and CPA illustrated the dose dependent cell death in MCF-7 cells. The addition of DOX (1 and 3 μg/ml) did not potentiate the toxicity of CHX. However, experiments involving CPA and DOX show that treating cells with varying concentrations of CPA in presence of DOX (1 and 3 μg/ml) results in increased cytotoxicity of CPA (Fig. 2B). Treatments with of 10 mM (approximate IC₅₀ value) concentration of CPA alone, about 55% cells were viable compared to untreated cells. When the cells were treated with 10 mM CPA in presence of 3 μg/ml DOX, less than 25% cells were viable. In the presence of 3 μg/ml DOX the IC₅₀ value further dropped to 2.5 mM. These data clearly
demonstrate the additive effect of DOX with CPA, which further enhances the cytotoxic potential of CPA on MCF-7 cells.

4.3.2 Effect of DOX on MCF-7 breast cancer cells as evaluated by MTT cytotoxicity assay

A dose response curve of the MCF-7 breast cancer cells with respect to various concentrations of DOX was generated (Fig. 3). In the dose dependent treatments of MCF-7 cells with DOX, it can be concluded that DOX is not cytotoxic to these cells. As shown in the representative graph, the MCF-7 cells were viable similar to untreated cells even at the concentration of 7 μg/ml of DOX. Hence, it was altogether concluded that DOX enhanced cytotoxicity of CPA on MCF-7 cells without being cytotoxic by itself. It needed further exploration that how this synergy was useful in enhancing therapeutic efficiency of CPA and whether this cytotoxicity depends on functional status of tumor suppressor p53 in these cells.

Fig. 3. In vitro toxicity assay for doxycycline treatment on MCF-7 breast cancer cell. MTT assay was performed as documented in materials and methods. Cells were incubated in presence of increasing concentrations of DOX for 24 h, and after 48 h of post drug incubation the dose dependent cell survival was determined by MTT metabolism. Cell survival (mean±standard deviation, n=5) is expressed as a fraction of untreated control cells.
4.3.3 DOX enhances apoptotic population of CPA treated MCF-7 breast cancer cells as evaluated by flowcytometry assay

To further examine whether DOX enhances CPA mediated cell killing via enhanced induction of apoptosis in CPA treated cells, MCF-7 cells were treated with CPA alone or in presence of DOX (3 µg/ml) for 48 h and percent PI stained cells were analysed by flowcytometry for cell phase analysis. Effect of CPA and CPA plus DOX on MCF-7 cells was determined by quantization of sub G0-G1 fraction (apoptotic fraction) of total cell population denoted as M1 in histograms obtained from flowcytometric analysis. Cells without any treatment or with DOX alone at a concentration of 3 µg/ml did not show any significant cell killing as shown by M1 population in Fig. 4 and tabulated in Table I where sub G0-G1 fraction is indicated as percent apoptotic population.

Fig.4: Effect of CPA and CPA plus DOX on MCF-7 cell apoptotic population determined by analysis of sub G0-G1 fraction (M1) as obtained from histograms of flowcytometry. MCF-7 cells were untreated (A), treated with DOX (B), treated with 10 mM CPA (C) and treated with CPA (10 mM) and 3 µg/ml DOX (D) for 24 h.
The percent cell killing by DOX (3 μg/ml) and CPA combination was 27.30±3.6 as compared to that of CPA alone which was 16.55±0.65. Hence addition of DOX, lead to enhancement of CPA mediated cellular apoptosis approximately by 11% (Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Apoptotic population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.69±0.05</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>5.2±0.35</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>16.55±0.65</td>
</tr>
<tr>
<td>Cyclophosphamide+Doxycycline</td>
<td>27.30±3.6</td>
</tr>
</tbody>
</table>

Table 1. Effect of CPA and CPA plus DOX on MCF-7 cells. Percent apoptotic population as determined by sub G0-G1 fraction by flow cytometric analysis. Values are expressed as means (standard deviation, n=4).

4.3.4 p53 and Bax are up-regulated following treatment of MCF-7 cells in vitro with a combination of DOX and CPA

The DNA damaging chemotherapeutic drugs are known to trigger nuclear accumulation of the tumor suppressor protein p53 resulting in apoptotic cell death. We examined whether p53 is involved in apoptotic cell death induced by CPA alone or by CPA and DOX together.

The MCF-7 cells, which contain functional wild type p53, were co-transfected with a reporter construct containing p53 response element (PG13CAT) along with a control β-galactosidase reporter plasmid (pCH110). As shown in Fig. 5A and 5B, p53 promoter is activated at least by 2 fold (P < 0.05) in the cells treated with CPA compared to untreated cells.
Addition of DOX further enhanced the CPA mediated increase in p53 reporter activity to 3 fold (P < 0.05). Thus DOX addition enhanced the CPA induced p53 reporter activity by at least 1.5 fold (Fig. 5B: inset). We also tested whether CPA or CPA plus DOX treatment activates expression of β-galactosidase gene under the control of cytomegalovirus immediate early promoter from a control co-transfected plasmid. As shown in Fig. 5B, no significant difference in β-galactosidase activity was detected in cellular extracts prepared from drugs treated and untreated cells.

Since CPA activated p53 reporter and CPA plus DOX combination further enhanced this activity, we investigated whether this could be ascribed to stabilization of p53 protein level alone or its transcript level as well. Therefore, RT-PCR analysis was performed on the RNA isolated from untreated cells, and either treated with DOX, CPA or with both together. As seen in Fig. 6A, p53 mRNA level was about 1.2 and 1.4 fold higher in cells treated with CPA and CPA plus DOX.
combination respectively compared to p53 mRNA level of untreated or DOX treated cells. Moreover, the increase in p53 transcript level also led to increase in p53 protein by 1.6 and 2.4 fold in cells treated with CPA and CPA plus DOX combination respectively (Fig. 6B). It is therefore likely that increase in p53 promoter activity is indeed due to increase in p53 transcript level and thereby stabilized protein level. No change in p21 transcript level was detected.

Fig. 6. Using total RNA isolated from MCF-7 cells treated with either carrier, DOX, CPA and CPA plus DOX, RT-PCR was performed with specific gene primers (A). MCF-7 cell lysates were electrophoresed, transferred, and immunoblotted with anti-human p53 antibody. Same blot was stripped and reprobed with anti human β-actin antibody as an internal control (B). Expression levels were measured by phosphorimager densitometry scanner analysis (Bio-rad, USA). Relative levels values are normalized with β-actin. Independent experiments were repeated at least three times.

p53 can up regulate Bax, p21 and down regulate Bcl-2. (EL-Deiry et al., 1993; Miyashita and Reed, 1995; Friedlander et al., 1996; Ludwig et al., 1996; Thomborrow and Manfredi, 1999), therefore we investigated the effect of CPA and CPA plus DOX on constitutive levels of Bax and Bcl-2 by RT-PCR and western blot analysis. Bax transcript levels increased 1.3 and 1.5 folds (Fig. 7A), whereas the protein increased by 1.45 and 1.75 fold (Fig. 7B), in CPA and CPA plus DOX treated cells compared to untreated control cells. No change in Bel-2 transcript level was detected.
Fig. 7. Bax up-regulation is involved in drugs mediated effect on MCF-7 cells in vitro. Using total RNA isolated from MCF-7 cells treated with either carrier, DOX, CPA and CPA plus DOX, RT-PCR was performed with specific gene primers for Bax and Bcl-2 and β-actin (A). MCF-7 cell lysates were electrophoresed, transferred, and immunoblotted with anti-human Bax antibody. Same blot was stripped and reprobed with anti-human β-actin antibody as an internal control (B). Expression levels were measured by phosphorimager densitometry scanner analysis (Bio-rad, USA). Relative levels values are normalized with β-actin. Independent experiments were repeated at least three times.

4.3.5 Enhanced cell death in CPA treated MCF-7 cells is DOX dose dependent

To further examine whether DOX enhances CPA mediated cell killing in a dose dependent manner, MCF-7 cells were treated with CPA alone or in presence of DOX (0.3 to 6 μg/ml) for 48 h and percent PI stained cells were analysed by flowcytometry as described in materials and methods. DOX at a concentration of 0.3 μg/ml did not further enhance the CPA mediated cell killing. The percent cell killing by DOX (0.3 μg/ml) and CPA combination was 47.5±4 compared to 46.75±3 for CPA alone. Increase in DOX concentration to 1, 3 and 6 mg/ml, lead to enhancement of CPA mediated cell killing approximately by 12, 23 and 27% respectively (Fig. 8).
Fig. 8. Enhanced cell death in CPA treated MCF-7 cells is DOX dose dependent. After dose dependent treatment of MCF-7 cells with DOX along with CPA (10 mM) for 48 h, cells were stained with 50 μg/ml PI and analyzed in a flow cytometer for total PI positive cells with acquisition of 10,000 cells (n=4). The representative % cell death is indicated by bar graph.

These results indicate that DOX in a dose dependent manner enhances CPA mediated killing of MCF-7 cells with maximum death being achieved with DOX concentration being 3 to 6 μg/ml. Under identical experimental conditions (see Fig. 3), DOX alone did not induce MCF-7 cell killing.

4.3.6 Downregulation of p53 prevents DOX enhanced MCF-7 cell killing by CPA

To examine DOX enhanced MCF-7 cell killing by CPA is indeed p53 dependent, we evaluated whether cell death induced by CPA and DOX together involved p53. MCF-7 cells were transiently transfected with either pTet-On or pTRErevp53 plasmids alone and also cells were transiently co-transfected with pTet-On and pTRErevp53 plasmids together. The transiently transfected cells were treated with combination of CPA and DOX for 48 h. Subsequently cells were stained with PI and analyzed for cell death. As shown in Fig. 9, DOX at 0.3 μg/ml did not affect cell killing by CPA. Increase in DOX to 3 μg/ml enhanced CPA mediated cell killing. Moreover transfection with pTet-On or pTRErevp53 plasmids did not in anyway alter the effect of drugs on MCF-7 cells.
MCF-7 cells in which pTet-On and pTREarcvp53 plasmids were co-transfected together, DOX addition failed to enhance CPA mediated cell killing. Cell death in these cells was comparable to CPA alone treated MCF-7 cells. These results suggest that p53 is involved in enhanced cell killing effect of CPA and DOX combination. To further verify the status of p53 protein in the cells which were transiently transfected and subsequently treated with the drugs, whole cell lysate from these cells were analyzed by western blot analysis for p53 expression. As shown in Fig. 9, p53 protein levels are significantly higher in CPA and DOX (3 μg/ml) treated MCF-7 cells compared to the cells which were treated with CPA and low non-effective dose of DOX (0.3 μg/ml). p53 protein levels were low in the cells in which antisense p53 was transiently expressed and subsequently exposed to CPA and DOX (3 μg/ml) together. As a control for western blot analysis same blot was stripped and reprobed for expression of actin and no significant difference were detected. Therefore, the p53 protein level in cells treated with drugs combination is reduced as a consequence of expression of antisense p53. These results suggest that downregulation of p53 protein partially rescues DOX enhanced MCF-7 cell killing by CPA.
4.3.7 Upregulation of Bcl-2 prevents DOX enhanced MCF-7 cell killing by CPA

Concurrent observation for the dramatic reduction of Bax to Bel-2 ratio in combination treatment causing cell death led to in vitro evaluation for role of Bcl-2 at molecular level in MCF-7 cell survival. To examine DOX enhanced MCF-7 cell killing by CPA is indeed Bax to Bcl-2 ratio dependent, we transiently transfected human Bcl-2 expression vector or control EGFPNI vector in the cells.

![Figure 10](image)

*Fig. 10. Transient over expression of Bcl-2 rescues MCF-7 cells from the toxicity induced by combination of the drugs. Bcl-2 was over expressed in MCF-7 cells before treating them with either CPA or DOX or with combination of both the drugs. Subsequently MTT assay was performed to evaluate cell survival, as described in materials and method.*

The transiently transfected cells were treated with combination of CPA and DOX for 48 h. As shown in Fig. 10, DOX at 3 μg/ml enhances cell killing by CPA. Moreover transfection with control vector did not alter the effect of drugs on MCF-7 cells. However, MCF-7 cells in which human Bcl-2 was overexpressed DOX addition failed to enhance CPA mediated cell killing. Cell death in these cells was comparable to CPA alone treated MCF-7 cells. These results suggest that Bcl-2 is involved in enhanced cell killing effect of CPA and DOX combination.
4.3.8 Enhanced reactive oxygen species (ROS) generation in CPA treated cells after DOX addition

The analysis for generation of reactive oxygen species after treatment with DOX and anticancer CPA has elucidated another point of synergy between these drugs which may be partly responsible for inducing cytotoxicity in MCF-7 cells.

Fig. 11. Intracellular DCF fluorescence as an index to quantify overall oxidative stress in cells. MCF-7 cells were untreated (A), treated with DOX (B), treated with 10 mM CPA (C) and treated with combination of CPA (10 mM) and 3 µg/ml DOX (D) for 24 h. Subsequently cells were labelled with DCF-DA to detect total intracellular ROS levels as described in Materials and Methods. Cells were photomicrographed by using confocal microscopy after DCF-DA treatment.

The comparisons for ROS generation after 24h treatment with either DOX or CPA alone, or with the combination of both revealed that the treatment with CPA alone induces ROS production in the cells but addition of DOX doubles the fold of this ROS generation as depicted in corresponding increase in DCF fluorescence intensity in Fig.11 and as illustrated by 2.5D histograms in Fig. 12.
ROS level in DOX treated cells were taken as one fold in the comparisons. Our data indicated the accumulation of ROS as supplementary to death-inducing increase in Bax protein levels and may be involved enhanced cell killing because the influence of induced reactive oxygen species in p53-mediated cell fate decisions has been well documented (Johnson et al., 1996).

4.3.9 Effect of doxycycline on the antitumor activity of cyclophosphamide

The effect of DOX on the antitumor activity of cyclophosphamide was evaluated by injecting breast cancer MCF-7 cells in athymic mice. Figure 13 shows experimental design as executed to generate MCF-7 xenograft tumor in nude mice. CPA, administered at 100 mg/kg/day ip dose in nude mice bearing tumor derived by injecting MCF-7 cells, resulted in decreased tumor size by fifty percent compared to tumors in untreated animals (Fig. 14).
Fig. 13. Experimental design and reference time frame. Human breast cancer cells (MCF-7) were implanted in athymic mice.

Fig. 14. Tumor growth regression activity of cyclophosphamide is potentiated by doxycycline in MCF-7 xenograft tumors. Tumors were surgically excised from the mice injected with carrier, CPA, and CPA plus DOX (A). The tumors excised from the mice were weighed. Values are expressed as means (standard deviation, n=6). Values between tumors from CPA and CPA plus DOX injected animals (B) are significantly different (P < 0.01).

DOX injection alone did not significantly induce tumor regression. The co-administration of DOX (5 mg/kg/day) along with CPA further enhanced the tumor regression by more than 25% (Figs. 14 A and B). Therefore it is clear that DOX, one
of the better-absorbed antibiotics with longer half-life can potentiate the antitumor activity of CPA in vivo.

To ascertain genes that may be involved in enhanced tumor regression following CPA and CPA plus DOX treatment. RT-PCR assay was carried out to monitor expression pattern of major cell growth regulatory genes. CPA treatment leads to increase in pro-apoptotic Bax by 1.2 fold that was further enhanced to 1.4 fold by DOX treatment. Concurrently the level of antiapoptotic gene Bcl-2 decreased significantly in CPA and CPA plus DOX treated tumors (Fig. 15). Moreover DOX treatment also enhances p53 transcript level without alteration in p21 gene transcript. These results suggest that slight but reproducible enhancement of p53 and Bax transcript levels may be responsible for DOX mediated potentiation of CPA induced tumor regression. Interestingly, drug treatments down regulated Bcl-2 gene in vivo, whereas no significant changes in Bcl-2 are detected in vitro (Fig 7).

Fig. 15. RT-PCR was performed for p53, p21, Bax and Bcl-2 with specific gene primers listed in materials and methods using total RNA isolated from treated and untreated MCF-7 xenograft tumors. Expression of β-actin mRNA was used as control for RNA integrity and normalization. Expression levels were measured by phosphorimager densitometry scanner analysis (Bio-rad, USA) and relative levels are given.
Fig. 16. Body weight profiles for the drug treated animals. In the tumorigenicity assay where tumor growth regression activity of cyclophosphamide is potentiated by doxycycline on human breast cancer cells (MCF-7) implanted in athymic mice, the toxicity on the host animals by additional doxycycline on cyclophosphamide dose was assessed by the change in animal weight over 21 days of the duration of the experiment before tumors were surgically removed. Groups receiving CPA and combination were weighed weekly. The weight of mice at the time of initial drug treatments (14th day) is 22 ± 1 g, which corresponds to normal adult weight of this mice strain.

The analyses of body weight changes (Fig.16) indicated that the treatment with CPA alone increases host toxicity as illustrated by body weight profiles and as reported in earlier studies (Jounaidi and Waxman, 2000). The host toxicity though was not further increased when CPA treatment was accompanied with DOX treatment. Hence, the potentiation of tumor regression activity of CPA by DOX is not on the cost of toxicity on host which is not enhanced further in the combination treatment.
4.4 DISCUSSION

In the present study, the effects of antimicrobial agent doxycycline (DOX) on cyclophosphamide (CPA) mediated MCF-7 breast carcinoma cell death, both in vivo and in vitro, were investigated. The results indicate that DOX potentiates in vivo tumor regression activity and also enhances in vitro cell killing of MCF-7 by CPA. In all the aspects studied, DOX enhanced CPA cytotoxic activity both in vivo and in vitro, appears identically regulated except for comparatively lowered Bcl-2 levels in nude mice model which could be due to additional effect of bioactivation of CPA by liver specific enzymes.

This is the first report demonstrating the potentiation of antitumor activity of CPA on solid tumor cells by a non-anticancer drug and one of the few on drugs combined with high dose regimen CPA to increase its therapeutic index. DOX, a water-soluble analogue of tetracycline, is used for treating a variety of infectious diseases in cancer patients (Moreno et al., 1994; Liston and Koehler, 1996). It is not only a well tolerated antibiotic but also has been exploited as inhibitor of proliferation and inducer of apoptosis and cell cycle arrest in cultured cells and as an antitumor agent in several tumor systems (van den Bogert et al., 1986; van den Bogert et al., 1988). DOX has been shown to inhibit the activity of 72- and 92-kDa type IV collagenase (MMPs) secreted by bone-metastasizing cells by 79-87%. Using tumor cell lines relevant to bone metastases, i.e. PC-3, MDA-MB-231, Hs696, B16/F1, it has been demonstrated that tetracycline and derivatives of tetracycline, namely DOX and minocycline, also induced cytotoxicity (Fife et al., 1998; Duivenvoorden et al., 2002). Moreover DOX treatment in an experimental bone metastasis mouse model of human breast cancer MDA-MB-231 cells resulted in a 70% reduction in total tumor burden compared with placebo control animals (Duivenvoorden et al., 2002), and it is also being investigated as an antiangiogenic agent for treatment of cancer (Guerin et al., 1992; Gilbertson-Beadling et al., 1996).

Therefore DOX could be of potential use in cancer therapeutics both alone and in combination with other anticancer drugs due to its relative nontoxicity to the host.
CPA is being utilized in treating breast cancers and the response of MCF-7 cell line, to CPA has been investigated earlier. Our results are consistent with the findings that CPA, even in its so-called 'bio-inert' form inhibits MCF-7 cell growth and the cytotoxic effect of CPA was lost by reducing its concentration to approximately $1 \times 10^{-3}$ M (Leung et al., 2000). Since very high dose regime of CPA is administered in cancer patients, its therapeutic effectiveness is potentially limited by the hematopoietic, renal, and cardiac toxicity that accompanies the systemic distribution of liver-derived activated drug metabolites (Chen et al., 1996; Schwartz and Waxman, 2000). Also, with proved carcinogenic and teratogenic side effects (Cohen et al., 1992), it has become applicable to target combination therapy to lower the dose of such drugs. Potentiation of antitumor and cytotoxic effect of CPA by addition of thalidomide, DOX and bis-indole alkaloid has been reported. Thalidomide and DOX have been shown to potentiate antitumor effect of CPA in murine tumor models using Colon 38 tumor cells and L 1210 leukemia cells (Pakulska 1992; Ding et al., 2002). In addition Yuehchukene, a bis-indole alkaloid is reported to potentiate the cytotoxic effect of CPA on MCF-7 cells (Leung et al., 2000). Our observation that DOX can potentiate CPA induced regression of solid tumor cells in mice further supports the usefulness of DOX as a potential candidate to be used in combination chemotherapeutic for cancer treatment.

An increasing number of genes that are involved in the evolutionary pathway for physiological cell death and its morphological counterpart, apoptosis, have been discovered. A number of these genes belong to Bcl-2 family. This family constitutes one of the most biologically relevant classes of apoptosis regulators acting at the effector stage (Kroemer, 1997) of apoptosis with some members functioning as suppressors of apoptosis and others as promoters of cell death. The ultimate vulnerability of cells to diverse apoptotic stimuli is determined by the relative ratio of various proapoptotic and antiapoptotic members of the Bcl-2 family (Oltvai et al., 1993; Yang and Korsmeyer, 1996). An oncogene-derived protein, Bcl-2, confers negative control in the pathway of cellular suicide machinery. A Bcl-2-homologous protein, Bax, promotes cell death by competing with Bcl-2. while Bax-Bax homodimers act as apoptosis inducers, Bax- Bcl-2 heterodimers formation evokes the survival signal for cells. Both these are transcriptional targets of tumor
suppressor p53. In all, a coordinate performance of these molecules is crucial for controlling life and death of a cell (Basu and Haldar, 1998). Our results indicate enhanced up-regulation of p53 and p53-regulated proapoptotic Bax upon treatment of MCF-7 cells with a combination of DOX and CPA in vitro and in vivo. Up-regulation of p53 and Bax correlated with tumor regression. Additionally, down-regulation of antiapoptotic Bcl-2 was observed in tumors derived from animals treated CPA and CPA plus DOX compared to untreated and DOX treated animals.

Stable transfection of Bax into the breast cancer cell line MCF-7 did not affect cell viability on its own but has been demonstrated to increase MCF-7 sensitivity to variety apoptosis inducing stimuli including CPA (Wagener et al., 1996). DOX enhanced cytotoxic activity of CPA in vitro results in increase in cellular population undergoing apoptosis. Also DOX in a dose dependent manner increases CPA mediated MCF-7 cell death. Activation of p53 promoter and increase in p53 protein following DOX plus CPA treatment together suggests the involvement of p53. Moreover expression of antisense p53 in MCF-7 cells also prevents DOX enhanced CPA mediated cell death indicating involvement of p53. Furthermore increase in mRNA as well as protein levels of pro-apoptotic Bax in these cells correlates with increased Bax mRNA detected in tumors of animals treated with DOX and CPA together. Therefore, enhancement of cytotoxic potential of CPA by DOX is due to increased p53 and Bax protein levels resulting in tumor regression by CPA in presence of DOX in vivo. This study also highlights relevance of p21 (WAF1/CIP1)-independent apoptosis and significance of alterations in Bax/Bcl-2 ratio favoring apoptosis and antitumor effect both in vitro as well as in vivo (Huang, et al., 2003). Though there was no significant decrease in Bcl-2 message in vitro following the combination therapy which could be explained by high stability of Bcl-2 protein in MCF-7 cells (Ogretmen and Safa, 1996). Our results are consistent with those reported for combination of chemotherapeutic agents with CPA against breast cancer cells (Priery et al., 2000). It is noteworthy that an inverse relationship exists between p53 and Bcl-2 in breast tumors and ability of p53 to downregulate Bcl-2 was noted in a panel of breast cancer cell lines (Leek et al., 1994; Haldar et al., 1994). We also observed that overexpression of Bcl-2 can block p53 dependent apoptosis (Basu and Haldar, 1998).
In complimentary experiments, we also investigated whether ROS generation is involved in this anticancer treatment regimen as free radicals are dangerous by-products of cellular metabolism of drugs that have direct effect on cell growth and development, cell survival, and have a significant role the pathogenesis of atherosclerosis, cancer, aging and several inflammatory diseases (Halliwell, 2000). In case of cancers, the higher the malignancy of tumor cells, lower the rate of lipid peroxidation and higher the degree of susceptibility to free radical-induced toxicity as free radicals can cause cell death by apoptosis (Das, 2002). Moreover, there are several reports suggesting that free radicals are powerful inducers of p53 activity and that they play a role in the execution of p53-dependent apoptosis (Martinez et al., 1997). Nonetheless, there are investigations reporting that ROS is one of the downstream mediators of p53-dependent apoptosis (Johnson et al., 1996). Inhibition of mitochondrial protein synthesis by doxycycline (DOX), a tetracycline analogue, has significant antitumor effects in several tumor systems. The tetracyclines specifically inhibit mitochondrial protein synthesis when present at the same low concentrations as used for their antibacterial action. Inhibition of mitochondrial protein synthesis leads to decrease in the oxidative energy-generating capacity of cells with generation of increased level of ROS in the cells. Additionally, it has been reported that CPA-induced genotoxicity is also due to the generation of reactive oxygen molecules during its bioactivation (Stankiewicz et al., 2002). These reports made us examine whether this mode of ROS generation is detectable and its additive for the enhanced cytotoxic effect in this combination of drugs. It has been shown that anticancer drug treatment increases intracellular ROS levels as reported earlier where curcumin has been used as an scavenger of ROS and affected therapeutic potential of CPA in vivo (Davis, Jr. et al., 2001; Somasundaram et al., 2002). We report that addition of DOX enhances this ROS generation further in the cells under CPA treatment. This illustrated that there is inherent role of ROS which therapeutically potentiate the toxicity of CPA by DOX in MCF-7 breast cancer cells.

These observations suggest that combination chemotherapy may derive its efficacy partly, through coordinated regulation of specific gene products associated with apoptosis. Moreover, characterization of other molecular events that underlie susceptibility of specific tumor cells to combination chemotherapeutic regimens
such as ROS generation may lead to additional improvements in treatment strategies for cancers.
4.5 References


