UNDERSTANDING THE MECHANISM OF DOXORUBICIN MEDIATED APOPTOSIS AND CHEMORESISTANCE

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Dedicated to

My Teachers and Parents

DECLARATION

I hereby declare that the work towards this thesis entitled "***Understanding the mechanism of Doxorubicin mediated apoptosis and chemoresistance***" has been carried out by me under the supervision of Dr. Sunil Kumar Manna, at the Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad. The work is original and has not been submitted in part or full for any other degree or diploma of any other Institute or University, earlier.

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CERTIFICATE

This is to certify that this thesis entitled ***"Understanding the mechanism of Doxorubicin mediated apoptosis and chemoresistance"***, submitted by **Ms. Charitha Gangadharan**, for the degree of Doctor of Philosophy to the Manipal University, and is based on the work carried out by her at the Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad. This work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution.

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ABBREVIATIONS

Abbreviations for Standard units (SI) of measurements are not included in the list below

ALLN	N-acetyl leucyl leucyl norleucinal
AP-1	activator protein-1
BAY 11-7082	3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile
BSA	bovine serum albumin
CD95/Fas	receptor for Fas ligand
CE	cytoplasmic extract
Cox2	cyclooxygenase 2
CXCR	chemokine receptor
DAPI	4'-6-diamidino-2-phenylindole
Dox	doxorubicin
DTT	dithiothreitol
E-64	L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol tetraacetic acid
EMSA	electrophoretic mobility shift assay
FasL	Fas ligand
FBS	fetal bovine serum
FKHR	forkhead transcription factor
GSH	glutathione
H-7	1-(5-Isoquinolinesulfonyl)-2-methylpiperazine
H-8	(methylamino)ethyl-5-isoquinolinesulfonamide
IAP	inhibitor of apoptosis protein
ICAM	intracellular adhesion molecule
IKK	IκBα kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IκBα	inhibitory subunit of kappa B
IκBα-DN	IκBα dominant negative

JNK	c-Jun –N terminal kinase
Kemptide	Leu-Arg-Arg-Ala-Ser-Leu-Gly
Mn-SOD	manganese superoxide dismutase
MSK	mitogen activated stress kinase
MTT	3-(4,5-Dimethyl-2-thiozolyl)-2,5-diphenyl-2H-tetrazolium bromide
NE	nuclear extract
NFAT	nuclear transcription factor of activated T-cells
NF-κB	nuclear transcription factor kappa B
NGF	nerve growth factor
NIK	NF-κB-inducing kinase
P_3-25	5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-Oxo-1,2,4-thiadiazolidine
PARP	poly(ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cells
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear neutrophils
PMSF	phenyl methyl sulphonyl fluoride
Rb	retinoblastoma
ROI	reactive oxygen intermediate
Rp-cAMPs	adenosine cyclic 3’, 5’-phosphorothioate tri ethyl ammonium salt
SA-LPS	serum activated lipopolysaccharide
SA-LPS/Jkt	serum activated LPS stimulated Jurkat cells
SEAP	secretory alkaline phosphatases
TBA	thiobarbituric acid
TNF	tumor necrosis factor
TRADD	TNF receptor associated death domain
TRAF 6	TNF receptor associated factor
TRAF6-BP	TRAF6 binding peptide
z-VAD-fmk	z-Val-Ala-Asp fluoromethyl ketone
γ-GCS	gamma glutamyl cysteinyl synthetase

PREFACE

The doxorubicin, an anthracycline is a major anti-tumor agent used in the treatment of a variety of cancers. Its intracellular effects include free radical formation, inhibition of DNA Topoisomerase II, DNA intercalation, resulting in inhibition of DNA replication and strand break related DNA damage, accumulation of iron in ferritin, and inhibition of its redistribution to other cellular compartments. Evidences demonstrate that cell response to doxorubicin is highly regulated by multiple signaling events, including generation of spingosine and activation of different caspases, c-Jun N-terminal Kinase (JNK), transcription factors like nuclear factor-kappaB (NF-κB), and Fas/Fas ligand system. Thus, doxorubicin mediated signaling events result in programmed cell death (apoptosis).

Attaining resistance to chemotherapy by several tumors is a common clinical problem in human cancer that may develop mostly by unknown mechanism. The development of cellular resistance mechanism to doxorubicin includes P-glycoprotein and Bcl-2 overexpression, altered topoisomerase II activity, loss of p53 function etc.

Chemotherapy has remained as one of the mainstays for tumor intervention. However, the development of resistance to certain chemotherapeutics by various forms of tumors has instigated the search for alternative agents that effectively kill cancer cells. The possible strategy to counter resistance would be the use of information regarding the metabolic pathway(s) mediated by a drug(s) in particular tumor type while selecting the chemotherapeutic regime. Hence, a thorough understanding of the molecular pathway(s) of apoptosis mediated by a particular chemotherapeutic agent is essential.

The use of anthracyclines has been reported to have beneficial effects in cancer patients. Also pre-clinical studies have demonstrated that doxorubicin has excellent activity against a variety of human tumor cell lines. However, a thorough understanding

of the antineoplastic activities of doxorubicin has not been addressed. Hence, the study to decipher the signal transduction pathway(s) mediated by doxorubicin in relation to its anti tumor activities is interesting. Also a comparative study on doxorubicin sensitive and resistant tumor will help to design combination therapy that increases the cytotoxic effects of doxorubicin and decrease the resistance of tumor.

Work done during my Ph. D. is divided into five chapters. I wish to present here a brief summary of the chapter wise contents of my thesis. **Chapter-I** reviews the current literature and attempts to present a comprehensive understanding of cancer and the signal transduction pathways mediated in cancer cells leading to tumorigenesis. Various topics concerning the role of certain transcription factors and the means of drug resistance in cancer are covered in this chapter. Moreover, notes on the combination therapy and the need to decipher signaling pathway(s) mediated by chemotherapeutic agents for countering the resistance phenomenon are included in this chapter. **Chapter-II** gives a detailed account of the materials and experimental procedures adopted for the various investigations carried out as documented in various chapters.

The transcription factor NF-κB mediates a major influence in tumorigenesis by giving a strong proliferative drive in tumor cells and also aiding them to abate apoptosis. Further NF-κB has been implicated in the development of drug resistance in cancer cells. **Chapter-III** describes the potential involvement of NF-κB in doxorubicin mediated apoptosis. We systematically examined the baseline levels of NF-κB activity in response to a challenge with doxorubicin. We demonstrate here that doxorubicin has a dual role in controlling the activity of NF-κB – an immediate activation, followed by a decreased DNA binding activity resulting from decreased amount of RelA/p65 subunit possibly by inducing the activity of proteasome, but not proteases. A subsequent induction of NF-κB was also observed through interleukin 8 (IL-8) that is expressed upon doxorubicin treatment. Increased amount of IL-8 induced apoptosis via increase in intracellular Ca^{2+}, activation of calcineurin, nuclear translocation of nuclear factor

activated T cell (NF-AT), NF-AT-dependent FasL expression and subsequent activation of caspases.

Conventional cytotoxic drugs frequently fail as a treatment modality due to the development of drug resistance that blocks their activity. We did a comparative study of doxorubicin resistant and sensitive cell line and found that doxorubicin-resistant cells show high basal level of NF-κB activity and bypasses apoptosis. This high NF-κB activity confers resistance to doxorubicin in doxorubicin-resistant cells and down regulation of NF-κB in these cells potentiated apoptosis. Hence **Chapter-IV** describes the role of NF-κB in chemoresistance and establishes NF-κB inhibition may have implication for refining systemic chemotherapy in future.

The RelA (p65) subunit of NF-κB actively participates in expression of NF-κB -dependent genes involved in inflammation and tumorigenesis. Hence, we hypothesized that-regulation of p65 subunit by a chemically synthesized dichlorophenyl derivative of 1,2,4-thiadiazolidine (known as P_3-25) should control those responses. We show that P_3-25 inhibits protein kinase A (PKA) activity, leading to decreased phosphorylation of p65. The resulting reduction in transcriptional activity of NF-κB leads to decreased antiapoptotic proteins that culminate in apoptosis in Dox-resistant cells and NF-κB-expressing cells. **Chapter-V** describes the role of P_3-25 as a potential chemotherapeutic agent to combat doxorubicin resistance by blocking NF-κB activation.

Over all the present study help to understand the role of various signaling pathways mediated by doxorubicin to counter or/and sensitize the survival of a cancer cell. This study may further help to design combination chemotherapy in intervene several tumors involving doxorubicin.

Chapter-1

Introduction and Review of Literature

A major feature of all higher eukaryotes is the defined life span of an organism, a property that extends to the individual somatic cells, whose growth and division are highly regulated. The nature of cells to divide and increase their number is called mitosis. Normally, cells grow and divide to form new cells to replace dead or worn-out cells. This orderly process is needed for repair of damaged tissues and growth of organs or the entire individual. An exception is provided by cancer cells, which arises as variants that have lost their usual growth control. Their ability to grow in inappropriate locations or to propagate indefinitely may be lethal for the individual organism in which they occur. Hence cancer is an abnormal, continuous multiplying of cells. The cells divide uncontrollably and may grow into adjacent tissue or spread to distant parts of the body called metastatic cancer. The mass of cancer cells eventually become large enough to produce lumps, masses, or tumors that can be detected, which can be benign or malignant. The smallest cancer that can be detected by examination, x-ray, or scan is slightly less than one-fourth of an inch in diameter and contains between a million to a billion cancer cells. Cancer is caused by both external factors (tobacco, infectious organisms, chemicals and radiation) and internal factors (inherited mutations, hormones, immune conditions and mutations that occur from metabolism). The causal factors may act together or in sequence to initiate or promote carcinogenesis. Cancer is treated with surgery, radiation, chemotherapy, hormone therapy, biological therapy and targeted therapy.

Cancer is a leading cause of death worldwide. The most frequent types of cancer differ between men and women. The disease accounted for 7.9 million deaths (or around 13% of all deaths worldwide) in 2007. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030. Overall cancer incidence rates decreased in the most recent time period in both men (1.8% per year from 2001 to 2005) and women (0.6% per year from 1998 to 2005), largely because of decreases in the three major cancer sites in men [lung, prostate, and colon and rectum (colorectum)] and in two major cancer sites in women (breast and

colorectum). Although progress has been made in reducing incidence and mortality rates and improving survival, cancer still accounts for more deaths than heart disease in persons younger than 85 years of age. Despite the good news that cancer incidence and death rates for men and women continue to decline, cancer is projected to become the leading cause of death worldwide in the year 2012, and low- and middle-income countries will feel the impact of higher cancer incidence and death rates more sharply than industrialized countries. Breast cancer incidences in India are also on rise: it is reported that one in 22 women in India is likely to suffer from breast cancer during her lifetime, while the figure is definitely more in America with one in eight being a victim of this deadly cancer. Studies indicate that India will face a potential breast cancer epidemic over the next decade because of changing lifestyles. A study conducted by the International Association of Cancer Research, based in Lyon, France, projected that there would be 250 000 cases of breast cancer in India by 2015. At least 30% of the estimated 10 million cancer cases diagnosed each year can be prevented. 30% of all cancer cases can be cured if diagnosed early. Between 25% and 30% of all cancer deaths are attributed to tobacco use. Halving tobacco consumption now would avert 150 million premature deaths by 2050.

1.1 Understanding the pathways of cancer

The tumorigenic process that leads from a normal cell to cancer can be divided into three phases: tumor initiation, tumor promotion and tumor progression. During the first phase of tumorigenesis, tumor initiation, the DNA of the cell is mutated by chemical or physical carcinogens, leading to the activation of oncogenes and/or the inactivation of tumor-suppressor genes (most commonly those that encode KRAS and p53, respectively). The second phase of tumorigenesis, tumor promotion, is characterized by the clonal expansion of initiated cells, owing to increased cell proliferation and/ or reduced cell death. Inflammation seems to be an important tumor promoter and several cytokines — such as interleukin-1 (IL-1), IL-6 and tumor-necrosis

factor — can promote tumor growth. Finally, invasion and metastasis, as well as an increase in tumor size, are the characteristics of the third phase of tumorigenesis, tumor progression. During this stage, additional mutations can be acquired, and this leads to the cancer cell gaining a further growth advantage and having a more malignant phenotype. Hence cancer is associated with genetic alterations that often result in abnormal chromosome sets (aneuploidy) and changes in the distribution of chromatin inside the nucleus. The complexity presented by cancer research ponders over a question as to how many distinct regulatory circuits within each type of cell must be disrupted in order for such a cell to become cancerous. Also does the same set of disruptions dictate transformation through wide variety of tissue repertoire and finally which of these circuits operate on a cell autonomous basis and which are coupled to environmental signals that the cell receives (Hanahan & Weinberg, 2000)? Indeed the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in normal cell physiology, which together define the progression of most human malignancies.

1.1.1 Self-sufficiency in growth signals:

Normal cell proliferation depends upon the presence of growth factors produced outside the cell. However, one of the key characteristics of tumor cell is its capacity for proliferation without dependence on external growth factors. Tumor cells may proliferate by either internal production of growth factors or by responding to levels of external growth factors not usually sufficient to produce proliferation in normal cells. Three common molecular strategies to achieve autonomy are evident, through involvement of alteration of extra cellular growth signals, transcellular transduction of those signals, or intercellular circuits that translate that signal into action (Hanahan & Weinberg, 2000). Tumor cells produce growth factors, which are used to signal itself in the autocrine mode thereby obviating dependence on growth factors from other cells within the tissue. The production of PDGF (platelet derived growth factor α) by

glioblastomas and sarcomas, respectively are two examples (Fedi et. al., 1997). The high basal level gene expression of thymidine phosphorylase (platelet derived endothelial cell growth factor) in colorectal tumors is found to be associated with development of a more aggressive and malignant tumor phenotype with increased resistance to cytotoxic agents (Brody et. al., 2009). The *EGFR* gene amplification with EGFR protein overexpression was detected in approximately 6% of breast carcinomas and may be candidates for trials of EGFR-targeted antibodies or small inhibitory molecules (Bhargava et. al., 2005).

1.1.2 Insensitivity of anti-growth signals:

In normal tissue, the stability of the cell population is maintained by a host of signals and factors inhibiting cell proliferation and differentiation. The growth of neoplastic cells cannot be explained only by an enhanced positive growth potential but rather by the balance between positive and negative growth factors which result from enhanced or uncontrolled expression of genes related to the opposing positive and negative proliferation signals in the process of neoplastic transformation. For cancer cells to survive and replicate these anti-growth signals must be avoided. Evidences suggest that there is progressive loss of sensitivity to endothelium-derived growth inhibitors expressed by human melanoma cells during tumor progression. Human melanoma development appears to be associated with progressive loss of sensitivity to growth inhibitory effects of IL-6 and other factors produced by endothelial cells (Rak et. al., 1994). Several cancer genes directly control transitions from a resting stage (G0 or G1) to a replicating phase (S) of the cell cycle (Sherr, 2000). The products of these genes include proteins as diverse as cdk4 (a kinase), cyclin D1 (which interacts with and activates cdk4), (Ortega et. al., 2002), retinoblastoma (Rb), a transcription factor, and p16 (which interacts with and inhibits cdk4). The genes encoding Rb and p16 are tumor-suppressor genes inactivated by mutation (Classon & Harlow, 2002), whereas those encoding cdk4 and cyclin D1 are oncogenes activated by mutation. Mutations or

down regulation of TGFRs also render the cancer cells to loss of sensitivity to the growth inhibitory signals.

1.1.3 Tissue invasion and metastasis:

Up to 90% cancer deaths are due to metastasis. The capability of invasion and metastasis enables cancer cells to escape the primary tumor mass and colonize new terrain in the body where nutrients and space are not limiting immediately. Several classes of proteins involved in the tethering of cells to their surroundings in a tissue are altered in cells possessing invasive or metastatic capabilities. The affected proteins include cell-cell adhesion molecules (CAMs) notably members of the immunoglobin and calcium dependent cadherin families, both of which link cells to extracellular matrix substrates. Carcinoma cells facilitate invasion by shifting their expression of integrins from those that favour the ECM present in normal epithelium to other integrins (eg. $\alpha 3\beta 1$ and $\alpha V\beta 3$) that preferentially bind the degraded stromal components produced by extracellular proteases (Varner & Cherish, 1996; Lukashev & Werb, 1998). In many types of carcinomas, matrix-degrading proteases are produced not by the epithelial cancer cells but rather by conscripted stromal and inflammatory cells (Vu & Werb, 2000). Once released by these cells, the carcinoma cells may wield them. Hence the activation of extra cellular proteases and the altered binding specificities of cadherins CAMs and integrins are clearly central to the acquisition of invasiveness and metastatic ability. The increased expression of matrix metalloproteases (MMP-2, MMP-9) and plasminogen fragmentation are associated with serious epithelial ovarian cancer progression. Proteases have been proposed to be involved in breast cancer metastasis (Murthy et. al., 2004). Several enzymes like type IV collagenases (MMP-2, MMP-9), interstitial collagenases (MMP-1) stromelysins (MMP-3, MMP-11), aspartate proteases (cathepsisn D), cysteine proteases (cathepsins B and L) are overexpressed or inappropriately regulated in breast cancer, but none has been proven to actually

mediate degradation of basement membrane or stromal matrix in the process of breast cancer metastasis in women with breast cancer (Dickson et. al., 1994).

1.1.4 Limitless potential for replication:

Cells have built-in check points that prevent oncogenes and tumorsuppressors from causing neoplasia. The senescence checkpoint is controlled by telomerase and the crisis checkpoint by pRb/p53. The maintenance of telomeric DNA underlies the ability of tumors to possess unlimited replicative potential, one of the hallmarks of cancer (Hanahan & Weinberg, 2000). Telomere length and structure are maintained by the reverse transcriptase telomerase and a multiprotein telomere complex termed shelterin. (Masutomi et. al., 2003). Telomerase activity is elevated in the vast majority of tumors, and telomeres are critically shortened in tumors versus normal tissues, thus providing a compelling rationale to target the telomerase/telomere pathway for broad-spectrum cancer therapy, most likely in combination with existing chemotherapeutic drugs. (Hahn et. al., 1999). Although there is relatively little to guide rational clinical combinations of anti telomerase/telomere therapeutics with other drugs at present (and although dose-limiting toxicities remain unknown), the association of telomerase/telomeres with DNA repair, particularly double-strand break repair pathways is suggestive of this new class of agent being usefully combined with cytotoxics that induce DNA damage (e.g., platins and topoisomerase inhibitors) or ionizing radiation (Kelland, 2007).

1.1.5 Sustained angiogenesis:

Angiogenesis, the growth of new blood vessels appears to be a midstage event in human cancer. Neo-vascularization is a pre-requisite to the rapid clonal expansion associated with macroscopic tumors. Tumor cells control angiogenesis regulators to their own ends. Counter balancing positive and negative signals encourage or block angiogenesis (Bergers et. al., 1999). The angiogenesis-initiating signals are exemplified

by vascular endothelial growth factors. Oncogenic ras and hypoxia induces VEGF, tumor cells expressing VEGF grow faster and their inhibitions by α-VEGF Abs inhibit tumor growth *in vivo* (Kim et. al., 1993). VEGF induces the expression of SDF-1 and synergises with bFGF for angiogenesis. A prototypical angiogenesis inhibitor is thrombospodin-1, which binds to CD36, a transmembrane receptor on endothelial cells coupled to intracellular Src-like tyrosine kinase suppresses angiogenisis (Bull et. al., 1994; Corey & Anderson, 1999). Thrombospondin-1 is regulated by p53: loss of p53 decreases thrombospondin-1 levels (Dameron et. al., 1994; Isenberg et. al., 2008). The chemokine Interleukin-8 (IL-8; CXCL8), a cytokine that is chemotactic for lymphocytes and neutrophils, is also angiogenic and exerts potent angiogenic properties on endothelial cells through interaction with its cognate receptors CXCR1 and CXCR2. Evidences suggest that IL-8 can directly enhance endothelial cell proliferation, survival, and MMP expression in CXCR1- and CXCR2-expressing endothelial cells and regulate angiogenesis. Malignant colonic epithelial cells overexpress IL-8, and CXCR2 blockade may be a novel target for anti-angiogenic therapy in colorectal adenocarcinoma (Li et. al., 2003).

1.1.6 Evading apoptosis:

In normal tissue, the stability of the cell population is maintained through a process of programmed cell death, or apoptosis, which is latent in virtually all cell types throughout the body. Broadly the molecular mechanism of apoptosis activation can be visualized as two pathways of caspase activation namely the death receptor or extrinsic pathway and the mitochondrial or intrinsic pathway. Both pathways branch into many pathways and converge on caspase activation, leading to DNA degradation and cell death. NF- κB has been shown to be constitutively activated in many types of cancer cells eg. Hodgkin lymphoma. There are several mechanisms by which NF-κB transcription factors are uncoupled from their normal modes of regulation, and these have been associated with cancer. The discovery of the Bcl-2 oncogene by its up

regulation via chromosomal transcription in follicular lymphoma (Korsmeyer, 1992; Huang et. al., 1996) and its recognition as having anti-apoptotic activity (Vaux et. al.,1988) opened up the investigation of apoptosis in cancer at the molecular level. Approximately in 50% of all cancers inactivation of the p53 tumor suppressor protein, leads to rapidly growing tumors containing low number of apoptotic cells (Symonds et. al., 1994; Brantley, 2000; Henriksson et. al., 2001; O'Shea et. al., 2004). The expression of soluble receptors that acts as decoys for death ligands interferes with death-receptor mediated apoptosis. Elevated expression of decoy receptors is seen in various tumors like colorectal and lung cancer (Pitti, 1998). The levels of ROS-scavenging enzymes such as SOD, glutathione peroxidase and peroxiredoxin have been shown to be significantly altered in malignant cells and in primary cancer tissues, suggesting aberrant regulation of redox homeostasis and stress adaptation in cancer cells. The increased intracellular antioxidant capacity, which can be conferred by GSH and thioredoxin as a result of redox adaptation, is a common phenomenon in tumour cells associated with resistance to many anticancer agents and radiation (Hu et. al., 2005; Murawaki et. al., 2008; Trachootham et. al., 2009).

1.2 Nuclear Transcription Factor κappaB

The eukaryotic transcription factor NF-κB was first identified by Sen and Baltimore (1986) 20 years ago as a protein bound to specific decameric DNA sequence (5'-GGGRNNYYCC-3', R: purine, N: any base, Y: pyrimidine) within the intronic enhancer of the immunoglobulin kappa light chain gene in mature B cells and plasma cells (Sen & Baltimore, 1986; Pasparakis et. al., 2006). Later it was shown to be expressed ubiquitously in the cytoplasm of all cell types, from Drosophila to man. It translocates to the nucleus only when activated, where it regulates the expression of over 200 immune, growth, and inflammation genes. The induction of NF-κB: DNA binding in response to exogenously applied stimuli is shown to be independent of *de novo* protein synthesis (Sen & Baltimore, 1986; Israel & Kroemer, 2006). The Rel/NF-κB transcription factor

family is composed of several structurally related proteins that exist in organisms ranging from insects to humans. The vertebrate family includes five cellular proteins: c-Rel, RelA, RelB, p50/p105, and p52/p100. These proteins can form homodimers or heterodimers that give diverse combinations of dimeric complexes, which inturn bind to DNA target sites known as κB sites, where they directly regulate gene expression. A commonly known NF-κB consists of a p50/RelA heterodimer (RelA is also referred to as p65). The different Rel/NF-κB proteins show a distinct ability to form dimers, distinct preferences for different κB sites, and distinct abilities to bind to inhibitory subunits known as IκBs (Ahn & Aggarwal, 2005). Thus, different Rel/NF-κB complexes can be induced in different cell types and, by means of distinct signals, interact in distinct ways with other transcription factors and regulatory proteins to regulate the expression of distinct gene sets.

1.2.1 Overview of the NF-κB pathway:

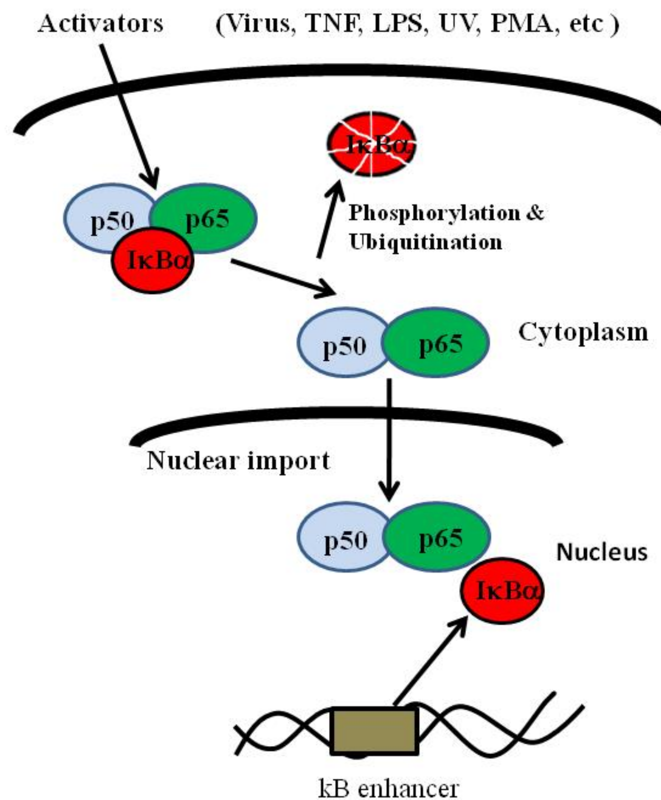
The five members of the mammalian NF-κB family, p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), exist in unstimulated cells as homo-or heterodimers bound to IκB family proteins. NF-κB proteins are characterized by the presence of a conserved 300-aminoacid Rel homology domain (RHD) that is located towards the N terminus of the protein and is responsible for dimerization, interaction with IκBs, and binding to DNA. Binding to IκB prevents the NF-κB:IκB complex from translocating to the nucleus, thereby maintaining NF-κB in an inactive state. NF-κB signaling is generally considered to occur through either the classical or alternative pathway (Bonizzi & Karin, 2004). In the classical pathway of NF-κB activation, for example, upon stimulation by the proinflammatory cytokine tumor necrosis factor α (TNF α), signaling pathways lead to activation of the β subunit of the IκB kinase (IKK) complex, which then phosphorylates IκB proteins on two N-terminal serine residues (IκBα on Ser32 and Ser36, and IκBβ on Ser19 and Ser23). In the alternative pathway,

IKKα is activated and phosphorylates p100. Phosphorylated IκBs are recognized by the ubiquitin ligase machinery, leading to their poly ubiquitination and subsequent degradation, or processing in the case of p100, by the proteasome (Karin & Ben-Neriah, 2000; Elia et. al., 2008). The freed NF-κB dimmers translocate to the nucleus, where they bind to specific sequences in the promoter or enhancer regions of target genes. Activated NF-κB can then be down-regulated through multiple mechanisms including the well characterized feedback pathway where by newly synthesized IκBα protein binds to nuclear NF-κB and exports it out to the cytosol.

There are seven IκB family members—IκBα, IκBβ, BCL-3, IκBε, IκBγ, and the precursor proteins p100 and p105—which are characterized by the presence of five to seven ankyrin repeats that assemble into elongated cylinders that bind the dimerization domain of NF-κB dimers (Hatada et. al., 1992; Jentsch, 2002). The crystallographic structures of IκBα and IκBβ bound to p65/p50 or p65/c-Rel dimers revealed that the IκB proteins mask only the nuclear localization sequence (NLS) of p65, whereas the NLS of p50 remains accessible (Haddad & Land, 2001). The presence of this accessible NLS on p50 coupled with nuclear export sequences (NES) that are present on IκBα and p65 results in constant shuttling of IκBα/NF-κB complexes between the nucleus and the cytoplasm, although the steady-state localization is in the cytosol (Kim et. al., 2006). The dynamic balance between cytosolic and nuclear localization is altered upon IκBα degradation, because it removes the contribution of the IκB NES and exposes the masked NLS of p65, resulting in predominantly nuclear localization of NF-κB.

NF-κB signaling proteins

Family/subfamily	Members	Domains
Rel	Rel A Rel B c-Rel	Rel Homology Domain, Trans Activation Doamin
NF-κB	p50/p105 p52/p100	Rel Homology domain, Ankyrin repeats
IκB	α,β,γ,ε,ζ,Bcl3	N-terminal 2 serine residues and ankyrin repeats



1.2.2 Signaling mechanisms of NF-κB that impact cancer development:

Cancer can be viewed as a chronic disease caused by defective genome surveillance and signal transduction mechanisms. If infection and inflammation enhance tumor development, they must do so through signal transduction mechanisms that impact those involved in either malignant conversion or cancer surveillance. The central role in inflammation and innate immunity is well known to be played by NF-κB transcription factor (Li & Verma, 2002). While needed for proper immune system function, inappropriate NF-κB activation plays a critical role in cancer development and progression and it may determine the response to therapy. NF-κB activation was shown to provide a critical mechanistic link between inflammation and cancer and is a major factor that controls the ability of both preneoplastic and malignant cells to resist apoptosis-based tumor surveillance mechanisms (Basseres & Baldwin, 2006).

NF-κB regulates numerous genes involved in cell survival, proliferation, angiogenesis, inflammation, invasion, and metastasis and play major role in each of the main phases of cancer development, which are known as initiation, promotion, and progression (Estrov et. al., 2003; Chen et. al., 2002). To quote a few examples, of the many gene targets NF- κB drives the expression of IL-2, granulocyte macrophage colony stimulating factor (GM-CSF) and CD40 ligand (CD40L) which encode growth factors that stimulate the proliferation of lymphoid and myeloid cells, (Karin, 2002), NF-κB site is present within the cyclin D1 promoter (Wang et. al., 1999b; Takebayashi et. al., 2003) and there is strong evidence that NF-κB dependent cyclin D1 is over-expressed in breast cancers. Leukemia and lymphoma-cancers of the bone marrow and lymph nodes respectively are caused by uncontrolled proliferation of blood cells. NF-κB is involved in the development of such cancers. Chromosomal rearrangements that affect the NF-κB locus at chromosomal region 10q24 have been associated with a variety of B and T cell lymphomas including chronic lymphocytic leukamia (CLL), multiple myelomas, T-cell lymphoma and cutaneous B and T cell lymphomas (Neri et. al., 1991; 1995; Migliazza et.

al., 1994). Numerous studies have documented elevated or constitutive NF-κB DNA binding activity both in mammary carcinoma cell lines and primary breast cancer cells of human and rodent origin (Sovak et. al., 1997; Cogswell et. al., 2000). Inflammation involves the interplay of several genes that include cytokines like TNF-α, IL-1, IL-6, IL-8, IL-12, and adhesion molecules like ICAM-1, VCAM and other factors like MMP-9, MIP-1α, growth factors. Many of these factors are also documented during tumor development, angiogenesis and metastasis. The production of these angiogenic factors is regulated by NF-κB activation (Meteoglu et. al., 2008). NF-κB mediates the upregulation of IL-8 and VEGF expression in bombesin-stimulated PC-3 cells (Zhang & Peng, 2007). NF-κB expression has been implicated in VEGF expression and the regulation of microvessel density in human colorectal cancer (Sakamoto et. al., 2009). Metastasis, mediated through the expression of various adhesion molecules, including ICAM-1, VCAM-1, and ELAM-1 (Nishikori, 2005), are in turn regulated by NF-κB. The inducible nitric oxide synthase (iNOS) has also been closely linked with metastatic ability of the tumor (Nishikori, 2005), and it too is regulated by NF-κB. Thus, inflammation and tumorigenesis seems to be closely linked, with NF-κB sandwiching them together.

Importantly, NF-κB and the signaling pathways that mediate its activation have become attractive targets for development of new chemopreventive and chemotherapeutic approaches. Most inflammatory agents mediate their effects through the activation of NF-κB and the latter is suppressed by anti-inflammatory agents. Similarly, most carcinogens and tumor promoters activate NF-κB, whereas chemopreventive agents suppress it, suggesting a strong linkage with cancer (Bharti & Aggarwal, 2002). Paradoxically most agents, including cytokines, chemotherapeutic agents, and radiation, that induce apoptosis also activate NF-κB (Beg & Baltimore, 1996), indicating that NF-κB is a part of the cells' autodefense mechanism and thus may mediate desensitization, chemoresistance, and radioresistance (Wang et. al., 1999a). Hence the promise of NF-κB as a therapeutic target can only be fulfilled through a better

understanding of the mechanistic differences that regulate specific NF-κB signaling pathways.

1.3 Anti cancer therapy

Cancer is deadly because it comes from within and evades the body's natural defenses. There are three primary ways of treating cancer at present. In the case of solid tumors, surgery can be used to cut out the cancerous tissue, while radiation therapy can kill the malignant cells, and chemotherapy stops them dividing. More than 100 drugs are used today for chemotherapy -- either alone or in combination with other drugs or treatments. Chemotherapy drugs can be divided into several groups based on factors such as how they work, their chemical structure, and their relationship to another drug. Knowing how the drug works is important in predicting side effects. *Alkylating agents* used in the treatment of different cancers (lymphoma, lung, breast, leukemia, and multiple myolema) directly damage the DNA and arrest the cancer cell in different phases of the cell cycle. *Antimetabolites* in treatment of leukemia, breast ovary cancers substitute for normal bases and hence interfere with DNA and RNA synthesis and damage the cells in S phase. *Topoisomerase inhibitors* kill cells by trapping topoisomerase enzyme intermediate termed the covalent complex, principle action by enzyme mediated DNA damage. Although most existing cancer drugs are anti-mitotic, they act not by targeting the specific lesions responsible for deregulated tumor growth, but by crudely interfering with the basic machinery of DNA synthesis and cell division.

1.3.1 Targeted cancer therapy:

In the recent years, the analysis of protein expression by cancer cells has led to the discovery of targets that are specific to tumors. Proteins have different functions in healthy cells, the same is true for neoplastic cells that depend on these molecules for proliferation and survival; in this case, these proteins are called oncoproteins. This new

generation cancer drugs interfere with a specific molecular target (typically a protein) that is believed to have a critical role in tumor growth or progression. The identification of appropriate targets is based on a detailed understanding of the molecular changes underlying cancer. This “Targeted approach” contrasts with the conventional, more empirical approach used to develop cytotoxic chemotherapeutics-the mainstay of cancer drug development in past decades (Sawyers, 2004).

Deregulated proliferation and inhibition of apoptosis drive the cancer cells to tumor and hence can also be the targets for therapeutic intervention. There are numerous mechanisms through which these two defects can occur, and the success of targeted therapy will depend to a large part on the molecular fingerprinting of individual tumors. Drugs designed to specifically inhibit growth-deregulating lesions include inhibitors of RTKs, Ras, downstream signaling kinases such as the mitogen-activate protein kinase and Akt pathway, and CDKs (Gibbs, 2000). By constitutive expression of death receptor ligands such as CD95L, tumors may adopt a killing mechanism from cytotoxic lymphocytes to delete the attacking anti-tumor T cells through the induction of apoptosis via CD95, CD95L interaction (Igney & Krammer, 2002). Among the earliest targeted therapies were antibodies directed against the cell surface markers cluster of differentiation 20 (CD20), CD33, and CD52, which are present on lymphoma and leukemia cells. Because CD20 is also present on normal lymphoid cells, targeting of this molecule affects over all immune function. This observation has led to the use of the anti-CD20 monoclonal antibody rituximab (Rituxan) for the treatment of autoimmune diseases such as rheumatoid arthritis (Silverman, 2007; Browning, 2006), in addition to non-Hodgkin’s lymphoma (Feugier et. al., 2005). The two main types of targeted therapy are monoclonal antibodies and small molecule inhibitors. The molecular pathways most often targeted in the treatment of solid tumors (e.g., breast, lung, and colorectal cancers) are those of the epidermal growth factor receptor (EGFR, also known as HER1), vascular endothelial

growth factor VEGF), and HER2/neu. Those proteins serve as bridge of information between the outside milieu and intracellular environment, fundamental for normal cellular growth and proliferation. When mutated, the genes responsible for coding of those receptors can produce permanently activated proteins (receptors), hypersensitive receptors, or cause protein over-expression ultimately causing carcinogenesis (Brennan et. al., 2000; Gazdar et. al., 2004; Krause et. al., 2005). Such pathways can be inhibited at multiple levels: by binding and neutralizing ligands (i. e. molecules that bind to specific receptor sites on cells); by occupying receptor binding sites (there by preventing ligand binding); by blocking receptor signaling within the cancer cells; or by interfering with downstream intracellular molecules. Trastuzumab, monoclonal antibody against EGFR2/ErbB2, has proven significant improvement in both adjuvant and metastatic breast cancer settings, and it is usually used in combination with taxanes (Slamon et. al., 2001; Hudis, 2007). Mammalian target of rapamycin is a kinase that when activated increases angiogenesis and cell proliferation and halts apoptosis (Easton et. al., 2006). Tyrosine kinase inhibitors, sorafinib and sunitinib, have changed the landscape and became standard of care for the past recent years for the first line treatment of metastatic clear cell carcinoma of the kidneys. Those are multi kinase inhibitors that block VEGFR2 and platelet-derived growth factor receptor (PDGFR) β. Ligands to these receptors are frequently upregulated in renal cell carcinoma promoting neo-angiogenesis and tumor growth (Easton et. al., 2006). Temsirolimus, an mTOR inhibitor, has also been recently tested in first-line metastatic renal cell carcinoma. A multitude of target drugs are available for clinical use with specificity to oncoproteins.

Table 1: **TARGETED THERAPY FOR PROVEN BENEFIT**

Trastuzumab/herceptin (monoclonal antibody)	Breast cancer
Bevacizumab/avastin (monoclonal antibody)	Breast cancer, colorectal cancer NSCLC
Cetuximab/erbitux (monoclonal antibody)	Colorectal cancer head and neck cancer
Panitumumab/vectibix (monoclonal antibody)	Colorectal cancer
Imatinib/gleevec (tyrosine kinase inhibitor)	Chronic myeloid leukemia, gastrointestinal stromal tumor
Erlotinib/tarceva (tyrosine kinase inhibitor)	NSCLC
Gefitinib/iressa (tyrosine kinase inhibitor)	NSCLC
Sunitinib/sutent (tyrosine kinase inhibitor)	Renal cell carcinoma
Sorafenib/nexavar (tyrosine kinase inhibitor)	Renal cell carcinoma

Targeted therapies, which include monoclonal antibodies and small molecule inhibitors, have significantly changed the treatment of cancer over the past 10 years. These drugs are now a component of therapy for many common malignancies, including breast, colorectal, lung, and pancreatic cancers, as well as lymphoma, leukemia, and multiple myeloma. The mechanisms of action and toxicities of targeted therapies differ from those of traditional cytotoxic chemotherapy. Targeted therapies are generally better tolerated than traditional chemotherapy, but they are associated with several adverse effects, such as acneiform rash, cardiac dysfunction, thrombosis, hypertension, and proteinuria. Small molecule inhibitors are metabolized by

cytochrome P450 enzymes and are subject to multiple drug interactions. Clearly, all forms of tumor therapy carry with them the danger of selection for resistance, a problem that may be exacerbated by the genomic plasticity inherent in most, if not all cancers. The most effective solution to this problem is almost certainly to attack simultaneously multiple lesions specific to individual tumors, in a much more sophisticated version of standard combined chemotherapies used at present. Evolution of cancer therapy is likely to remain a combination of design and error, but the development of mechanisms to target the mission-critical events that are common to all cancers provides a glimpse of therapeutic potential hitherto unimaginable.

1.4 Drug resistance in cancer

Most human tumors, when they do respond, contain subclones that become drug resistant. Under the selective pressure of a toxic therapy, the genetic diversity within most human tumors leads to rapid outgrowth of drug-resistant cells. A vast array of resistance mechanisms, involving mutations or amplification of the target enzyme, overexpression of drug transporters, or mutations in cell-death pathways, can defeat single agents, no matter how well designed and targeted — this was also observed by Gilman and colleagues earlier (1946). Elucidation of cellular resistance mechanisms holds the promise of leading to better treatments for cancer patients. The past few years have seen notable growth in the identification of resistance mechanisms. Resistance mechanisms can operate to either prevent agents from entering cells, as in loss of plasma membrane carriers for nucleoside analogs (hENT1), antifolates (RFC1) and cisplatin (CTR1), or enhance their extrusion, as exemplified by energy-dependent pumps such as ABC transporters (Pgp, MRPs, ABCG2) and ATB7b, a copper pump that is able to mediate the extrusion of cisplatin. As assessed in drug-resistant cell lines, ATP-dependent pumps appear to be most relevant to agents that enter cells by passive diffusion, such as natural product chemotherapeutic agents. On the other hand, cells are more likely to acquire resistance to agents that require plasma membrane carriers by

decreasing the expression of the carriers, as opposed to deploying energy-dependent pumps for these agents or their metabolites. Once inside the cell, the activity of drugs that require activation can be impaired by loss of specific enzymatic activities involved in their metabolic activation, as in the case of the metabolism of nucleoside analogs to cytotoxic nucleotide analogs (e.g. AraC). Certain agents can also be enzymatically inactivated, as in the case of methylation of thiopurine nucleobase analogs by thiopurine methyl transferase (TPMT). Cellular targets can also be altered, either by increased expression levels, or by mutations, such that they are less susceptible to inhibition, as in the case of camptothecins (topo isomeraseI), epipodophyllotoxins (topo isomeraseII) anti-folates (dihydrofolate reductase), fluoropyrimidines (thymidylate synthetase) and Gleevec (BCR-ABL). Once a cellular target is damaged, resistance factors can attenuate the capacity of a cell to undergo apoptosis (i.e. loss of p53 and cell cycle check points). Another class of resistance involves factors that influence the ability of the cell to repair damaged macromolecules. Examples of this include loss of mismatch repair enzymes, which appears to confer resistance to fluoropyrimidines, or inactivation of MGMT, which confers enhanced sensitivity to certain alkylating agents. Cellular mechanisms of drug resistance have been intensively studied, as experimental models can be easily generated by *in-vitro* selection with cytotoxic agents. Cancer cells in culture can become resistant to a single drug, or a class of drugs with a similar mechanism of action, by altering the drug's cellular target or by increasing repair of drug-induced damage, frequently to DNA. After selection for resistance to a single drug, cells might show cross-resistance to a structurally and mechanistically unrelated drug(s) - a phenomenon that is known as 'multidrug resistance'. This might explain why treatment regimens that combine multiple agents with different targets are not effective. Some of the mechanisms, which underline drug resistance in cancers, are detailed.

Glutathione-S-transferases (GSTs) are a family of Phase II detoxification enzymes that catalyse the conjugation of glutathione (GSH) to a wide variety of

endogenous and exogenous electrophilic compounds. GSTs are divided into two distinct super-family members: the membrane-bound microsomal and cytosolic family members. GSTs have been implicated in the development of resistance toward chemotherapy agents (Beaumont et. al., 1998; Li et. al., 2010). It is plausible that GSTs serve two distinct roles in the development of drug resistance via direct detoxification (Kauvar et. al., 1998) as well as acting as an inhibitor of the MAP kinase pathway (Cumming et. al., 2001; Townsend & Tew, 2003). The link between GSTs and the MAP kinase pathway provides a rationale as to why in many cases the drugs used to select for resistance are neither subject to conjugation with GSH, nor substrates for GSTs. GSTs have emerged as a promising therapeutic target because specific isozymes are over-expressed in a wide variety of tumors and may play a role in the etiology of other diseases, including neurodegenerative diseases, multiple sclerosis, and asthma. Additionally, the regulatory properties of GSTπ in kinase cascades have provided a translational opportunity to target GSTs in myeloproliferative pathways, with the consequent clinical testing of new agents in myelodysplastic syndrome.

Tumor cells can acquire resistance to apoptosis by various mechanisms that interfere at different levels of apoptosis signaling. Different studies have demonstrated changes that could affect either the death receptor pathway or the Bcl-2-inhibitable pathway in a wide variety of neoplasms. Along the mitochondrial pathway, for example, Bcl-2 upregulation is observed in indolent lymphomas and a variety of additional neoplasms (Reed, 1999), Bax mutations have been observed in mismatch repair-deficient colon and gastric cancers, (Rampino et. al., 1997), and loss of Apaf-1 expression (Soengas et. al., 2001) as well as overexpression of the IAPML-IAP/Livin (Liu, et. al., 2009; Abd-Elrahman, et. al., 2009) has been observed in melanomas. Tumors can acquire apoptosis resistance by down regulating or mutating pro-apoptotic molecules. In certain types of cancer, the pro-apoptotic Bcl-2 family member Bax is mutated that lead to loss of expression, and mutations in the BH domains leading to loss of function (Rampino et. al., 1997; Meijerink et. al., 1998; Molenaar et. al., 1998).

Reduced Bax expression is associated with a poor response rate to chemotherapy and shorter survival in some situations (Krajewski et. al., 1995). As p53 has a central function in apoptosis induction alterations of the p53 pathway influence the sensitivity of tumors to apoptosis (Lowe et. al., 2000). Tumors that are deficient in p53 in SCID mice and cell lineages showed a poor response to chemotherapy (Geisler et. al., 2001). Specific mutations in the gene encoding p53 have been linked to primary resistance to doxorubicin treatment and early relapse in patients with breast cancer (Fichtner et. al., 2004).

The mechanism by which many chemotherapeutic drugs are taken up by cells is unknown. Alterations in drug efflux due to ABC transporter proteins such as P-glycoprotein (P-gp) and multidrug resistance protein (MRP) have been demonstrated *in-vitro* (Gottesman et. al., 2002; Ambudkar et. al., 1999). These proteins can directly transport drugs out of cells and particularly target natural hydrophobic drugs such as taxanes, anthracyclins, and vinca alkaloids (Thomas & Coley, 2003; Krishna & Mayer, 2000). Physiological roles for MRP1 in protecting certain tissues from the effects of chemotherapeutic agents, and in inflammation and dendritic cell function, have been identified, and MRP2 is involved in the hepatobiliary elimination of bilirubin glueuronide and many pharmaceutical agents. Both of these pumps are potent resistance factors for natural product chemotherapeutic agents. MRP3 is able to confer resistance to epipodophyllotoxins and may have a role in the disposition of bile acids in pathological conditions. MRP1, MRP2, MRP3 and MRP4 are resistance factors for methotrexate, and MRP1 may be involved in folate homeostasis. MRP4, MRP5 and MRP8 are cyclic nucleotide efflux pumps that can be deployed for the purpose of conferring resistance to nucleotide analogs.

The cell death-suppressive function of NF-κB plays a critical role in oncogenesis and cancer progression. Numerous reports have shown that various types of tumor cells become resistant to proapoptotic drugs through constitutive activation of NF-κB.

In addition, a study analyzing NF-κB inhibition in association with radiation indicated that NF-κB activation leads to radiation resistance in experimental colorectal tumors (Baldwin, 2001). Based on these studies, clinical trials utilizing certain chemotherapies in conjunction with NF-κB inhibitors (proteasome inhibitors, thalidomide, etc.) are presently underway. Primary prostate cancer tissue and prostate cancer cell lines also show constitutive NF-κB activity that is necessary for cell resistance to apoptosis (Rayet et. al., 1999; Fujioka et. al., 2003). The inhibition of NF-κB also decreased prostate cancer cell growth *in-vitro* and led to a marked reduction in the incidence and growth of tumors in nude mice (Suh et. al., 2002). As with other tumors, these results suggest an important role for NF-κB in the onset of prostate cancer or in its progression to invasion and metastasis. Wang and collaborators showed that the delivery of a super-repressor form of IκBα in chemoresistant tumors in mice enhanced the apoptotic response to chemotherapeutic agents and induced tumor regression (Wang et. al., 1999a). The main mechanisms of resistance against 5-fluorouracil in colorectal carcinoma are due to overexpression of dihydropyrimidine dehydrogenase, MRP8, thymidylate synthase and NF-κB p65 (Tegze et. al., 2005). Imatinib represents at present the most attractive therapy for BCR-ABL positive leukemias, even though a percentage of CML patients develop resistance to this compound. Inhibition of a specific kinase involved in NF-κB pathway (IKK) by PS1145 inhibits the proliferation of CML cell and its resistant patients (Cilloni et. al., 2006), hence a new therapeutic approach, which combines Imatinib and the IKK inhibitor PS1145 in CML resistant patients.

1.5 Strategy to overcome chemoresistance: Combination therapy

Chemotherapy drugs are most effective when given in combination (combination chemotherapy). The rationale for combination chemotherapy is to use drugs that work by different mechanisms of action, thereby decreasing the likelihood that resistant cancer cells will develop. When drugs with different effects are combined, each drug can be used at its optimal dose, without intolerable side effects. For some cancers, the best approach is a combination of surgery, radiation therapy, and chemotherapy. Surgery or radiation therapy treats cancer that is confined locally, while chemotherapy also kills the cancer cells that have spread to distant sites. Sometimes radiation therapy or chemotherapy is given before surgery to shrink a tumor, thereby improving the opportunity for complete surgical removal. Radiation therapy and low-dose chemotherapy after surgery help to destroy any remaining cancer cells. The stage of the cancer often determines whether single therapy or a combination is needed. For example, early-stage breast cancer may be treated with surgery alone or surgery combined with radiation therapy, chemotherapy, or with all three treatments, depending on the size of the tumor and the risk of recurrence. Locally advanced breast cancer is usually treated with chemotherapy, radiation therapy, and surgery.

By 1987 there were several different anti-cancer drugs available and, with some of them, a greater effect was achieved by giving a combination of two or more drugs at the same time. Professor Alan Bennett from Kings College London discovered that two particular drugs methotrexate and indomethecin, when used together were more effective at killing cancer cells when tested in the laboratory. Before this combination could be tried out on patients why this happened was to be studied. Subsequent research revealed that indomethecin made human breast cancer cells take up more of the methotrexate drug. It was also found that this effect extended to a number of other anti-cancer drugs. This research stimulated the testing of new combinations of anti-

cancer drugs, such as the cyclophosphamide, methotrexate and fluorouracil combination that is now used to treat breast cancer. Researchers at the Cold spring harbor laboratories (2004) achieved an experimental breakthrough in the simultaneous use of two or more agents for treating cancer. A two-drug (rapamycin and doxorubicin) combination therapy, led to the complete remission of a mouse model of B-cell lymphoma in all of the treated animals. In contrast, animals treated with either drug alone rarely experienced complete remission. Because most traditional chemotherapy agents act by triggering programmed cell death, such agents are frequently ineffective against tumors that lack a functional programmed cell death mechanism. Such tumors are said to be chemotherapy-resistant. The evasion of treatment-induced programmed cell death by chemotherapy-resistant tumors has been a major impediment to successful therapeutic outcomes for human cancer. Genetic lesions that aberrantly activate the Akt protein or other components of the Akt-mediated growth control pathway (e.g. mTOR or another protein called PI3 kinase) are common in leukemia, lymphoma, and in a variety of solid tumors. The drug used here, rapamycin blocks the action of an Akt "effector" protein called mTOR and thereby restores the programmed cell death mechanism in Akt-positive lymphomas. The drug chosen to trigger the programmed cell death mechanism was doxorubicin. With the programmed cell death mechanism restored by rapamycin treatment, triggering the mechanism by doxorubicin treated delivered the decisive, knockout blow to Akt-positive lymphomas. There was massive death of lymphoma cells observed when treated with both the drugs and the animals rapidly became tumor-free, and their period of tumor-free survival was greatly extended compared to that of mice bearing genetically different, non-responsive "Bcl-2-positive" lymphomas, and when compared to mice bearing Akt-positive lymphomas that were either untreated or treated with rapamycin or doxorubicin alone. In patients with breast cancer, both doxorubicin and epirubicin are used in treatment of advanced disease and as the basis of adjuvant regimes in some patients with early disease (Xu et. al., 2005). A meta-analysis suggested that doxorubicin is associated with significantly improved survival in

patients with advanced ovarian cancer. 22 to 36% of patients with gastric cancer respond to doxorubicin therapy and response rates to doxorubicin-or epirubicin based combination regimes are substantially higher. Anthracyclines have limited activity against prostate cancer; however, mitoxantrone has been approved for use in combination with steroids to treat pain in men with hormone-refractory prostate cancer. The combination of doxorubicin with cyclophosphamide, vincristine, prednisone (CHOP) has become standard therapy for most patients with diffuse cell non-Hodgkin's lymphomas. In patients with Hodgkin's lymphoma, the combination of doxorubicin plus bleomycin, vincristine and dacarbazine (ABVD) is an effective and well tolerated regime. Therefore, the combination therapy outlined by several studies is a promising general strategy for treating many cancers that are refractory to current therapies.

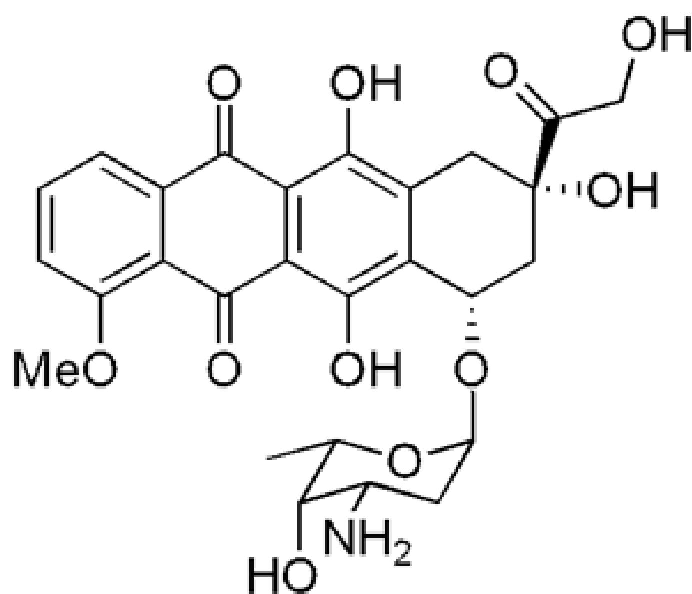
1.6 Doxorubicin as an anticancer drug

Anthracyclines are potent antineoplastic agents used extensively to treat a range of cancers, including leukemias, lymphomas, sarcomas, and carcinomas (Gewirtz, 1999). The first identified anthracyclines, daunorubicin and doxorubicin, were isolated from pigment producing *Streptomyces* spp. In the early 1960s and remain in widespread clinical use today. Chemically, all anthracylines consist of an aglycone ring coupled with an amino sugar. Anthracycline analogues have been developed to improve antitumor activities or reduce the toxicity. Doxorubicin, daunorubicin, and epirubicin are clinically used anthracyclines. The intricate and complex cellular responses to anthracyclines hinder efforts to unveil the mechanisms involved in their cytostatic and cytotoxic actions. However, anthracyclines are proposed to disrupt macromolecular biosynthesis by various mechanisms, including DNA intercalation and the inhibition of DNA polymerase and topoisomerase II (Gewirtz, 1999). Anthracyclines can also induce DNA damage by the generation of free radicals that react with a variety of macromolecules, thus inhibiting cellular proliferation or causing apoptosis (Gewirtz,

1999). In general, the antitumor effect of anthracyclines is mainly attributed to their DNA-binding and-damaging abilities (Myers, 1998; Minotti et. al., 2004).

Evidences also demonstrate that cell response to doxorubicin is highly regulated by multiple signaling events, including generation of sphingosine and activation of different caspases, c-Jun N-terminal Kinase (JNK), transcription factors like nuclear factor-kappaB (NF-κB), and Fas/Fas ligand system. Thus, doxorubicin mediated signaling events result in programmed cell death (apoptosis). Attaining resistance to chemotherapy by several tumors is a common clinical problem in human cancer that may develop mostly by unknown mechanism. The development of cellular resistance mechanism to doxorubicin includes P-glycoprotein and Bcl-2 over expression, altered topoisomerase II activity, loss of p53 function etc. Chemotherapy has remained as one of the mainstays for tumor intervention; however the development of resistance to certain chemotherapeutics by various forms of tumors has instigated the search for alternative agents that effectively kill cancer cells. The possible strategy to counter resistance would be to use the information regarding the metabolic pathway(s) mediated by a drug(s) in particular tumor type while selecting the chemotherapeutic regime. Hence, a thorough understanding of the molecular pathway(s) of apoptosis mediated by a particular chemotherapeutic agent is essential.

CHEMICAL STRUCTURE OF DOXORUBICIN



The use of anthracyclines has been reported to have beneficial effects in cancer patients. Also pre-clinical studies have demonstrated that doxorubicin has excellent activity against a variety of human tumor cell lines. However, a thorough understanding of the antineoplastic activities of doxorubicin has not been addressed. Hence, the study to decipher the signal transduction pathway(s) mediated by doxorubicin in relation to its anti tumor activities is interesting. Also a comparative study on doxorubicin sensitive and resistant tumor will help to design combination therapy that increases the cytotoxic effects of doxorubicin and decrease the resistance of tumor.

Chapter-II

Materials and Methods

2.1 Materials:

2.1.1 Chemicals and Antibodies used:

Doxorubicin, etoposide, cis-platin, vincristine, adriamycin, taxol, MTT [3-(4,5- dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)], glycine, acrylamide, bis-acrylamide, 4-methyl umbelliferyl phosphate (4-MUP), ortho-phenylenediamine (OPD), *p*-nitrophenyl phosphate, EDTA, EGTA, PMSF, Leupeptin, E-64, BAPTA-AM, RII peptide, Fura-2AM, N-acetyl leucyl leucyl norleucinal (ALLN), lactacystine, MG132, Ac-DVED-pNA, Ac-ITED-pNA, propidium iodide (PI), forskolin, H-7 [1-(5-Isoquinolinesulfonyl)-2-methylpiperazine], PKA catalytic subunit and anti-tubulin antibody were obtained from **Sigma** (St Louis, MO, USA). DMEM and fetal bovine serum (FBS) were obtained from **Life Technologies** (Grand Island, NY, USA). Dihydrorhodamine, DAPI, goat anti-rabbit IgG-Alexa Flour and FITC were purchased from **Molecular Probes**, (The Netherlands). Penicillin, streptomycin, and RPMI-1640 medium were obtained from **Invitrogen Corporation** (Carlsbad, CA, USA). M-MuLV reverse transcriptase, DNA-polymerase, RNase inhibitor, dNTPs and $MgCl_2$ were also obtained from **Invitrogen Corporation** (Carlsbad, CA, USA). Phosphate free DMEM, Opti-MEM medium, trypsin-EDTA, antibiotic-antimycotic, freezing medium, fungizone, L-glutamine, (FBS) and TRIzol were obtained from **Gibco BRL** (Grand Island, NY, USA). Dibutyryl cAMP (DB-cAMP), Adenosine cyclic 3', 5'-phosphorothioate triethylammonium salt (Rp-cAMPS), H-8 [(methylamino)ethyl-5-isoquinolinesulfonamide, hydrochloride], cAMP assay kit, BAY 11-7082 (designated as BAY), poly ADP-ribose polymerase (PARP) and phospho-IκBα antibodies were obtained from **Calbiochem** (San Diego, CA, USA). Recombinant TNFα, recombinant human IL-8, were obtained from **Pepro Tech Inc**. (Rocky Hill, NJ, USA). Oct1 and CREB double-stranded oligonucleotide and antibodies against p65, IκBα, p50, NF-AT, cyclin D1, c-Rel, tumor necrosis factor (TNF), interleukin-8 (IL-8), FasL, cyclooxygenase (Cox)2, transglutaminase (TGase) 2, multi-drug resistance protein (Mdr), intercellular adhesion molecule (ICAM) 1, superoxide dismutase (SOD) 1, CRM1, Bcl-2, Bcl-xL, IKKα, IKKβ, CRM1, MSK1, surviving, IAP-1, PKAα, goat-anti-rabbit IgG

conjugated with HRP, ShRNA for PKA, were obtained from **Santa Cruz Biotechnology** (Santa Cruz, CA, USA). The SuperFect transfection reagent was purchased from **Qiagen** (Hilden, Germany). The SignaTECT®cAMP-dependent PKA assay kit was purchased from **Promega** (Madison, USA). Antibody against phospho-p65 was obtained from **Cell Signaling Technologies** (Danvers, MA). The 'Live & Dead' cells assay kit was purchased from **Molecular Probe** (Eugene, OR). TRAF6 binding peptide (TRAF6-BP) and its mutant form [TRAF6-BP (Mut)] were synthesized in **German Center for Biotechnology** (GBF, Braunschweig, Germany). The plasmid construct for *p65*, dominant negative *TRAF2* and *6*, *NF-κB-SEAP, NF-κB-luciferase, FasL-luciferase*, *NF-AT-luciferase, Cox-2 luciferase* and green fluorescence protein (GFP) were kind gift from **Prof. B. B. Aggarwal** of the University of Texas, M. D. Anderson Cancer Center (Houston, TX). The 5-(4-Methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine (P_3-25) was synthesized and provided by **Prof. K. K. Narang**, Dept. of Applied Chemistry, Banaras Hindu University, Varanasi, India.

2.1.2 Cell lines:

The U-937 (human histiocytic lymphoma), HuT-78 (cutaneous T-cell lymphoma) MCF-7 (human breast carcinoma) cells, HeLa (cervical epithelial cells), Jurkat (T cells), A549, were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 (for U-937, A549) or DMEM medium (HuT-78, MCF-7, Jurkat, HeLa) containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). All cells were free from mycoplasma, as detected by Gen-Probe mycoplasma detection kit (Fisher Scientific, PA, USA). Doxorubicin resistant and revertant MCF-7 cells were obtained from **Prof. Kapil Mehta**, MD Anderson Cancer Center, Houston, USA. Doxorubicin resistant cells were cultured in presence of 2 µM doxorubicin. Cells were cultured in MEM (for doxorubicin-resistant, and -revertant) medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml).

2.2 Buffers and Media :

2.2.1 ELECROPHORECTIC MOBILITY SHIFT ASSAY

Buffers	**Composition**
Cytoplasmic Extraction Buffer	1 M HEPES (pH 7.9) 2 M KCl 0.1 M EGTA (pH 7) 0.5 M EDTA (pH 8)
Nuclear Extraction Buffer :	1 M HEPES (pH 7.9) 5 M NaCl 0.1 M EGTA (pH 7) 0.5 M EDTA (pH 8)
6x EMSA sample loading dye [10 ml] :	25 mg of Xylene Cyanol FF 25 mg of Bromophenol Blue 7.0 ml of Milli-Q water 3.0 ml of Glycerol
10X EMSA Binding Buffer :	200 mM HEPES (pH 7.9) 4 mM EDTA (pH 8.0) 40 mM DTT 50% glycerol
5x EMSA buffer :	0.25 M Tris 2.0 M Glycine 0.01 M EDTA (pH 8.5) pH made upto 8.5
EMSA Gel Mix (50ml) :	28.66 ml of Milli-Q water 10 ml of 5X EMSA buffer 11 ml of 30% Acrylamide: bisacrylamide (29:1) mix 400 µl of 10% APS 40 µl of TEMED
NFAT-EMSA Binding Buffer :	10 mM Tris (pH 7.5) 30 mM NaCl 0.5 mM EDTA 1 mM DTT 5% glycerol

2.2.2 SDS-PAGE

Buffers	**Composition**
Whole cell lysis buffer	20 mM HEPES (pH 7.9) 250 mM NaCl 1% NP-40 2 mM EDTA 1 mM DTT 2 µg/ml leupeptin 1 µg/ml Aprotinin 100 µg/ml PMSF (1 mM DTT, leupeptin, Aprotinin, PMSF: To be added just before use)
Stacking Gel Mix (10ml)	2.5 ml of 0.5M Tris-Cl (pH 6.8) 1.66 ml of 30% Acrylamide ; bisacrylamide (29:1) Mix 100 µl of 10% SDS 5.63 ml of Milli-Q water 100 µl of 10% APS 10 µl of TEMED.
9% Resolving Gel Mix (10ml)	2.5 ml of 1.5 M Tris-Cl (pH 6.8) 3 ml of 30% Acrylamide; bisacrylamide (29:1) mix 100 µl of 10% SDS 4.29 ml of Milli-Q water 100 µl of 10% APS 10 µl of TEMED.
2X SDS loading Buffer	130 mM Tris-Cl (pH 8.0) 20% (v/v) Glycerol 4.6% (w/v) SDS 0.02% Bromophenol blue 2% DTT
10X SDS-PAGE Running Buffer	0.25 M Trizma Base 1.92 M Glycine 1% SDS

2.2.3 WESTERN BLOT

Buffers	Composition
10X Blotting Buffer	0.25 M Trizma Base 1.92 M Glycine 1% SDS
2 litres of 1X Blotting Buffer	400 ml of methanol 200 ml of 10X blotting buffer 1400 ml of Milli-Q water.
Phosphate Buffered Saline	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 2 mM KH_2PO_4
Blocking Buffer :	5% Fat free milk or 3% BSA 0.05% Tween Make up volume with PBS.
Stripping Buffer :	100 mM β-mercaptoethanol 2% (w/v) SDS 62.5 mM Tris-HCl (pH6.7)

2.2.4 KINASE ASSAY

Buffers	Composition
Assay Buffer (10X)	20 mM HEPES (pH 7.4) 10 mM $MgCl_2$ 1 mM DTT
Wash Buffer	20 mM HEPES (pH 7.4) 25 mM NaCl 1 mM DTT

2.2.5 CALCINEURIN ASSAY

Buffers	**Composition**
Assay Buffer (2X)	200mM NaCl 100mM Tris (pH 7.5) 1 mM $CaCl_2$ 12 mM $MgCl_2$

2.3 Experimental Procedures:

2.3.1 Isolation of PBMCs and neutrophils from human blood:

Neutrophils were separated from fresh peripheral human blood of normal healthy donors by dextran T-500 sedimentation followed by ficoll-paque (Histopaque-1077) density gradient centrifugation method. The oxalated blood was immediately incubated with 3% dextran solution in saline with 1:1 ratio at 37°C for 30 minutes. The erythrocytes (RBCs) form rouleaux and were sedimented at 400xg for 30 minutes at room temperature. The PBMC layer formed above the ficoll level was removed by aspiration and suspended in phosphate buffer saline later to be centrifuged and the pellet suspended in RPMI medium for culture. After removal of the ficoll paque the granulocyte enriched pellet was washed with D-PBS and RBC contaminants in neutrophils were lysed by addition of 0.2% NaCl solution. Then, 1.6% NaCl was added to bring the correct osmolarity of the cell suspension. After centrifugation at low speed for 5 minutes, the pellet was taken and suspended in medium. The purity of the separated cells was examined by staining the cells with giesma stain and observed under phase contrast microscope. The isolated cells were up to 94-96% neutrophils. The viability of the cells was checked with trypan blue exclusion test, wherein, the viable cells exclude the dye and the non-viable cells take-up the dye giving a blue coloration. The diluted cell suspension was taken in 0.2% trypan blue dye in saline and dye positive and negative cells were counted separately under microscope using hemocytometer, which were expressed in percentage. The cells were found to be 98% viable by trypan blue dye exclusion test.

2.3.2 Preparation of cytoplasmic and nuclear extracts:

To the fresh cell pellet in a micro centrifuge tube 200 µl of ice-cold cytoplasmic extract buffer was added and the pellet was suspended. The cell suspension was incubated on ice for 30 min. The buffer is hypotonic in nature, and cells are allowed to swell on ice. 6.2 µl of

10 % triton X 100 (freshly prepared) for every 200 µl of cell suspension was added to each microfuge tube. The cell suspension was vortexed vigorously for 15 seconds for the rupture of the plasma membrane. The contents were centrifuged for 1 min at 10000 rpm, supernatant (Cytoplasmic Extract) is removed. If the cytoplasmic extract is to be saved then it is transferred to a pre-chilled microfuge tube and the cytoplasmic extract was stored at -70^0C. 25 µl of ice-cold nuclear extract buffer was added to the pellet (nuclear pellet + membrane) in the microfuge tube. This was incubated on ice for 45 min with intermittent vortexing, finally centrifuged for 5 min at 10,000xg. The supernatant (nuclear extract) was stored at -70^0C.

2.3.3 Protein estimation by Bradford method:

In the first well of a 96-well plate 50µl of milli-Q water was added in duplicate. To the subsequent wells 50 µl BSA standards (i.e. 50µg/ml, 100µg/ml) was added in duplicate. To the other wells diluted unknown protein sample (122.5µl of water + 2.5 µl of unknown protein extract) were added in duplicates. Then 200 µl of Bio-Rad reagent (1 ml reagent + 4.5 µl of milli-Q and mixed) was added to each of the well including blank. The concentration of protein was read using the ELISA reader at 590 nm.

2.3.4 Radioactive End Labelling of oligos:

The oligonucleotides, γ32-ATP, T_4 polynucleotide kinase buffer were thawed out. The following components were mixed in a microfuge tube on ice:-

Chemicals	**Volume pipetted**
oligo (1 p mole/μl)	3 μl
10 x T_4 Polynucleotide kinase buffer	1 μl
Sterile dist. H_2O	2.66 μl
γ32-ATP	3 μl
T_4 Polynucleotide kinase	0.33 μl
Total reaction volume	10 μl

This was mixed by tapping, pulse spin and incubated at 37^0C for 45 min so that enzymatic reaction occurs. The reaction was stopped by adding 2 μl of 0.5 M EDTA pH-8.0. The column was prepared by inserting first glass wool in the 1 ml syringe and swollen sephadex G-50 was added into the syringe. Now the column was taken and transferred to another improvised stand that contains a microfuge tube for the collection of elute. Then the reaction mixture was loaded on the top of the G-50 column. The eluting fractions were collected in the microfuge tube by loading 0.2 ml milli-Q water on the top of the column. After collecting 5 to 6 fractions, the tubes were analyzed for amount of radioactivity using a GM counter. The tube having between 7-10 x 10^6 counts was selected. That means the specific activity of the oligo would be between 3.5-4.5 x 10^6 cpm/pmoles. A labeling between 3.5-4.5 x 10^6 cpm/pmole would correspond to efficiency between 50 to 65 %. To the selected tube 100 pM of the complimentary strand of the oligo was added and the content boiled at 95°C for 5 minutes and allowed to anneal at room temperature for 3 to 4 hours.

2.3.5 Electrophoretic Mobility Shift Assay:

The binding reaction-mixture was prepared. The constituents of which are as following:-

Stock solutions	**Volume pipetted**
H_2O	6µl
10 x Binding buffer	2 µl
Poly dI : dC (1 µg/µl)	2 µl
^{32}P-ds oligo(4 fmoles/µl)	4 µl
10 % Triton X 100	2 µl
Total Reaction Volume	16 µl

4 µl of nuclear extract was added to the above mixture, mixed well and incubated at 37^{0}C in water bath for 1 hour. Tubes are transferred on ice; to it is added 4 µl of DNA-loading dye and mixed well by tapping. Pulsed spin to collect everything to the bottom and the tubes were transferred on ice. The samples were loaded into a native PAGE gel which was pre-run at constant voltage for 15 minutes. Electrophoresis was performed at 150 V (40 mA), till the bromophenol blue migrated 1-2 cm from bottom of the gel. A thin wedge-shaped article between the glass plates was inserted from one corner of the gel-mould and applied a twisting pressure to lift one of the glass plate (usually the top smaller one) carefully without disturbing the gel. A piece of Whatmann (3 mm thickness) paper was cut according to the size of the gel and was put on the gel. The paper was pressed gently and carefully and the gel was lifted, that is now firmly stuck on the paper. Then the gel was covered with saran wrap and kept on the gel-dryer at 80^{0}C for 1 hour under suction. After drying the gel, it was removed from the gel-dryer; the corners were taped and were exposed on a Molecular-Dynamics Phosphorimager Screen to read the protein of interest.

2.3.6 Western Blot:

The proteins were resolved in a denaturing SDS-PAGE gel and after completion of the run the gel was over laid on a nitrocellulose paper cut to the size of gel and kept in the blotting cassette in the presence of blotting buffer. Finally the cassette was put in the mini transblot apparatus (Bio Rad) and blotting was done for 4 hours at a constant voltage of 60 V. Then the membrane was taken out and rinsed in PBS-T (Phosphate Buffer Saline – Tween) for 5 minutes by gentle shaking. Later the membrane was immersed in 5% non-fat milk solution in PBS-T with gentle shaking for 1 hour at 37°C. The membrane was washed off from the traces of the fat free milk with PBS-T and the membrane was over laid with primary antibody diluted in PBS-T for 3 hours at 4°C with shaking. After incubation the membrane was washed with PBS-T and layered with secondary antibody (conjugated with horse-radish peroxidase) diluted in 5% fat free milk solution (in PBS-T) and incubated for 45 minutes at room temperature. After incubation the membrane was washed and processed for the protein bands of interest using ECL-plus detection reagent (Amersham Biosciences) followed by development of the bands using X-ray film (Hyperfilm-ECL, Amersham Biosciences).

2.3.7 Enzyme release assay:

Neutrophils (1 x 10^7 cells/ml) were suspended in D-PBS containing glucose (1 mg/ml) and BSA (5 mg/ml). Cells were treated with different modulator (s) and then washed and suspended in fresh medium. Cytochalasin B (5 μg/ml) was added for 5 minutes at 37°C. The supernatant was taken for alkaline phosphatase enzyme assay. For alkaline phosphatase assay the 50 μl supernatant was added with 100 μl substrate solution (4 mg/ml p-nitrophenyl phosphate and 0.5 mM $MgCl_2$ in 1 M diethanolamine buffer, pH 9.8) the reaction proceeded at 37°C for 30 minutes. The absorbance was taken at 405 nm after stopping the reaction by addition of 100 μl 1 N NaOH . For β–D-glucoronidase assay, 100 μl supernatant was added with 100μl of substrate solution containing 0.01 M p-nitrophenyl β–D-glucoronidase in 0.1 M sodium acetate buffer, pH 4. After incubation

for 18 hours at 37°C, the reaction was stopped by adding 100 µl of 0.4 M glycine and absorbance was measured at 400 nm.

2.3.8 Calcineurin activity assay:

After different treatments, cells (2 x 10^6) were extracted and extracts were passed through sephadex G-25 column to eliminate the free phosphate and proteins fractions were pulled together and used for calcineurin activity assay. The extract (10 µg proteins) was incubated with 25 µl of 2x assay buffer (200 mM NaCl, 100 mM Tris [pH 7.5], 12 mM $MgCl_2$, 1 mM $CaCl_2$). The mixture was incubated without or with RII phosphopeptide (5 µM) for 10 min at 30°C. Reaction was terminated by addition of 100 µl of Malachite green mix (3 volume of 0.045% Malachite green and 1 volume of 4.2% ammonium molybdate in 4N HCl) and allowed for 30 min incubation at 30^0C. The absorbance was read at 660 nm. The calcineurin activity (fold) was calculated in terms of inorganic phosphate release from the total protein and unstimulated cells value was considered as one fold.

2.3.9 Kinase assay:

After treatment of cells (3x10^6/ml) with the drug for required time interval, cell extracts were prepared by lysing cells in buffer containig 20 mM HEPES, (pH 7.4), 2 mM EDTA, 250 mM NaCl, 1% Nonidet- P-40, 2µg/ml leupeptin , 2µg/ml aprotinin, 1 mM PMSF, 0.5µg/ml benzamidine and 1 mM DTT . Cell extracts (250µg/sample) were immunoprecipitated with 0.3µg of anti-PKAα, anti-MSK and anti IKK for 3 hours at 4°C. Immunocomplexes were collected by incubation with protein A/G-Sepharose beads for 1 hour at 4°C the beads were extensively washed with lysis buffer (4x400 µl) and kinase buffer (2x400 µl, 20 mM HEPES (pH 7.4) , 1 mM DTT, 25 mM NaCl). Kinase assays were performed for different time intervals at 30°C with GST-p65 as substrates in 20 mM HEPES (pH7.4) , 10 mM MgCl2, 1 mM DTT, and 10 µCi of [γ32 P] ATP. Reactions were stopped with the addition of 20 µl of 2x SDS sample buffer, boiled for 5 min and

subjected to SDS-PAGE (10%). GST-p65 was visualized by staining with coomassie blue and the dried gel was analysed by a Phosphor Imager.

2.3.10 Total RNA isolation from cultured mammalian cells:

To the cell pellet (approximately 2 million cells) 1ml of TRIzol was added and the cells suspended in it by repeated pipetting. Later 200µl of choloroform was added followed by vortexing for about 30 seconds. The tube was centrifuged at maximum speed for 5 minutes. The upper aqueous phase was transferred into a fresh microcentrifuge tube and 400 µl of ice-cold isopropanol added, this was incubated at -20°C for 1 hour. The RNA was pelleted by centrifugation at maximum speed for 15 minutes at room temperature. The supernatant was decanted and the pellet washed with 200 µl of 70% ethanol followed by centrifugation at maximum speed for 10 minutes. The supernatant was removed and the pellet dried under vacuum for about 5 minutes. The pellet was re-solubilized in 30-50 µl RNase free deionized (DEPC-treated milli-Q) water and aliquots were stored at -70°C.

2.3.11 Reverse transcriptase (RT)-PCR:

Total RNA from each specimen was extracted using TRIzol (Gibco BRL) and 1 µg of total RNA was reverse-transcribed using poly-T oligonucleotide and M-MuLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The PCR war performed using primers for gene of interest using gene specific primers. Following PCR, the amplicons were analyzed by gel electrophoresis with ethidium bromide staining. The expression of the investigated genes was determined by normalizing their expression against the expression of housekeeping GAPDH/ Actin gene.

2.3.12 NF-κB-dependent reporter gene transcription:

Cells were transiently co-transfected by the lipofection method using 0.5 μg required plasmid DNA(s) with the protein of interest, a plasmid bearing NF-κB promoter DNA linked to the heat-stable secretory alkaline phosphatase (SEAP) gene and β-galactosidase expression plasmid (Promega, Madison, USA). By adding control plasmid pCMVFLAG1 DNA, the total amount of DNA was maintained to 3 μg for each transfection. The cells were cultured for 12 hours and required stimulations done. Twelve hours later, cell culture-conditioned medium was harvested, and 25 μl was analyzed for SEAP activity essentially as per the CLONTECH protocol (Palo Alto, CA). β-Galactosidase activity was measured simultaneously using a β-galactosidase assay kit (Promega, Madison, USA). The relative promoter activity was normalized with β-galactosidase activity as the transfection efficiency.

2.3.13 FasL and NF-AT-dependent luciferase gene transcription assay:

The expression of FasL-, and NF-AT-dependent luciferase reporter gene was carried out. Cells were transiently transfected with SuperFect transfection reagent containing 0.5 μg of each reporter plasmid containing FasL, or NF-AT binding site cloned upstream of luciferase (designated as *FasL-luciferase*, or *NF-AT-luciferase*) and *GFP* constructs. After 3 h of transfection, cells were washed and cultured for 12 h followed by treatment with Doxorubicin for different times. GFP positive cells were counted. The cell pellets were extracted with lysis buffer (part of luciferase assay kit from Promega, Madison, USA) and the extracts were incubated with the firefly luciferin (substrate)(Promega Madison, USA). Light emission was monitored with a Luminometer and values were calculated as fold of activation over vector-transfected value.

2.3.14 Intracellular Ca^{2+} release assay:

Intracellular Ca^{2+} mobilization was measured using the Fura-2AM method. MCF-7 cells were incubated in low Calcium Buffer A (136 mM NaCI, 2.7 mM KCI, 2 mM $MgCI_2$, 5 mM HEPES, 5.6 mM glucose, 0.42 mM NaH_2PO_4 and 0.5 mM EGTA buffer, pH 6.6). Cells were washed and incubated in high Calcium Buffer B (same composition as buffer A, except $CaCl_2$ 0.16 mM and without EGTA, pH 7.4) and incubated with 2.5 μM Fura-2AM at 37°C for 30 min. Then the cells were washed, scrapped, and suspended in Buffer B and fluorescence intensity was monitored at a fixed excitation wavelength at 330 nm and an emission wavelength at 510 nm in a Perkin Elmner spectrofluorometer.

2.3.15 Cytokines assay by Two-site sandwich ELISA:

MCF-7 cells, plated at 1 x 10^5 cells/well in a 96-well plate were incubated with 1 μM of doxorubicin for different times at 37°C. Culture supernatants were examined for concentrations of TNFα and IL-8 following the manufacturers' instructions (BD Biosciences Pharmingen, San Diego, CA). 96-well polyvinyl chloride micro titer plates were coated and incubated overnight with purified anti-TNFα or -IL-8 antibody at a 2 μg/ml concentration. The plates were washed and then blocked for an hour at 37°C with 2% bovine serum albumin in PBS and incubated for 3 h with various culture supernatants followed by incubation with biotin-conjugated secondary antibody and streptavidin-HRP for an hour. The HRP activity was detected using *o*-phenylenediamine tetra hydrochloride (OPD) as substrate and the reaction was stopped with 1N H_2SO_4. The absorbance was read at 492 nm. Cytokines concentrations in the samples were calculated with a standard curve generated from recombinant IL-8 and TNF-α.

2.3.16 Immunocytochemistry :

The levels of different proteins (p65, NF-AT, Mdr) were examined by the immunocytochemical method with slight modifications. Cells were plated on a poly-L-lysine-coated glass slide air-dried, fixed with 3% formaldehyde, and permeabilized with

0.1% of Triton X-100. Slides were incubated with antibodies for specific proteins for 6 h followed by incubation with anti-rabbit IgG-Alexa Fluor for 1 h. Slides were mounted with mounting medium with DAPI and analyzed under a fluorescence microscope.

2.3.17 MTT assay:

The drug-induced cytotoxicity was measured by the MTT assay. 5×10^3 cells per well (of 96 well plate) were incubated in the presence or absence of the indicated test sample in a final volume of 0.1 ml for 72 hours at 37^0C in triplicate assays. Thereafter 0.025 ml of MTT solution (5 mg/ml in PBS) was added to each well. After two hours of incubation at 37^0C 0.1 ml of extraction buffer (20% SDS in 50% Dimethlylformamide) was added. After an overnight incubation at 37^0C, the absorbance at 570 nm was measured using at 96 well plate reader (Bio Rad, Hercules, CA) with the extraction buffer as blank.

2.3.18 Live and dead assay:

The cytotoxic effects were also determined by the Live/Dead assay (Molecular Probes, Eugene, OR). After different treatment 1×10^5 cells were stained with Live/Dead reagent (5 μM ethidium homodimer, 5 μM calcein-AM) and then incubated at 37^0C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan).

2.3.19 Measurement of reactive oxygen intermediates:

The production of reactive intermediates mainly oxygen and nitrogen species upon treatment of cells with the inducer was determined by flow cytometry. Cells were exposed to dihydrorhodamine 123 (5 mM stock in DMSO) and then stimulated with doxorubicin at 37°C for different time/ concentration, scrapped, washed and resuspended in 1 ml D-PBS at 0.5×10^6 concentration. Rhodamine 123 fluorescence intensity resulting from dihydrorhodamine 123 oxidation was measured by a FACScan

flow cytometer with excitation at 488 nm and was detected between 515 and 550 nm. Data analysis was performed using LYSYSII software (Becton Dickinson).

2.3.20 Determination of lipid peroxidation:

Drug-induced lipid peroxidation was determined by detection of thiobarbituric acid (TBA)-reactive substances, which are the end products of polyunsaturated fatty acids and related esters due to peroxidation. Cells (3×10^6/ml) pretreated without or with drug for required time were stimulated with stimulant for the required time. Then cells were washed with PBS and underwent three cycles of freeze thawing in 200 µl of water. A 20 µl of aliquot was removed, assayed for protein with Bradford protein determination and remaining samples were mixed with 800 µl of assay mix [0.4% (w/v) thiobarbituric acid, 0.5% (w/v) SDS, 9.4% (v/v) acetic acid, pH 3.5]. Samples were incubated for 60 min at 95°C, cooled to room temperature, and centrifuged at 14,000 × g for 10 min, and the absorbance of the supernatants was read at 532 nm against a standard curve prepared using the MDA standard (10 mM 1,1,3,3-tetramethoxypropane in 20 mM Tris-HCl, pH 7.4). Results were calculated as nmol of TBARs equivalents/mg of protein expressed as a percentage of thiobarbituric acid-reactive substances above control values. Untreated cells showed 0.568 ± 0.08 nmol of TBA-reactive substances/mg protein (subtracting the background absorbance obtained by heating 800 µl of assay mix plus 200 µl water).

2.3.21 DNA and nuclear fragmentation assay:

DNA was extracted with phenol: chloroform: isoamylalcohol (25:24:1,v/v/v) and precipitated by 100% ethanol at -20^0C overnight and ran on 1% agarose gel. DNA fragments were visualized as ladder with ethidium bromide under UV light. The cells were pelleted and washed with PBS thoroughly. To the pellet ice-cold 80% methanol was added drop wise and gradually the cells were suspended in it. This was incubated for 6 hours at 4°C. The cells were pelleted at the methanol discarded. To the pellet 80-

100 µl of PI staining mix was added and the cells gradually dispersed in it followed by incubation at 37°C for 30 minutes in dark. Taking equal volumes of the cell suspension and mounting medium prepared slides. The slides were viewed under phase contrast and fluorescent modes.

2.4 Oligonucleotide Used for EMSA

Name	**Sequence (in 5' - 3' direction)**
NF-κB	5' TGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG 3'
CREB	5' AGAGATTGCCTGACGTCAGAGAGCTAG 3'
NFAT	5' CGCCCAAAGAGGAAAATTTGTTTCATA 3'
OCT-1	5' TGTCGAATGCAAATCACTAGAA 3'
Sp1	5' ATTCGATCGGGGCGGGGCGAGC 3'

2.5 Primers Used for RT-PCR

Name	**Sequence (in 5' - 3' direction)**
Cox-2	5' TTCAAATGAGATTGTGGGAAAAT 3' 5' AGATCATCTCTGCCTGAGTATCTT 3'
Bax	5' ATGGACGGGTCCGGGGAG 3' 5' TGGAAGAAGATGGGCTGA 3'
IL-8	5' CAGACAGAGCTCTCTTCCAT 3' 5' GCAGCTCTGTGTGAAGGTGCA 3'
TNF	5' CGAGTGACAAGCCTGTAGCC 3' 5' CATACCAGGGCTTGGCCTCA 3'
Cyclin D1	5' TGTTTGCAAGCAGGACTTTG 3' 5' ACGTCAGCCTCCACACTCTT 3';
ICAM	5' AGGGAGGCTCCGTGCTGGTGA 3' 5' TCAGTGCGGCACGAGAAATTG 3'
FasL	5' GGATTGGGCCTGGGGATGTTTCA 3' 5' TTGTGGCTCAGGGGCAGGTTGTTG 3'
p65	5' GGAATTCCATATGGTCGACAACTCCGA 3' 5'CCGCTCGAGTAAGGAGCTGATCTGACT 3'
Actin	5' CCAACCGTGAAAAGATGACC 3' 5'GCAGTAATCTCCTTCTGCATCC 3'.

Chapter-III

Late phase Activation of Nuclear Transcription Factor kappaB by Doxorubicin is Mediated by Interleukin-8 and Induction of Apoptosis via FasL.

Introduction

Breast cancer is still a leading cause of cancer death in women in the United States and Europe (O'Shaughnessy, 2002). Adjuvant chemotherapy has been shown to provide disease-free and overall survival benefits for patients. The development of drugs and strategies to improve relapse-free and overall survival therefore remains a high priority. Anthracyclines are among the most active chemotherapeutic drugs for the treatment of breast cancer and anthracycline-containing regimens have an impact, albeit modest, on patient survival (Piccart, 2003). Doxorubicin is an anthracycline whose therapeutic efficacy is limited by the possible development of severe cardiotoxicity. It has been suggested that both iron and reactive oxygen species (ROS) mediate cardiotoxicity induced by doxorubicin, but the mechanisms through which iron and ROS interact and damage cardiac cells are still debated. (Corna et. al., 2004). Development of resistance to chemotherapeutic agents is another major problem in treatment of cancer. Doxorubicin is one of the most effective chemotherapeutic agents available to treat leukemia and breast cancer patients. The drug is able to induce regression of metastatic breast cancer (Blum et. al., 1974; Harris et. al., 1994). Development of resistance is known to hinder effectiveness of doxorubicin and several other anticancer drugs. A feature that is often associated with the development of drug resistance is expression of *mdr*, a gene that encodes for P-glycoprotein (P-gp), an integral trans membrane protein (Chen et. al., 2002). Similarly, altered expressions of other enzymes, such as type I topoisomerase (Pessian, 1993), glutathione-related enzymes (O'Brien et. al., 1996), and protein kinase C (PKC) (Blobe et. al., 1993) have been observed during the transition from drug sensitivity to drug resistance. Overcoming resistance and side effects to doxorubicin would represent a major advance in the effective management of breast cancer. The possible strategy to counter side effects and resistance would be to use the information regarding the metabolic pathways mediated by a drug in particular tumor type while selecting the chemotherapeutic regime. Hence, a thorough understanding of the molecular pathways of apoptosis mediated by a particular chemotherapeutic agent is essential.

Several reports suggest that doxorubicin generates oxidative stress that leads to DNA damage and culminates cell death through mitotic catastrophe (Eom et. al.,

2005; Ecuyer et. al., 2006; Yeh et. al., 2007). Free radical formation and DNA damages via inhibition of topoisomeraseII may be primarily responsible for the cytotoxic effects of doxorubicin (Mizutani et. al., 2005). Doxorubicin induces apoptosis via the activation of caspases and disruption of mitochondrial membrane potential (Gamen et. al., 2000). Doxorubicin also exerts its effects in part by modulating the expression of several members of the Bcl-2 family members: it causes decrease in Bcl-2 expression and increase in Bax expression (Corna et. al., 2004). Despite the wide spread clinical use of doxorubicin, its antiproliferative and death-inducing signal cascades are not yet fully understood. Different doses of doxorubicin activate different regulatory mechanisms to induce either apoptosis or cell death through mitotic catastrophe. p38, c-Jun N-terminal kinase (JNK), mitogen-activated protein (MAP) kinases, and NF-κB are significantly activated during high dose doxorubicin-induced apoptosis but not low dose doxorubicin-induced cell death through mitotic catastrophe. Studies further suggest that NF-κB is transiently activated during the initial phase of doxorubicin–induced apoptosis (Eom et. al., 2005).

Aberrantly active NF-κB complexes can contribute in tumorigenesis by regulating genes that promote the growth and survival of cancer cells (Barre et. al., 2007, Giri et. al., 1998, Manna et. al., 1999a). Higher levels and transcription potential of constitutive nuclear NF-κB can be obtained in many tumor cells in response to certain types of chemotherapeutic compounds (Chuang et. al., 2002). Upregulated NF-κB activity by a transient exposure to a small dose of anticancer drugs may induce drug resistance in some cancer cells (Yeh et. al., 2003). NF-κB, a heterodimer of two subunits p50 (NF-κB1) and p65 (RelA), is normally present in the cytoplasm in an inactive state in complex with an inhibitory subunit of kappa B (IκBα). Upon phosphorylation and subsequent degradation of IκBα, a nuclear localization signal on the p50–p65 heterodimer is exposed leading to nuclear translocation of NF-κB. The p50–p65 heterodimer binds with a specific sequence in DNA, resulting gene transcription. Phosphorylation and acetylation of p65 subunit potentiates NF-κB-dependent gene transcription, a hallmark for tumorigenic response (Manna et. al., 2007; Clarke et. al., 2008). Expression of several cytokines,

adhesion molecules, cyclins, Bax, Bcl-2 etc is NF-κB-dependent. Among the NF-κB regulated genes involved in managing the response to inflammation and carcinogenesis are those encoding for iNOS, Cox-2, TNFα, IL-1, IL-6, IL-8, and others. Cytokine IL-8 induces signal through its cell surface receptors (IL-8Rs) via recruitment of TNF receptor associated factor (TRAF) 6 (Manna et. al., 2005). TRAF6 binding peptide (TRAF6-BP) which is permeable to cells and binds with the TRAF6 inhibits IL-8-mediated NF-κB activation (Ye et. al., 2002).

Caspases are evolutionarily conserved family of aspartate specific cystein-dependent proteases with central functions in apoptotic and inflammatory signaling pathways (Coux et. al., 1996). Uncontrolled or deregulated activity of caspases has been observed in several pathological conditions including Alzheimer's disease, muscular dystrophies, and tumorigenesis (Wood et. al., 1999; Howley et. al., 2008). A peptidylprolyl isomerase, Pin 1, binds and isomerises specific phosphorylated serine/threonine residues of p65 and thereby facilitates proteolysis (Ryo et. al., 2003). Several cytotoxic drugs mediate the induction of apoptosis by activation of CD95/APO-1/Fas system, activation of caspases, or alterations in mitochondrial function (Fellenberg et. al., 2000). Activation of caspase8 preceded by activation of caspase3 was reported after treatment of mature and immature B-lymphoid cells with epirubicin or taxol (Wieder et. al., 2001). The observation of caspase activity and the identification of caspase substrate in the absence of cell death revealed non apoptotic functions of caspases. High levels of caspase activation achieved with or without mitochondrial amplification clearly lead to caspase-dependent apoptosis. In contrast, limited caspase activation may reveal mainly the non-apoptotic functions (proliferation, differentiation, intercellular communication through cytokine release and NF-κB activation). These non-apoptotic functions partly involve prodomain-mediated and/or caspase activity-dependent activation of NF-κB. In turn, NF-κB could then augment the anti-apoptotic status of the cell, allowing the non-apoptotic functions of caspases to operate.

Calcineurin is a eukaryotic Ca^{2+}–and calmodulin-dependent serine/threonine protein phosphatase. It is a heterodimeric protein consisting of a catalytic subunit calcineurinA, which contains an active site dinuclear metal center, and a tightly

associated, myristoylated, Ca^{2+}-binding subunit, calcineurinB. As a serine/threonine protein phosphatase, calcineurin participates in a number of cellular processes and Ca^{2+} dependent signal transduction pathways. Calcineurin is potently inhibited by immunosuppressant drugs, cyclosporine and FK506, in the presence of their respective cytoplasmic immunophilin proteins, cyclophilin and FK506-binding protein. Many studies have used these immunosuppressant drugs and/or modern genetic techniques to disrupt calcineurin in model organisms such as yeast, filamentous fungi, plants, vertebrates, and mammals to explore its biological function. Many physiologic processes such as neuronal death, T-cell activation, and cardiac and skeletal myocytes differentiation were found to be regulated by calcineurin (Rusnak et. al., 2000). Calcineurin activity was found to decrease significantly in tissues of cervical carcinoma patients (Crabtree et. al., 2002). Calcineurin signaling is prominent in transplant rejection and autoimmune disease, where the inhibitors CsA and FK506 are used clinically, and is being studied for its contribution to myocardial hypertrophy and to virulence in fungal pathogens. Calcineurin is a downstream target of intracellular Ca^{2+} signaling (Klee et. al., 1998). Sustained elevation of calcium in cells keeps high calcineurin activity. Family of transcription factors of nuclear factor of activated T lymphocytes (NF-AT) is the target substrates for calcineurin (Rusnak et. al., 2000; Kingsbury et. al., 2000; Abbasi et. al., 2005). Substrate selection is determined in part by a docking site in calcineurin that recognizes the consensus PxIxIT motif in NF-AT and some other substrates (Jayanthi et. al., 2004). Egr, Nur77, AP-1 transcription factors, FasL expression are responsive to NFATs (Jayanthi et. al., 2004). The NFAT Proteins belong to a family of transcription factors that are involved in the regulation of cytokine expression (Jayanthi et. al., 2004). At present, five members of the NFAT family have been identified. These include NFAT1 (NFATp or NFATc2), NFAT2 (NFATc1), NFAT3 (NFATc4), NFAT4 (NFATc3 or NFATx), and NFAT5 (Jayanthi et. al., 2004). Although originally described in T cells, the NFATs are now known to participate in the regulation of calcium-and calcineurin- mediated transcriptional activity. Upon stimulation of calcineurin several residues at regulatory domain of NF-AT are dephosphorylated and this leads to nuclear translocation of NF-AT and activation of target genes of AP-1 (Liu et. al., 1997), MEF2 (Srivastava, et. al., 1999),

FasL (Molkentin et. al., 1998; Raghavendra et. al., 2007), and GATA (Zhu et. al., 1998) proteins. The nuclear translocation of NF-AT and activity of calcineurin are blocked by CsA which blocks calcineurin activity affecting downstream signals and cell death (Molkentin et. al., 1998).

Doxorubicin has shown to induce reactive oxygen species which often causes DNA damage leading to mitochondrial potential change followed by cell death (Eom et. al., 2005; Ecuyer et. al., 2006; Yeh et. al., 2007). NF-κB has been implicated in the development of drug resistance in cancer cells. Constant presence of doxorubicin during culture of MCF-7 cells gave high basal expression of NF-κB and NF-κB-dependent genes and the cells attained resistance against it (Manna et. al., 2007; Manna &, Gangadharan, 2009; Gangadharan et. al., 2009). We systematically examined the baseline levels of NF-κB activity in response to a challenge with doxorubicin. Surprisingly we found a biphasic induction of NF-κB on treatment with doxorubicin in a time dependent manner. The signaling mechanisms involved in NF-κB mediated chemoresistance have not yet been completely elucidated. In fact, some studies have challenged the notion of chemoresistance, suggesting that NF-κB can play a proapoptotic role in response to chemotherapy agents (Ashikawa et. al., 2004; Campbell et. al., 2004; Campbell et. al., 2006; Bednarski et. al., 2008; Gangadharan et. al., 2009). Hence delineating the role of NF-κB in doxorubicin mediated apoptosis will help to design selective inhibitory strategies for NF-κB pathway and also help us to understand the signaling steps leading to NF-κB mediated chemoresistance. In this study, we used human breast cancer epithelial cell line to examine the potential involvement of NF-κB in doxorubicin mediated apoptosis. We further elucidate that the biphasic induction of NF-κB by doxorubicin is proapoptotic in this experimental setting. In this report we are providing evidences for the first time that doxorubicin-mediated cell death proceeds through the signaling cascades: early induction of NF-κB → increased IL-8 expression → increased intracellular Ca^{2+} → activation of calcineurin → nuclear translocation of NF-AT → expression of NF-AT-dependent FasL → FasL-mediated caspases activation → induction of cell death. Thus, this study suggests the novel pathway for doxorubicin-mediated cell death which might help to formulate it with other molecules for combination therapy.

Results

In this study the effects of doxorubicin in MCF-7 cells were examined. The doxorubicin was used as a solution in DMSO at 10 mM concentration. Further dilution was carried out in cell culture medium.

Doxorubicin increases biphasic DNA binding to NF-κB in MCF-7 cells.

MCF-7 cells were plated in 60 mm petridish and treated with 1 μM doxorubicin at 40% confluent state for different times, nuclear extracts were prepared, and assayed for NF-κB by gel shift assay. As shown in Fig.1A, NF-κB DNA binding increased gradually till 3 h and decreased by 12 h, followed by an increase till 36 h of doxorubicin treatment suggesting doxorubicin-induced biphasic induction of NF-κB. The experiment was repeated 5 times and similar results were obtained.

To detect the composition and specificity of the retarded band visualized by EMSA, nuclear extracts from doxorubicin-treated MCF-7 cells were incubated with antibodies (Abs) p50 (NF-κBI), p65 (Rel A), or in combination and 50 fold excess of cold NF-κB then conducted EMSA. Abs to either subunit of NF-κB shifted the band to a higher molecular weight (Fig.1B), thus suggesting that the retarded complex consisted of p50 and p65 subunits. Neither preimmune serum nor irrelevant Abs such as anti-c-Rel or anti-cyclin D1 had any effect on the mobility of NF-κB. The complex completely disappeared in presence of 50-fold molar excess of cold NF-κB and was unable to bind with mutant oligonucleotides, indicating its specificity NF-κB.

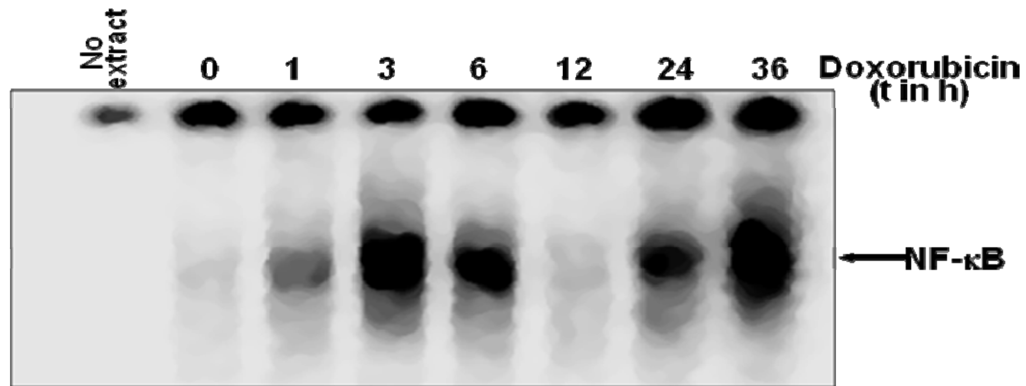


Fig 1A: Effect of doxorubicin on NF-κB activation. Cells were incubated at 37°C with 1 μM doxorubicin for different time periods as indicated and then tested for NF-κB activation by EMSA from nuclear extracts.

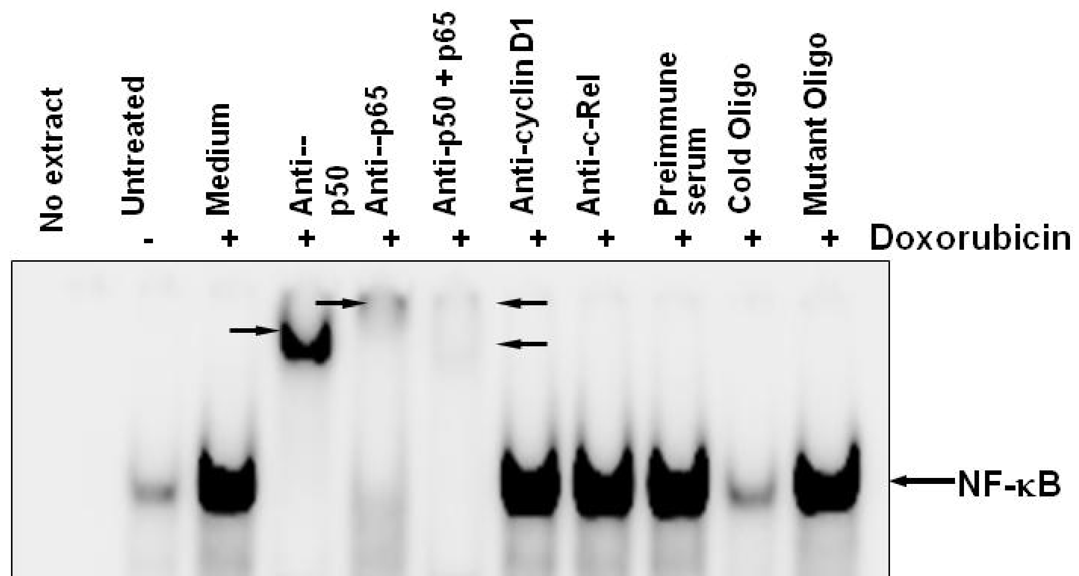


Fig 1B: Supershift of NF-κB band. Nuclear extracts were prepared from untreated or doxorubicin-treated MCF-7cells, incubated for 15 min with different Abs and cold NF-κB oligo and mutated NF-κB oligonucleotides (50-fold), and then assayed for NF-κB, as described in Materials and Methods.

Doxorubicin decreases amount of p65.

The doxorubicin-mediated biphasic induction of p65 was also detected in doxorubicin treated MCF-7 cells by immunofluorescence using anti-p65 antibody

followed by Alexa Fluor-labeled anti-rabbit IgG. As shown in Figure 1C, the p65 level was detected in the cytoplasm in un-stimulated cells whereas increased in the nucleus within 6 h, decreased from cytoplasm and nucleus at 12 and 24 h, followed by an increase in both cytoplasm and nucleus at 36 h upon doxorubicin treatment. The levels were gradually decreased and then increased with time of doxorubicin treatment further suggesting the biphasic induction of NF-κB.

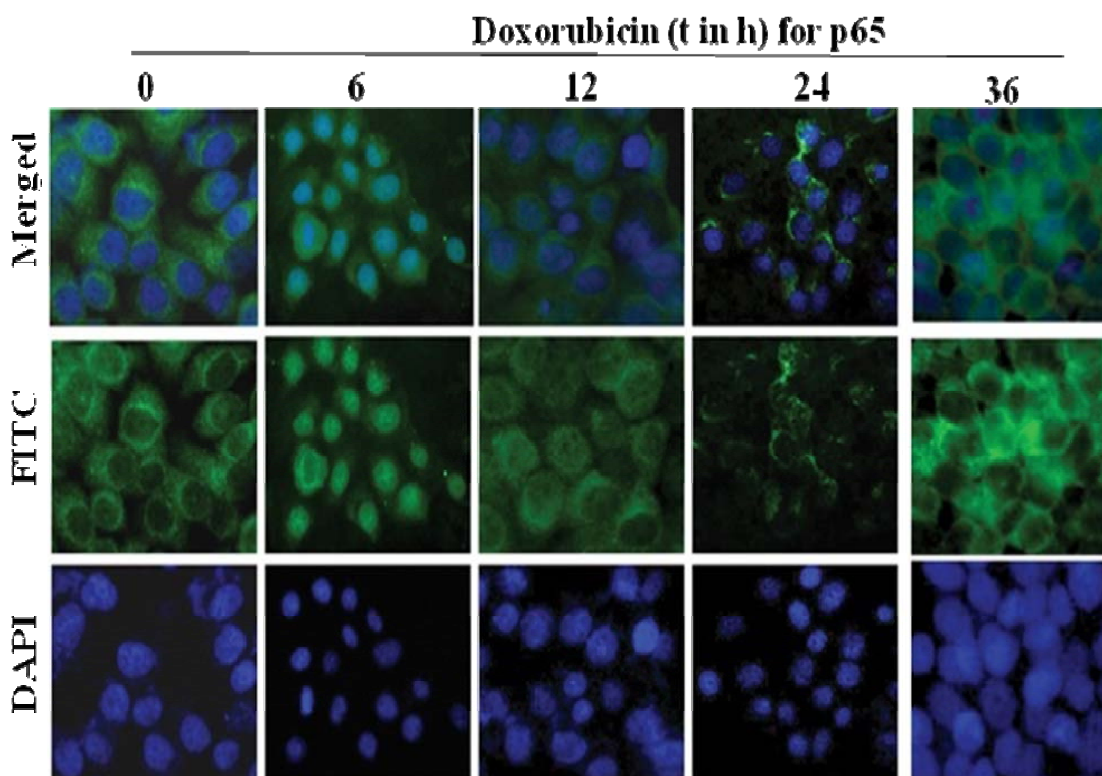


Fig 1C: Effect of doxorubicin on p65 level in MCF-7 cells. Cells were treated with 1μM doxorubicin for indicated time periods. The cells were fixed, incubated with anti-p65 antibody (1:100) followed by Alexa Fluor-labeled anti-rabbit IgG (1:1000) and then mounted with mounting medium containing DAPI. The cells were visualized under fluorescent microscope.

Doxorubicin treatment shows biphasic increase in NF-κB DNA binding and decrease in the amount of p65 in different cell types.

As cell signaling pathways differ in different cell types we studied the effects of doxorubicin treatment in other cell types. Doxorubicin increased biphasic NF-κB

DNA binding almost equally in U-937, A549, and HeLa cells (Fig.2A). The amount of p65 decreased at 12 h in doxorubicin treated cells extract (Fig.2B). These data suggest that the effect of doxorubicin is not restricted to any specific cell types.

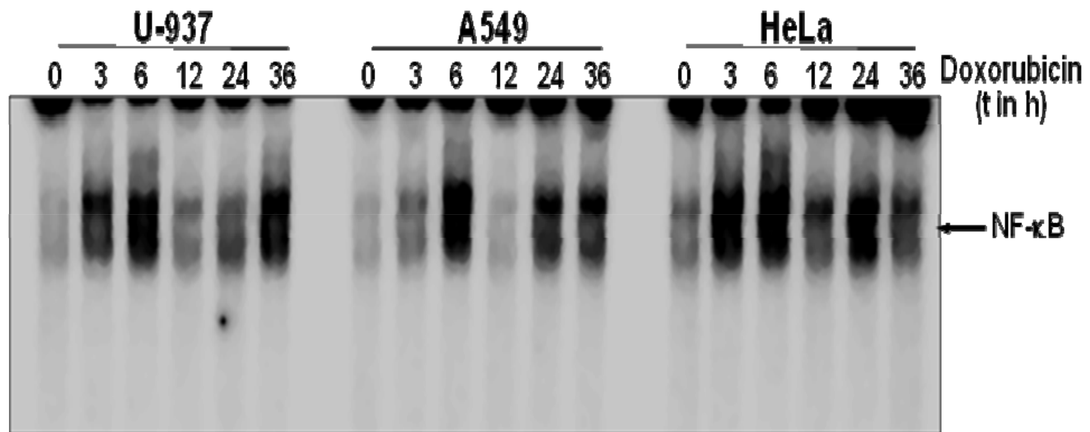


Fig 2A: Effect of doxorubicin on activation of NF-κB in different cell lines. U-937, A549, HeLa cells, were incubated at 37°C with 1 μM doxorubicin for different time intervals, nuclear extracts were prepared and then assayed for NF-κB.

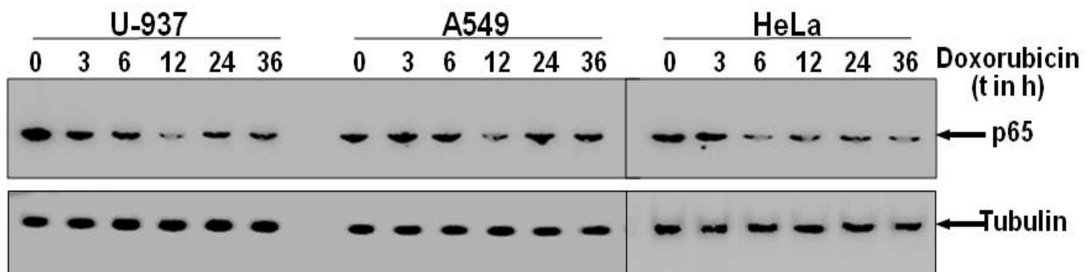


Fig 2B: Effect of doxorubicin on p65 in different cell lines. Cells were incubated at 37°C with 1μM doxorubicin for different times, as indicated and then whole cell extracts were assayed for p65 and tubulin by Western blot analysis.

Proteasome inhibitors, but not protease inhibitors protect doxorubicin-mediated decrease in the amount of p65.

The cells were pretreated with different protease inhibitors and pan caspase inhibitor for 4 h followed by treatment with doxorubicin (1 μM) for 12 h. These results indicated that doxorubicin-mediated decrease in the amount of p65 was not protected by EDTA, EGTA, PMSF, E-64, cocktail of proteases inhibitors, or pan

caspase inhibitor (Fig.3A). Proteasome inhibitors like MG132, but not ALLN or lactacystine protected doxorubicin-mediated decrease in the amount of p65 (Fig.3B). MG132 at 100 μM concentration almost completely prevented doxorubicin-mediated decrease in the amount of p65 (Fig.3B). These data suggest that doxorubicin may be inducing cleavage of p65 in the proteasome.

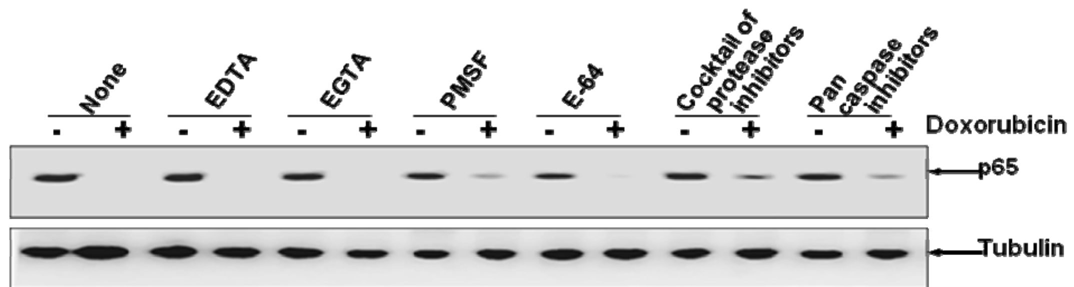


Fig 3A: Effect of protease inhibitors on doxorubicin-mediated decrease in the amount of p65. MCF-7 cells, pretreated with EDTA (100 μM), EGTA (100 μM), PMSF (100 μM), E-64 (50 μM), cocktail of protease inhibitors, or z-VAD-fmk (50 μM) for 4 h were treated with 1 μM doxorubicin for 12 h. The amount of p65 was measured from whole cell extracts by western blot.

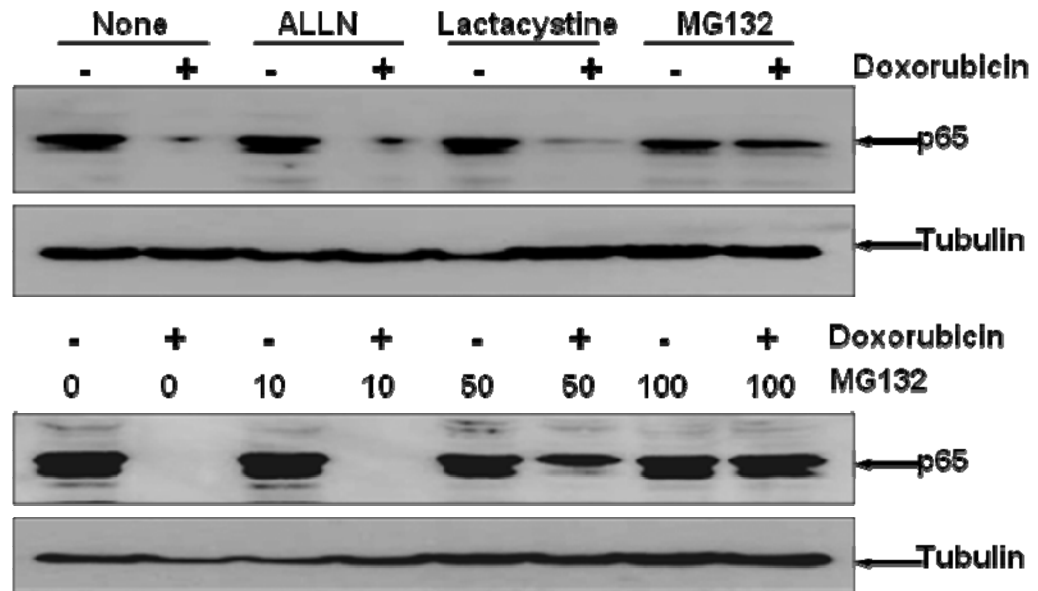


Fig3B: Effect of proteasome inhibitors on doxorubicin-mediated decrease in the amount of p65. MCF-7 cells, pretreated with ALLN (50 μM), lactacystine (10 μM), or MG132 (50 μM) for 2 h were treated with 1 μM doxorubicin for 12 h. Cells, pretreated with different concentrations of MG132 for 2 h were treated with 1 μM doxorubicin for 12 h. The amount of p65 in both experiments was measured from whole cell extract by Western blot.

Role of p65 on doxorubicin-mediated cell death.

MCF-7 cells, transfected with *p65* construct were treated with doxorubicin for 12 h and the amount of p65 was measured from whole cell extracts. Doxorubicin treatment did not decrease amount of p65 significantly in *p65*-transfected cells (Fig.3C). Doxorubicin alone induced 60% ($p<0.001$) cell death. Pretreatment of cells with MG132 followed by doxorubicin showed 44% ($p<0.005$) cell death as detected by MTT dye conversion assay (Fig.3C). In p65-transfected cells, doxorubicin induced 37% ($p<0.001$) cell death and MG132 pretreatment did not interfere further cell death. These data suggest that p65 overexpression might partially block doxorubicin-mediated cell death and proteasome inhibitors does not interfere doxorubicin-mediated cell death.

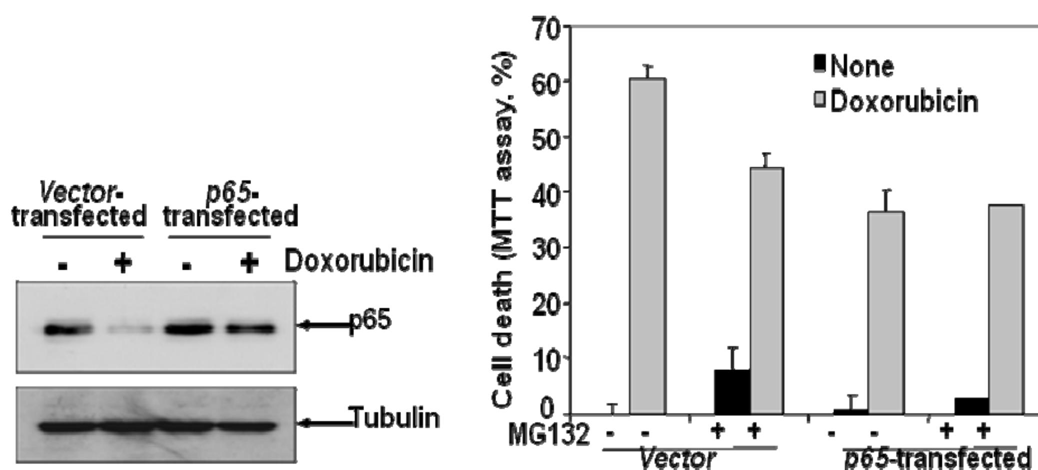


Fig 3C: Effect of doxorubicin on p65 and cell viability in p65 transfected cells. MCF-7 cells, transfected with vector and *p65* constructs for 3 h by Superfect transfection reagent, washed and cultured for 12 h were treated with 1 μM doxorubicin for 12 h. The amount of p65 was measured from whole cell extracts by Western blot. Transfected cells, pretreated with 50 μM MG132 for 2 h were treated with 1 μM doxorubicin for 48 h. Cell viability was detected by MTT dye conversion assay from duplicate samples and results indicated in percentage of cell death.

Doxorubicin induces caspases activation.

Caspase activation is a crucial early event in the commitment of a cell to undergo programmed cell death. Caspases are aspartate-specific cysteine proteases

that when activated cleave numerous cellular proteins, leading to disassembly of the cell. The specificity of caspase activity comes from the unique amino acid sequence each caspase recognizes and cleaves. Depending on the particular apoptotic pathway triggered, specific caspases can be activated. In this study, we found the activity of caspase 8 increased in cells treated with doxorubicin significantly from 24 h (Fig.3D). This result further suggests that proteasome has no role in doxorubicin-mediated cells death, but caspases activation may be taking part.

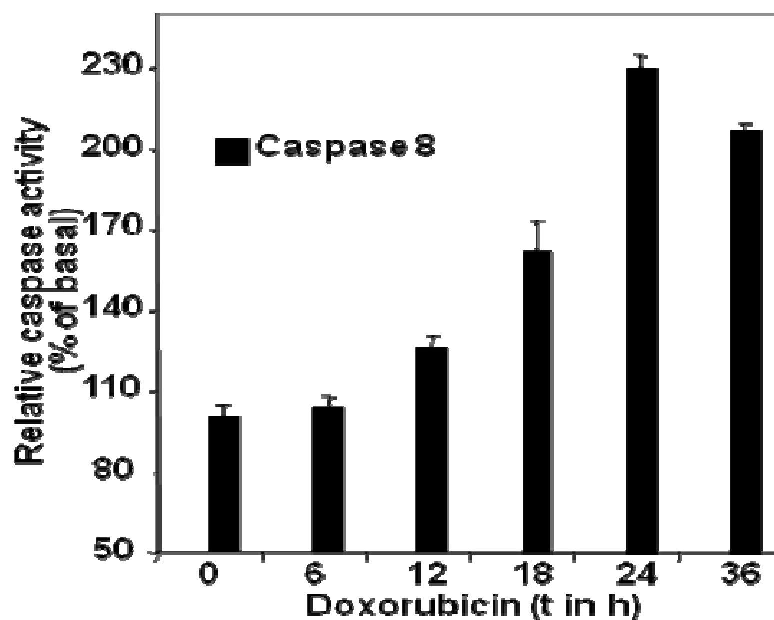


Fig 3D: Effect of caspases on doxorubicin mediated cell death. MCF-7 cells were treated with 1 μM doxorubicin for different times in triplicate samples. Caspase8 activity was measured by incubating the cell extracts with Ac-ITED-pNA as substrate. The absorbance was recorded at 405 nm. Caspase8 activity was indicated in percentage considering unstimulated cells as 100%.

Doxorubicin induces IL-8, FasL and Bax expression.

As doxorubicin-mediated cell death occurs through activation of caspases, the upstream signaling molecules such as FasL, TNF, Bax etc those induce apoptosis pathway were measured. Surprisingly, doxorubicin increased amount of IL-8, but not TNF, in the culture supernatant (Fig.4A).

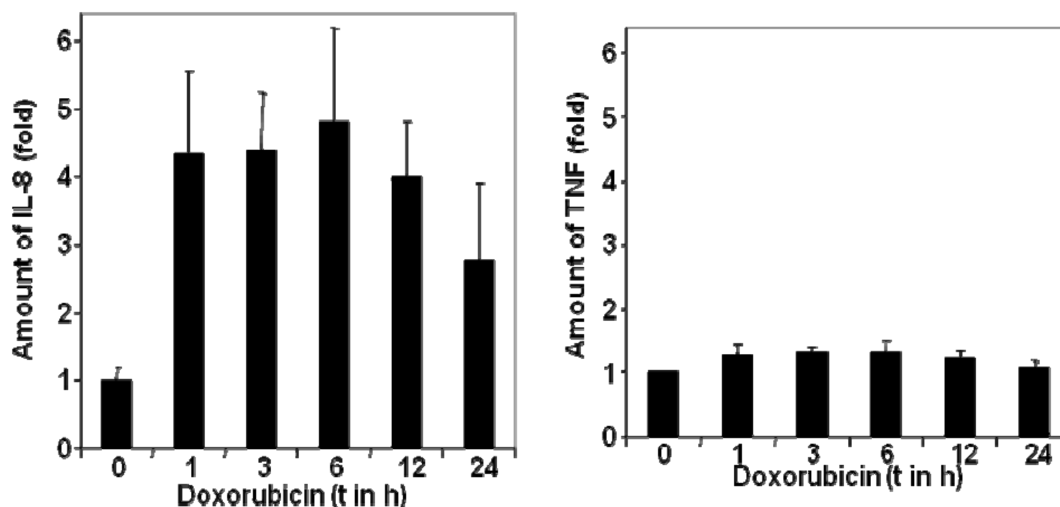


Fig 4A: Effect of doxorubicin on expression of IL-8, TNF. MCF-7 cells were treated with 1 μM doxorubicin for different times in phenol-free medium in triplicate. The supernatant was collected and used to measure IL-8 and TNF from culture supernatant using specific cytokine assay kits and represented as pg/10^6 cells.

As doxorubicin induced biphasic activation in NF-κB DNA binding activity and a decrease in the p65 protein level, the levels of p65 mRNA were also determined by RT-PCR. Also some of the NF-κB dependent genes expressions like IL-8 and TNF (cytokines), cyclin D1 and ICAM 1 (Tumor promotion genes), FasL and Bax (apoptotic), were also estimated by RT-PCR. The basal expression of p65, IL-8, and cyclin D1 decreased between 6-12 h of doxorubicin treatment (Fig.4B) in conformity with biphasic response of NF-κB. Doxorubicin treatment for 24 h decreased the basal expression of TNF or ICAM1 (Fig.4B). The expression of FasL and Bax increased from 6 h and decrease at 24 h of doxorubicin treatment (Fig.4B). These data suggest that doxorubicin induces expression of FasL and Bax; increases IL-8 in culture supernatant at early time of treatment but does not interfere with the amount of TNF.

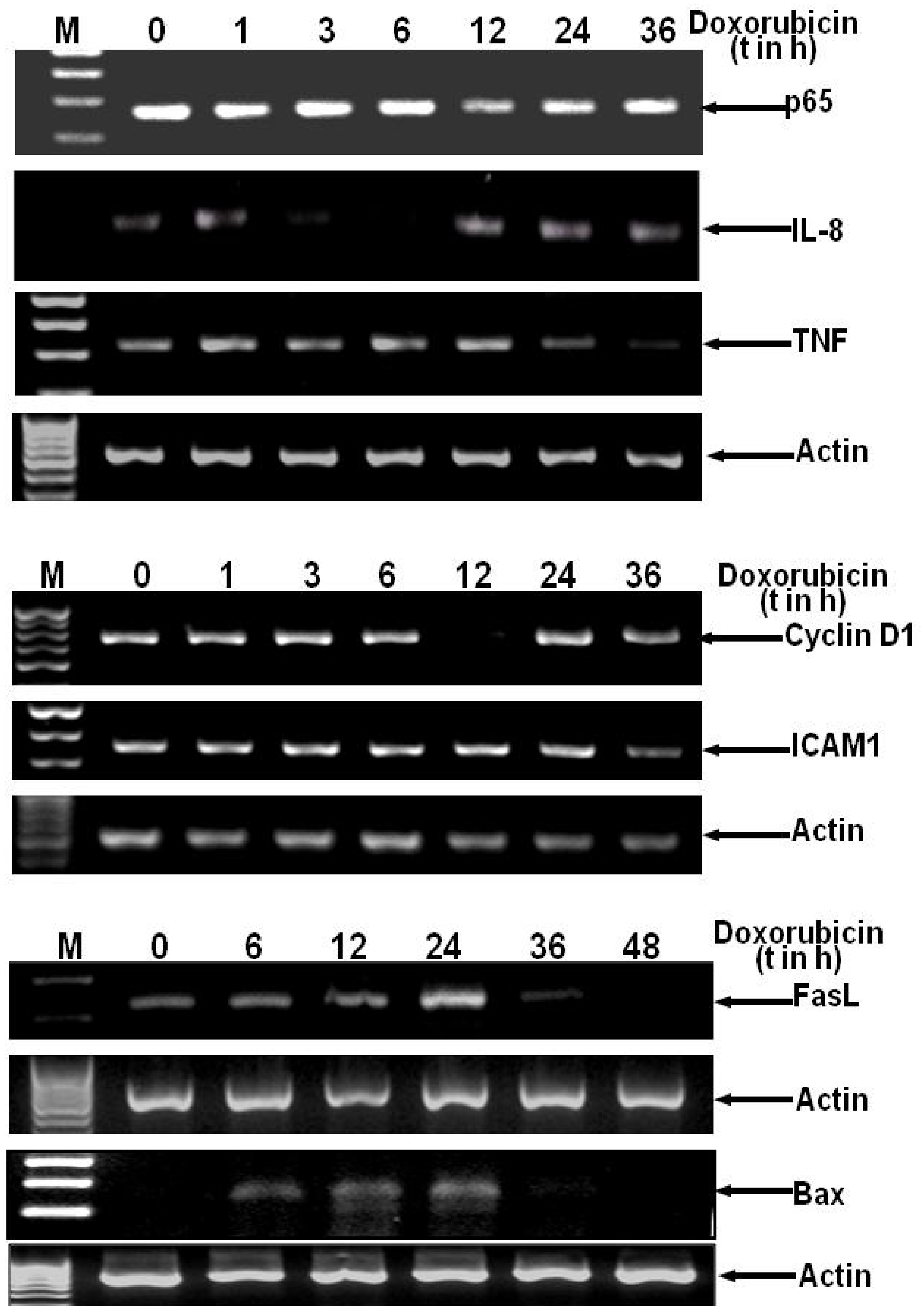


Fig 4B: Effect of doxorubicin on p65 mRNA and NF-κB dependent genes. Cell pellets, after treatment with doxorubicin (1 μM) for different times were extracted for RNA. The isolated RNA was used to measure p65, IL-8, TNF, cyclin D1, ICAM1, FasL, Bax, and actin using specific primers by semiquantitative RT-PCR. Products were detected in 2% agarose gel.

Anti-IL-8 antibody and dominant negative TRAF6 prevent doxorubicin-mediated late induction of NF-κB.

As doxorubicin treatment increases amount of IL-8, a potent inducer of NF-κB (Manna et. al., 2005), we tested the effect of anti-IL-8 Ab on doxorubicin-mediated biphasic activation of NF-κB. Anti-IL-8 Ab, but not anti-TNF Ab, inhibited doxorubicin-mediated late induction of NF-κB DNA binding (Fig.5A).

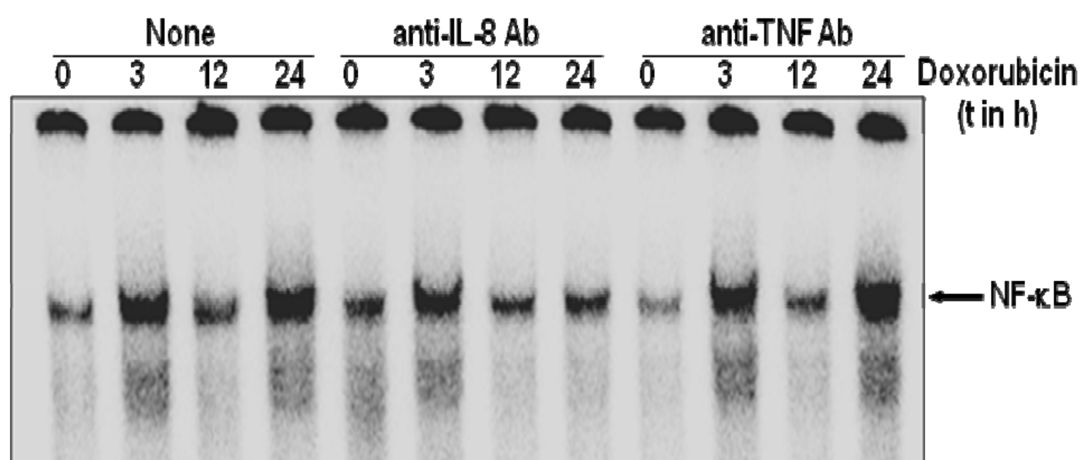


Fig 5A: Effect of anti-IL-8 and anti-TNF antibody on doxorubicin-mediated NF-κB activation. MCF-7 cells were incubated with anti-IL-8 or -TNF Ab (1 μg/ml each) for 2 h and then treated with 1 μM doxorubicin for different times. Nuclear extracts were prepared and assayed for NF-κB DNA binding.

Cytokines, a group of small secreted proteins, are involved in mediation and regulation of immunity, inflammation. Cytokine action is initiated by binding to specific membrane receptors which then signal the cell via second messengers to alter cellular behavior, including gene expression. A known receptor for IL-8 mediated action is CRCR1 whose expression seen on neutrophils and Natural Killer cells. As IL-8 induces NF-κB in cells by recruiting TRAF6 (Manna et. al., 2005), the role of IL-8 in doxorubicin-mediated late induction of NF-κB has tested. The cells transfected with dominant negative *TRAF6* (*TRAF6-DN*), but not *TRAF2* (*TRAF2-DN*) was able to inhibit doxorubicin-mediated late induction of NF-κB DNA binding (Fig.5B) and NF-κB-dependent luciferase expression (Fig.5C). These results

suggested that doxorubicin-mediated late phase activation of NF-κB is due to induction of IL-8 and is mediated through TRAF6.

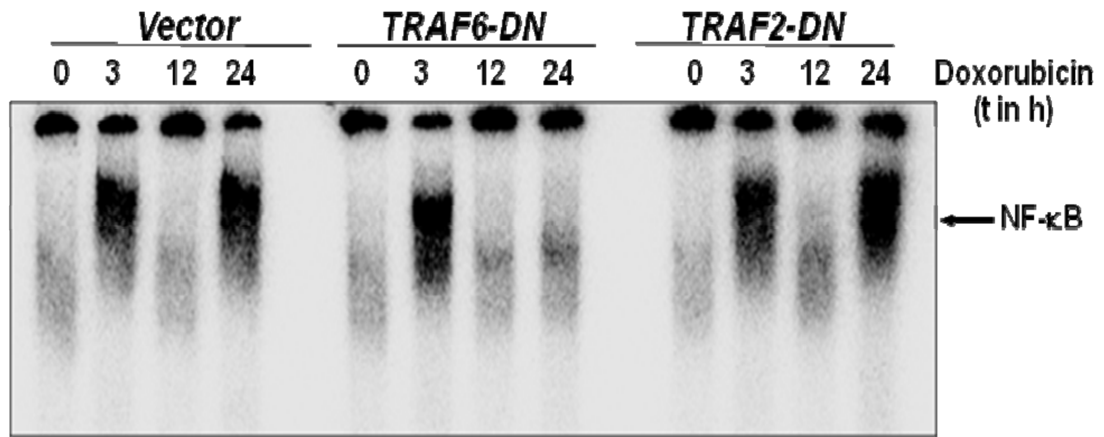


Fig 5B: Role of TRAF6 and TRAF2 on doxorubicin mediated NF-κB DNA binding. MCF-7 cells were transfected with constructs of dominant negative *TRAF2* or *TRAF6* (*TRAF2-DN* or *TRAF6-DN*) for 3 h by Qiagen SuperFect reagent and then treated with 1 μM doxorubicin for different times. NF-κB was assayed from nuclear extracts.

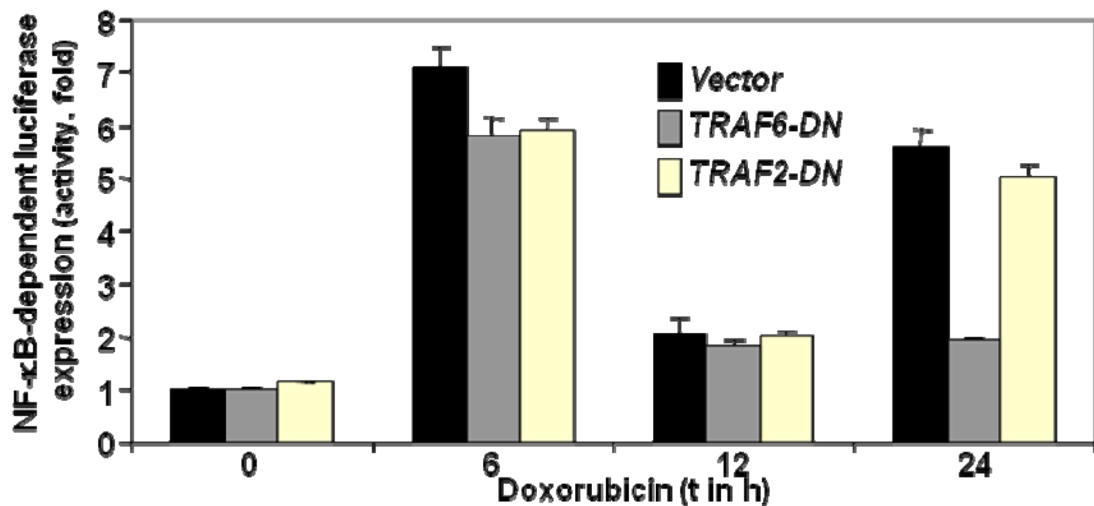


Fig 5C: Role of TRAF6 and TRAF2 on doxorubicin mediated NF-κB dependent luciferase expression. MCF-7 cells were transfected with constructs of dominant negative *TRAF2* or *TRAF6* (*TRAF2-DN* or *TRAF6-DN*) and *NF-κB-luciferase* for 3 h by Qiagen SuperFect reagent and then treated with 1 μM doxorubicin for different times. NF-κB dependent luciferase activity was assayed from whole cell extracts. Fold of luciferase activity was indicated in the figure.

Dominant negative TRAF6, but not TRAF2 protects doxorubicin-mediated cell death.

To define the role of IL-8 in doxorubicin-mediated cell death, cells transfected with *TRAF2-DN* and *TRAF6-DN* were treated with doxorubicin different times and tested for cell death by MTT dye conversion assay. Doxorubicin caused cell death in a time-dependent manner in vector- and *TRAF2-DN*-transfected cells. In *TRAF6-DN*-transfected cells, doxorubicin treatment was unable to induce cell death (Fig.5D). These data further suggested that doxorubicin-mediated cell death is specifically TRAF6 dependent and its action is premeditated by IL-8-TRAF6 pathway.

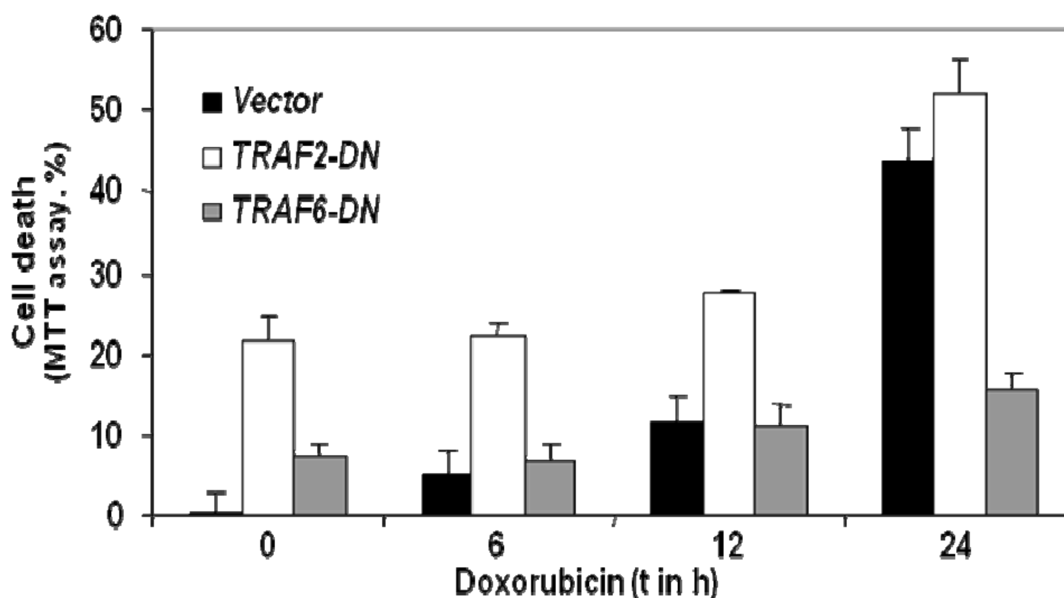


Fig 5D: Role of TRAF6 and TRAF2 on doxorubicin mediated apoptosis. Cell viability was assayed in TRAF6 and TRAF2 transfected cells upon treatment with 1 μM doxorubicin for different times in triplicate samples. The inhibition of cell viability (cell death) was indicated in the figure. The figure represented one of three independent experiments.

TRAF6 binding peptide (TRAF6-BP) protects doxorubicin-mediated late induction of NF-κB and cell death.

To support further, the role of TRAF6 on doxorubicin-mediated cell signaling that leads to late phase activation of NF-κB and induction of cell death, the cells were pretreated with TRAF6-BP and then incubated with doxorubicin for different times.

Doxorubicin showed a biphasic increase in NF-κB DNA binding. Late phase increase in NF-κB DNA binding was inhibited by TRAF6-BP, but not by TRAF6 mutant peptide [TRAF6-BP (Mut)] (Fig.6A). This was further validated with the data obtained on the activity of NF-κB-dependent reporter gene, luciferase for similar treatments (Fig.6B).

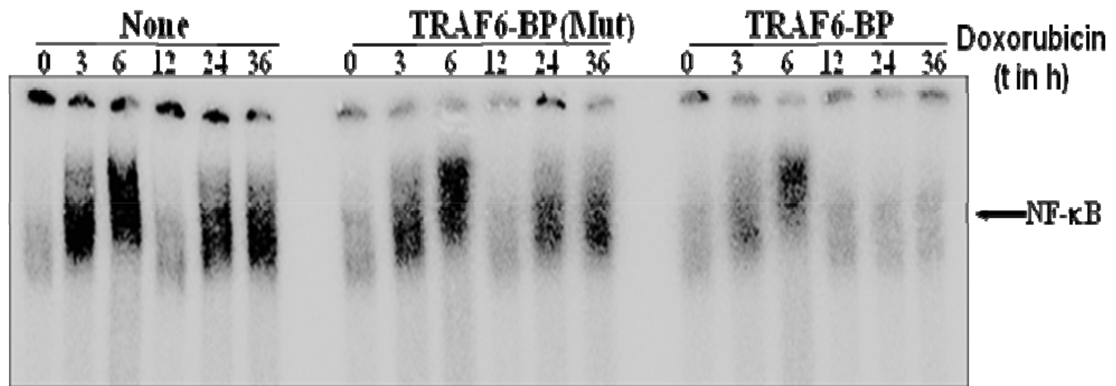


Fig 6A: Effect of TRAF6 binding peptide (TRAF6-BP) on doxorubicin-mediated NF-κB DNA binding. MCF-7 cells were incubated with 200 μM each of TRAF6-BP and mutant peptide [TRAF6-BP(Mut)] for 2 h and then treated with 1 μM doxorubicin for different times. Nuclear extracts were prepared and assayed for NF-κB DNA binding.

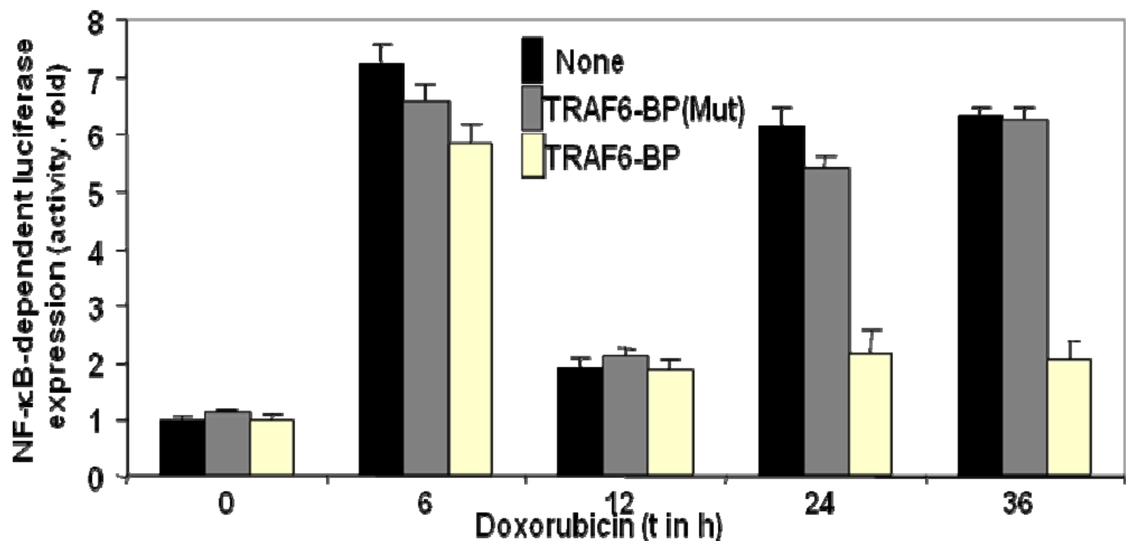


Fig 6B: Effect of TRAF6 binding peptide (TRAF6-BP) on doxorubicin-mediated NF-κB dependent luciferase expression. MCF-7 cells were incubated with 200 μM each of TRAF6-BP and mutant peptide [TRAF6-BP(Mut)] for 2 h and then treated with 1 μM doxorubicin for different times. Cells were transfected with *NF-κB-luciferase* construct for 3 h by Qiagen SuperFect reagent and then treated with 1 μM doxorubicin for different times. Luciferase activity was measured from whole cell extracts. Fold of luciferase activity was indicated in the figure.

IL-8 mediated NF-κB activation and cell death was shown earlier by EMSA as shown in Fig 5C, 5D and 6C. *TRAF6-DN* plasmids were able to inhibit the late phase induction of NF-κB and apoptosis. TRAF6 BP was also able to inhibit late phase of NF-κB activation. Also incubating cells with TRAF6-BP prevented doxorubicin-mediated cell death significantly in a time dependent manner (Fig.6C). These results substantiate the role of TRAF6 in doxorubicin-mediated NF-κB activation and cell death.

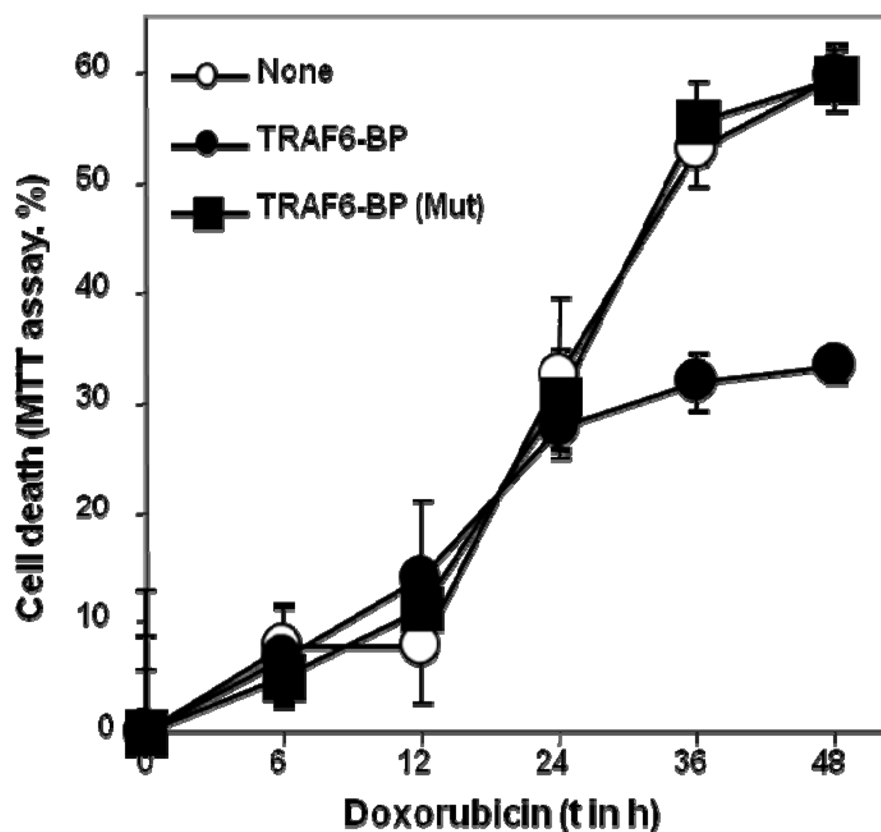


Fig 6C: Effect of TRAF6 binding peptide (TRAF6-BP) on doxorubicin-mediated apoptosis. Cell viability was assayed in MCF-7 cells, pretreated with 200 μM each of TRAF6-BP and TRAF6-BP(Mut) for 2 h and then treated with 1 μM doxorubicin for different times in triplicate samples. The inhibition of cell viability as detected by MTT dye conversion assay (cell death) was indicated in the figure. The figure represented one of three independent experiments.

Doxorubicin increases intracellular Ca^{2+}, calcineurin activity, nuclear translocation of NF-AT, and FasL expression.

Doxorubicin is a very effective anticancer drug producing, among other side effects, a dose-dependent cardiotoxicity which has been related to alterations in intracellular calcium homeostasis. IL-8 is a potent inducer of calcium. The putative action of doxorubicin on intracellular Ca^{2+} fluxes and expression of IL-8 by doxorubicin prompted us to investigate its role in doxorubicin mediated apoptosis. Intracellular Ca^{2+}, as measured by Fura-2AM fluorescence dye increased in cells treated with doxorubicin (0.5 and 1 μM) and IL-8 (100 ng/ml) (Fig.7A).

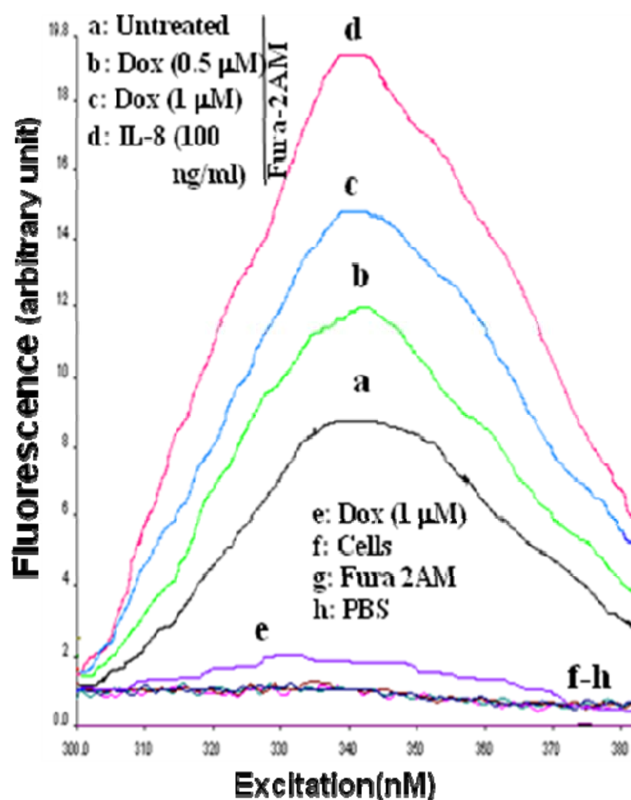


Fig 7A: Effect of doxorubicin on intracellular Ca^{2+} release. MCF-7 cells were treated with 0.5 and 1 μM doxorubicin and 100 ng/ml IL-8 for 12 h. Intracellular free Ca^{2+} measured using Fura-2AM as fluorescent probe in Fluorimeter.

Calcineurin or PP2B (protein tyrosine phosphatase 2B) is a calcium-dependent phosphatase that is activated by a sustained elevation in intracellular

calcium. Calcineurin has been shown to be both necessary and sufficient to induce cardiac hypertrophy *in-vivo* and *in-vitro*. Treatment with the antineoplastic agent doxorubicin was shown to activate calcineurin signaling in H9c2 rat cardiac muscle cells. Doxorubicin treatment also increased calcineurin activity in epithelial MCF-7 cells in time-dependent manner and addition of cyclosporine A (CsA) completely inhibited this effect suggesting the increase in intracellular calcium upon doxorubicin treatment, activated calcineurin (Fig.7B).

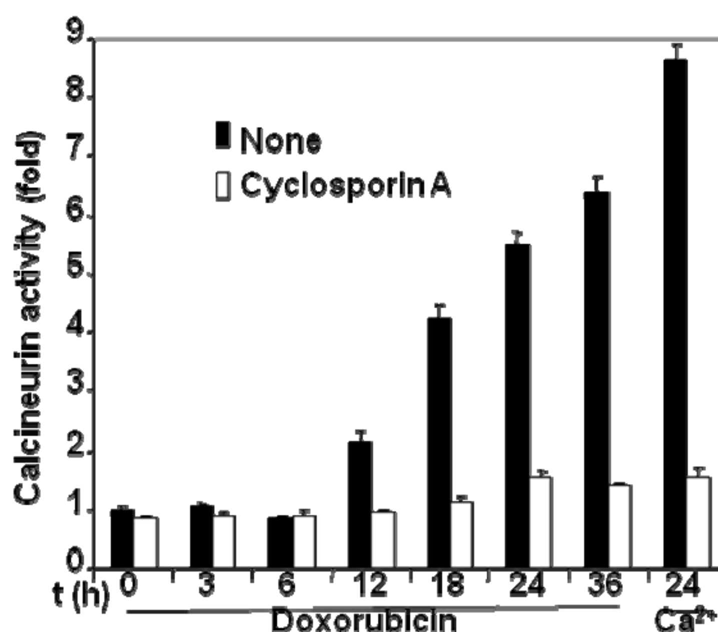


Fig 7B: Effect of doxorubicin on calcineurin activation. MCF-7 cells were treated with doxorubicin (1 μM) alone and with CsA (2.5 μM) for 2 h and then treated with doxorubicin (1 μM) for different times. Calcineurin activity assayed from whole cell extracts.

It was already shown that doxorubicin treatment to cells induces calcium efflux into the cytosol and activates calcineurin (Fig. 7A and 7B). Presence of calcium in the cytosol activates calcineurin, which dephosphorylates the transcription factor, NF-AT allowing the nuclear localization and transcriptional activation of the transcription factor. Also, sequence analysis of the upstream region of FasL gene has revealed potential binding sites for NF-AT. Hence it was appropriate to elucidate the role of NF-AT in doxorubicin treated cells. A gel retardation assay or EMSA was done, where in the nuclear extracts of MCF-7 cells untreated or doxorubicin treated for various time intervals was incubated with ^{32}P labeled NF-AT binding oligo.

According to results as shown in Fig. 7C, doxorubicin treatment increased nuclear NF-AT DNA binding activity in a time-dependent manner and NF-AT-dependent reporter gene, luciferase activity (Fig.7C). Tanslocation of NF-AT from cytoplasm to nucleus was also shown by immunofluorescence data using Alexa-Fluor conjugated anti-NF-AT antibody (Fig.7C).

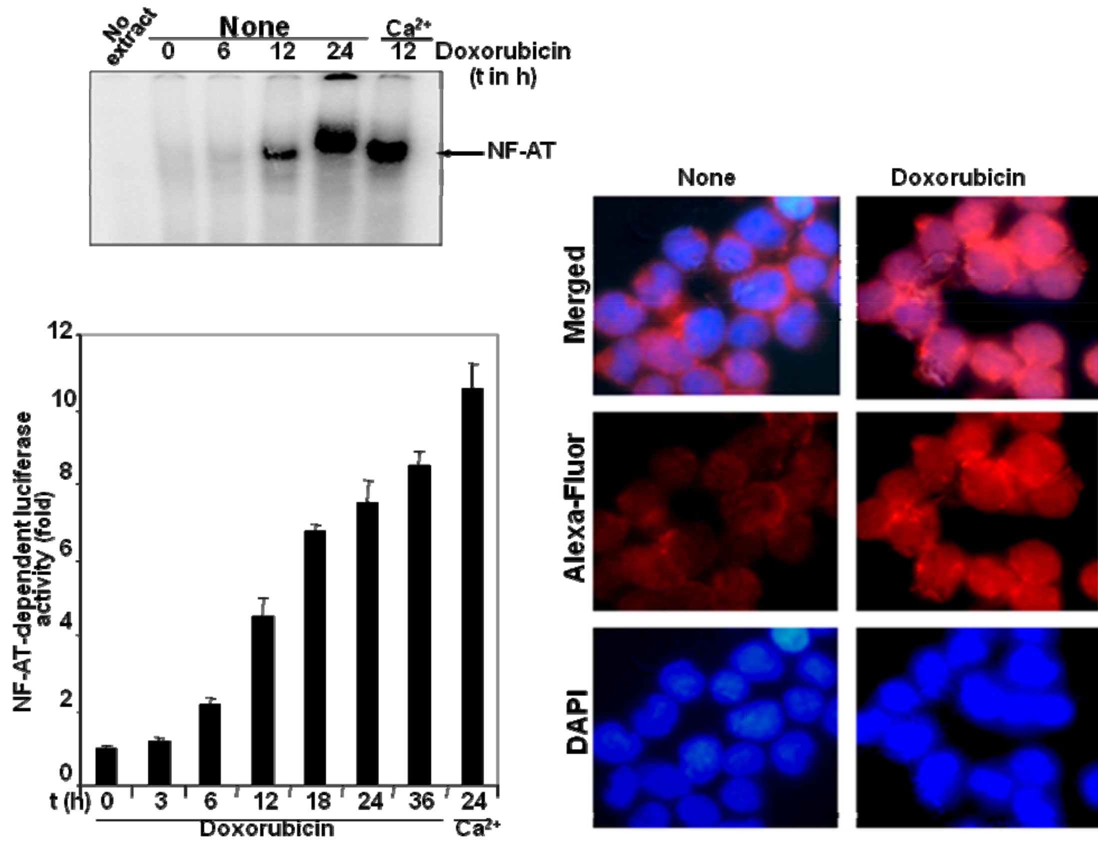


Fig 7C: Effect of doxorubicin on nuclear translocation of NF-AT and NF-AT dependent reporter gene expression. MCF-7 cells were treated with doxorubicin (1 μM) for different times or $CaCl_2$ (10 μM) for 12 h and nuclear extracts were assayed for NF-AT DNA binding by EMSA. Cells were also transfected with Qiagen SuperFect reagent for 3 h with plasmids for NF-AT promoter DNA that had been linked to luciferase (*NF-AT-luciferase*) and GFP. After washing, cells were cultured for 12 h. The GFP positive cells were counted and transfection efficiency was calculated. After treatment with doxorubicin (1 μM) for different times, proteins were extracted and measured the luciferase activity as per Promega protocol. Further MCF-7 cells either treated with doxorubicin for 12 h or untreated were stained with FITC conjugated anti-NF-AT antibodies. These were further viewed under fluorescent microscope.

The addition of doxorubicin (1 μM) to MCF-7 cells induced a significant nuclear translocation of NF-AT after 12-24 h, as monitored by EMSA (Fig 7C). Treatment of cells with $CaCl_2$ caused nuclear translocation of NF-AT in MCF-7 cells (positive control). Results from RT-PCR experiments showed the transcription of FasL mRNA increased during doxorubicin treatment (Fig 4B), reaching a maximum after 24 h. A similar trend was also observed with FasL promoter activity under these conditions (Fig 7D). Doxorubicin treatment increased the amount of FasL-dependent luciferase activity. All these data suggested that doxorubicin increases intracellular Ca^{2+} that leads to activation of calcineurin, nuclear translocation of NF-AT, and NF-AT-dependent FasL expression.

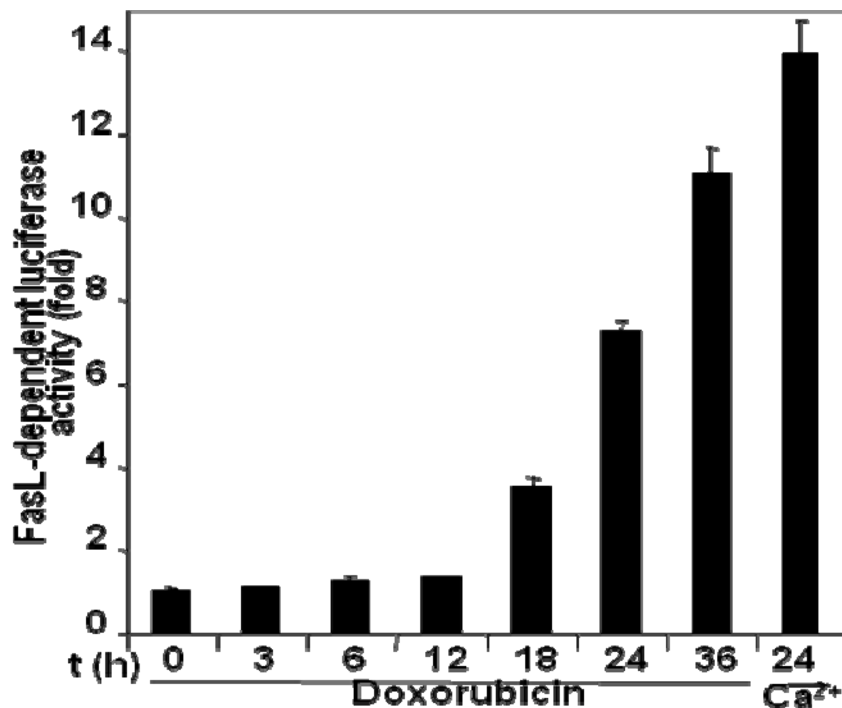


Fig 7D: Doxorubicin induced FasL expression. MCF-7 cells were transfected with Qiagen SuperFect reagent for 3 h with plasmids for FasL promoter DNA that had been linked to luciferase (*FasL-luciferase*) and GFP. After washing, cells were cultured for 12 h. The GFP positive cells were counted and transfection efficiency was calculated. Cells, treated with doxorubicin (1 μM) for different times were extracted and the luciferase activity measured as per Promega protocol.

Depletion of Ca^{2+}, inhibition of calcineurin, or incubation of anti-IL-8 or anti-FasL Ab partially protects doxorubicin-mediated cell death.

Doxorubicin induced apoptosis was significantly attenuated when MCF-7 cells were pretreated with CsA (a potent inhibitor of calcineurin), or BAPTA-AM (an intracellular calcium chelator). A similar trend was also noticed when cells were pre-incubated with IL-8 or FasL neutralizing antibodies. Treatment with CsA, BAPTA-AM, anti-IL-8 Ab, or anti-FasL Ab protected cell death substantially at any time of doxorubicin treatment (Fig.7E). These results imply that doxorubicin treatment induces a calcium/calcineurin-dependent increase in NF-AT translocation into the nucleus, transcription of FasL and subsequently activation of caspases in doxorubicin treated cells and it is not the only pathway that doxorubicin mediates its apoptotic effect. Further confirming that doxorubicin activates multiple signaling pathways to induce apoptosis.

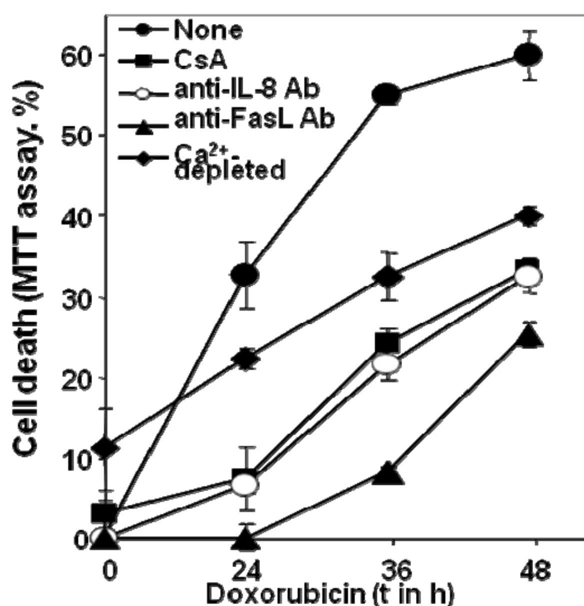


Fig 7E: Effect of CsA, BAPTA-AM, anti-IL-8 Ab, or anti-FasL Ab on doxorubicin mediated apoptosis. MCF-7 cells, incubated with cyclosporin A (2.5 μM), BAPTA-AM (2.5 μM), anti-IL-8 Ab (1 μg/ml), or anti-FasL Ab (1 μM) for 4 h and then were treated with doxorubicin (1 μM) for different times in triplicate. The inhibition of cell viability as detected by MTT dye conversion assay (cell death) was indicated in the figure. The figure represented one of three independent experiments.

Discussion

Doxorubicin is widely used chemotherapeutic drug for treating breast cancer and many other tumors. This study provides evidence in support of the concept that doxorubicin-mediated cell signaling leads to cell death. Doxorubicin treatment often causes DNA damage and oxidative stress that leads to change in mitochondrial membrane potential followed by cell death (Eom et. al., 2005; Ecuyer et. al., 2006; Yeh et. al., 2007). In terms of cell signaling, it increases DNA binding activity of NF-κB known for its proapoptotic activity mediated by activation of proapoptotic factors like Bax (Ostrakhovitch et. al., 2005). However, most of the effects of NF-κB are cell proliferative. We analysed the pattern of NF-κB induction upon doxorubicin treatment. Interestingly, doxorubicin-induced NF-κB DNA-binding activity was bimodal with its strongest peak at 3 to 6 h post treatment and a reproducible second increase in DNA-binding noted at 24 to 36 h. Others have found that NF-κB activity can oscillate over time in response to the TNF-α as a function of the levels of IκB proteins in the cell, but this work has not been reported previously when cells have been exposed to chemotherapy. NF-κB activation is redox-sentitive (Anddrassy et. al., 2002, Shimokawa, 2004, Fischer et. al., 2006). Doxorubicin is known to increase reactive oxygen species by reacting with cellular iron (Kotamraju et. al., 2002) and this may cause the initial activation of NF-κB. Late phase activation of NF-κB is very much surprising as doxorubicin induces cell death at this time of incubation. As such, it is possible that the source for delayed/ second phase induction of NF-κB is the expression of NF-κB dependent cytokines by the early phase NF-κB. To better understand the bimodal NF-κB activation we looked into the mechanisms leading to the decrease in p65. The decrease in p65 protein at 12 h of doxorubicin treatment from whole cell extract may be due to inhibition of p65 synthesis or action of proteases. RT-PCR data for p65 ruled out the inhibition of p65 synthesis. Several protease inhibitors did not protect, but proteasome inhibitors, especially MG132 protected doxorubicin-mediated decrease in the amount of p65. How doxorubicin induces proteasome and decreases in the amount of p65 need to be studied further. Though doxorubicin treatment decreases p65 subunit of NF-κB at 12 h, it has no significant impact on cell death as shown in *p65*-overexpressed cells. This suggested that other factors were involved in cell death mediated by doxorubicin.

Apoptosis is a suicide program that is involved in the elimination of superfluous or traumatized cells and is mediated by several members of the caspase family of proteolytic enzymes. Doxorubicin treatment also increased activities of caspases. Recruitment of proteins containing death domain is a prerequisite for activation of caspases. Recruitment of these proteins is often preceded by interaction of death ligands like FasL, TNF, and TRAIL with their respective receptors. Several studies have shown that commonly used chemotherapeutic drugs can stimulate the expression of NF-κB -dependent inflammatory cytokines, such as TNF, IL-1, FGF, G-CSF, VEGF, and IL-6 (Ujhazy et. al., 2003, Levina et. al., 2008). We found that doxorubicin activated caspases and induced the production of IL-8. Doxorubicin treatment did not enhance TNF expression another NF-κB dependent gene, from early hours of treatment. Several reports showing that signal from some cytokines might select induction of apoptosis in target cells suggested the role of IL-8 in doxorubicin mediated apoptosis (Terui et. al., 1998). The basal expression of some of the NF-κB-dependent genes such as IL-8 and cyclin D1, but not TNF and ICAM1, decreased between 6 to 12 h of doxorubicin treatment. Some of these genes are dependent on activator protein 1 (AP-1) also. It is also reported that doxorubicin-mediated cardiotoxicity was attenuated by soluble Fas by suppressing induction of proinflammatory cytokines, such as TNF, IL-1 and IL-6 (Niu et. al., 2009). Increased expression of FasL by doxorubicin treatment suggested the doxorubicin-mediated cell death proceeds through FasL-Fas mediated pathway that leads to late activation of caspases. Expression of proapoptotic Bax at early time of doxorubicin treatment proves it as an NF-κB-dependent gene product which may facilitate FasL-dependent apoptosis through caspase-dependent pathway. Surprisingly, IL-8-mediated cell signaling proved to be the important determinant for doxorubicin-mediated cell death. Substantial abrogation of doxorubicin-mediated cell death and late phase of NF-κB activation on preincubation with anti-IL-8 Ab, affirmed the role of IL-8 as an intermediate factor in doxorubicin-mediated cell signaling. IL-8 induces NF-κB through recruitment of TRAF6 (Manna et. al., 2005). Doxorubicin-mediated late phase activation of NF-κB was inhibited by dominant negative TRAF6, but not TRAF2 transfected cells and anti-IL-8 Ab, but not -TNF Ab incubated cells. IL-8-mediated cell signaling via recruitment of TRAF6 was

further supported by the inhibition of late phase activation of NF-κB with TRAF6 binding peptide (TRAF6-BP). Protection from doxorubicin-mediated cell death by TRAF6-BP indicates the involvement of IL-8. Govindaraju et. al. (2008) demonstrated that airway smooth muscle from healthy individuals expressed CXCR1 and CXCR2 and that IL-8 increases intracellular calcium and triggers contraction, suggested the role of IL-8 to be a potent inducer of intracellular Ca^{2+}(IL-8 potent inducer of calcium). We also observed that doxorubicin treatment increased intracellular Ca^{2+} in MCF-7 cells. More over BAPTA-AM, an intracellular Ca^{2+} chelator, substantially inhibited doxorubicin-mediated cell death, further suggesting the role of calcium. In the signaling routes of apoptosis, Ca^{2+}-dependent serine threonine phosphatase calcineurin play an important role by blocking Bcl-2: calcineurin dephosphorylates and activates BAD further enhancing BAD heterodimerization with BCl-XL and promoting apoptosis (Rizzuto et. al., 2003). Regulation of acetylcholinesterase expression by calcineurin and its target NFAT in calcium ionophore A23187-induced apoptosis further confirmed the role of calcineurin in apoptosis (Zhu et. al., 2007). Doxorubicin treatment increased calcineurin activity in epithelial MCF-7 cells in time-dependent manner and addition of cyclosporine A (CsA) completely inhibited this effect. Further doxorubicin treatment induced a significant nuclear translocation of NF-AT after 12-24 h, as monitored by EMSA in MCF-7 cells. Late induction of caspases and anti-FasL Ab-mediated inhibition of doxorubicin-induced cell death suggested the role of FasL in doxorubicin-mediated cell signaling. These results imply that the activation of transcription factor NF-κB upon doxorubicin treatment was proapoptotic and is mediated through IL-8.

One major obstacle in chemotherapy is resistance of tumor cells against cytostatic drugs. Doxorubicin is one of the most important anticancer drugs for the treatment of solid tumors and it has been shown to induce apoptosis (Hortobagyi, 1997). Unfortunately, repetitive doxorubicin treatment induces resistance of cancer cells, resulting in therapeutic failure (Rittierodt & Harada, 2003; Puhlmann et. al., 2005). Therefore, a better understanding of doxorubicin-induced signaling pathways in cancer cells is needed to overcome resistance and to enhance the efficacy of anthracycline treatment. Also a detailed study of the mechanism of action

would help to design the chemotherapeutic drugs for effective combination therapy with doxorubicin. We found that NF-κB DNA binding activity was initially induced by doxorubicin and this NF-κB expresses several NF-κB -dependent genes. IL-8 is one of these factors which induced cell death through calcineurin-NF-AT-FasL pathway. We provide evidences for the first time that doxorubicin-mediated cell death is proceeding through expression of IL-8, IL-8-mediated calcification that leads to calcineurin activation, calcineurin-mediated shuttling of NF-AT from cytosol to nucleus, with associated increase in the expression of FasL, in turn induces cell death via caspase-dependent pathway. This study will help to understand doxorubicin-mediated chemoresistance and further designing other molecules for combination chemotherapy to intervene several tumors involving doxorubicin.

Chapter-IV

Inhibition of Constitutive Activity of Nuclear Transcription Factor kappa B Sensitizes Doxorubicin-Resistant Cells to Apoptosis.

Introduction

As many as 30% of women diagnosed with early breast cancer will eventually progress to or relapse with locally advanced or metastatic breast cancer. Resistance to the commonly used chemotherapies (anthracyclines), as well as the approval of new pharmacologic options for treating breast cancer, presents important clinical, cost-effective, and societal challenges. Doxorubicin is an anthracycline antibiotic, which remains an important agent in many chemotherapy regimens. The drug is able to induce regression of metastatic breast cancer alone (Blum et. al., 1974; Harris et. al., 1993) and constitutes the core of most combination chemotherapeutic regimes currently used to treat breast cancer. Although doxorubicin is currently considered to be one of the most effective agents in the treatment of breast cancer, resistance and cadiotoxic side effects leads to an unsuccessful out come in many patients. Multiple molecular, cellular, micro-environmental and systemic causes of anticancer drug resistance have been identified during the last 25 years. At the same time, genome-wide analysis of human tumor tissues has made it possible to assess the expression of critical genes or mutations that determine the response of an individual patient's tumor to drug treatment. The identification of novel biomarkers, which correlate with treatment response, would allow therapy to be tailored on an individual patient basis. Biomarkers may also provide information on new drug targets for future therapeutic intervention. Overcoming resistance to doxorubicin would represent a major advance in the effective management of breast cancer. Although the mechanisms remain unclear, it is accepted that the development of drug resistance is a multi-factorial phenomenon.

Conventional cytotoxic drugs frequently fail as a treatment modality due to the development of drug resistance that blocks their activity. Acquired drug resistance is a multi-factorial phenomenon, involving multiple genetic and epigenetic mechanisms (Stavrovskaya, 2000; Gottesman, 2002; Fojo, 2007). Combination chemotherapy has been shown to be more effective than single-agent therapy for many types of cancer, but both are known to induce drug resistance in cancer cells. Two major culprits in the development of drug resistance are nuclear

factor-kappaB (NF-κappaB) and the multidrug resistance (MDR) gene. Aberrantly active NF-κB complexes can contribute in tumorigenesis by regulating genes that promote the growth and survival of cancer cells (Giri et. al., 1998; Manna et. al., 1999a; Barre et. al., 2007). NF-κB activation is known to regulate an array of downstream antiapoptotic genes, such as cIAP-2 and Bcl-xL (Hinz et. al., 2001; Wang et. al., 2003). It is this antiapoptotic function of NF-κB that has implicated this family of transcription factors in the development of cancers, chemoresistance, and potential target in treatment of many cancers. Overexpression of NF-κB or mutation of its natural inhibitor, IκBα, has been found in several tumors (Gilmore et. al., 1996; Rayet & Gelinas, 1999; Cabannes et. al., 1999). Furthermore, NF-κB activation is closely related to drug-resistance of tumor cells, partly due to its associated increase of the apoptosis threshold (Wang et. al., 1996; Van Antwerp et. al., 1996; Patel et. al., 1996). Although many tumor cells display some level of constitutive activity of nuclear NF-κB, the transcription potential of it can be further enhanced in response to certain types of chemotherapy (Chuang et. al., 2002). Constitutive expression of NF-κB has been implicated as one of the causes for drug resistance in tumors and suppression of NF-κB activation may inhibit tumor growth and increase the efficacy of chemotherapy (Bargou et. al., 1997; Cusack et. al., 1999; Duffey et. al., 1999; Wang et. al., 1999a; Wang et. al., 1999b; Yamamoto & Gaynor, 2001). This study indicates that NF-κB plays an important role in chemoresistance and establishes NF-κB inhibition may have implication for refining systemic chemotherapy in future.

NF-κB is a heterodimer of two subunits p50 (NF-κB1) and p65 (RelA). It is normally present in the cytoplasm in an inactive state in complex with an inhibitory subunit of kappa B (IκBα). Upon phosphorylation and subsequent degradation of IκBα, a nuclear localization signal on the p50-p65 heterodimer is exposed, leading to nuclear translocation of NF-κB. The p50–p65 heterodimer binds with a specific sequence in DNA, which in turn results in gene transcription. Phosphorylation of p65 of NF-κB (RelA) is required for effective NF-κB-dependent gene transcription (Jobin et. al., 2000; Bonizzi et. al., 2004; Hayden et. al., 2004). NF-κB induction can be abrogated using NF-κB inhibitors which may function by various mechanisms including the inhibition of IκB phosphorylation, inhibition of IκB proteasome-

mediated degradation, and inhibition of translocation of activated NF-κB dimers to the nucleus. BAY 11-7082 ((*E*)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile) is an irreversible inhibitor of IκBα phosphorylation which results in the down-regulation of the cytokine-induced NF-κB activation (Woods et. al., 2000; Keller et. al., 2000) and apoptosis (Izban et. al., 2000). Treatment of BAY 11-7082 has shown to inhibit IκBα phosphorylation (Izban et. al., 2000; Remacle-Bonnet et. al., 2000; Mori et. al., 2002) thereby blocks proteasomal degradation of IκBα, and hence sequestering NF-κB in the cytoplasm in an inactivated state. P_3-25 is shown to block p65 phosphorylation and to inhibit NF-κB -dependent reporter gene transcription (Goffi et. al., 2005; Manna et. al., 2007).

Doxorubicin, an anthracycline is a major anti-tumor agent used in the treatment of a variety of cancers. The mechanisms by which doxorubicin drives cancer cells into apoptosis are related to its ability to disturb DNA function and to induce DNA damage. Doxorubicin can intercalate into the DNA double helix, inhibit topoisomerase II and cross-link DNA strands (Gewirtz, 1999). Furthermore it is known, that doxorubicin produces reactive oxygen species (ROS) via one-electron reduction to the corresponding semiquinone free radicals which then react rapidly with oxygen to generate superoxide radical anions (Navarro et. al., 2006). At moderate concentrations doxorubicin induced ROS can act as important effectors modulating diverse signalling pathways like the redox-sensitive NFκB pathway (Chovolou et. al., 2007), the p53 tumor suppressor pathway (Wang et. al., 2004) and the PI3K/AKT pathway (Yu, et. al., 2007; Lüpertz et. al., 2008). Evidences demonstrate that cell response to doxorubicin is highly regulated by multiple signaling events, including generation of sphingosine (Reynoldsa et. al., 2004; Jurisicova et. al., 2006) and activation of different caspases, c-Jun N-terminal kinase (JNK) (Mikami et. al., 2006), transcription factors (Chuang et. al., 2002), and Fas/Fas ligand system (Kalivendi et. al., 2005). Drug resistance to chemotherapy agents such as doxorubicin appears to be an important cause of therapeutic failure in cancer treatment. The development of cellular resistance mechanism to chemotherapeutic drugs includes altered expression of different proteins like multi-drug resistant genes, transglutaminase (TGase), Bcl-2, and deficient intracellular calcium pools,

and loss of p53 function (Chen et. al., 2002; Jin et. al., 2007; Verma et. al., 2007). Resistance to doxorubicin is also "multi-factorial", decreased drug uptake, decreased formation of DNA single- and double-strand breaks, increased GST activity, earlier onset of DNA repair, as well as elevated P-glycoprotein are all characteristic of doxorubicin resistance (Deffie et. al., 1998). Unfortunately, clinically acquired resistance to a single anticancer agent such as doxorubicin is frequently accompanied by the development of cross-resistance to multiple drugs. Various agents such as verapamil have been used to overcome the doxorubicin resistance in various clinical studies but were unsuccessful. Much remains to be learned about the mechanisms of doxorubicin induced apoptosis and resistance against it.

Administration of suboptimal doses of anticancer drugs not only fails to control tumor but often results in increased drug resistance of tumor cells. In the laboratory conditions, development of doxorubicin resistance in a human breast cancer cell line (MCF-7) is a result of propagation of an inherently resistant sub clone. Doxorubicin resistant and revertant MCF-7 cells were a gift from Dr. Kapil Mehta, MD Anderson Cancer Centre, Houston, Texas, USA. Doxorubicin resistant cells were cultured in presence of 2 μM doxorubicin. The drug resistant subline was established from the parental MCF-7 cells by culturing in continuous presence of 2 μM doxorubicin. During the first week of culture, the drug killed majority of the cells. Nevertheless, we continued maintaining the cultures by changing drug-containing medium every 3-4 days. After about 2 weeks of culture, few actively dividing cells were observed in the flask that grew to confluency in about 2 weeks. We then used these cells to characterize the drug-resistance phenomenon. The parental MCF-7 cells thus survived and grew in presence of doxorubicin (Dox-resistant), consistently exhibited 60 to 80-fold resistance to doxorubicin compared with the parental MCF-7 cells. A revertant MCF-7 cells subline was also established by culturing the drug-resistant subline in the absence of doxorubicin for 6 months (Devarajan et. al., 2002). While culturing, these cells might change some genetic make-up that leads to decrease in several molecules involved in resistance against doxorubicin behaves like wild type cells.

As NF-κB is currently being used as a target for cancer therapy, it is of interest to see the possible interplay of NF-κB and doxorubicin resistance. Interestingly, doxorubicin, like several chemotherapeutic agents taxol, vincristine also causes NF-κB upregulation. Several reports indicate that constitutive or induced NF-κB expression leads to resistance of apoptosis in tumor cells stimulated by a wide variety of agents. Moreover inhibiting NF-κB by adenoviral IκBα or proteosome inhibitors are currently being tested to overcome chemotherapy-induced resistance. In this study, we demonstrate that doxorubicin induces NF-κB in wild type and Dox-revertant, but not in Dox-resistant MCF-7 cells. Dox-resistant cells show high basal NF-κB activity and expression of the genes dependent on NF-κB. We also demonstrate that high NF-κB activity confers resistance to Dox-resistant cells and down regulation of NF-κB in these cells (by transfecting with *IκBα-DN* construct or treating with BAY 11-7082 (known as BAY), an IκBα kinase (IKK) inhibitor and P_3-25 [5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine], a synthetic derivative of thiadiazolidine sensitizes the cells to apoptosis. High basal NF-κB activity in doxorubicin-resistant cells confers basal expression of Bcl-2, superoxide dismutase (SOD), multi-drug resistance gene (Mdr), and transglutaminase. Basal amounts of reactive oxygen intermediates (ROI) might maintain high basal activity of NF-κB in Dox-resistant cells that leads to expression of its dependent genes like Bcl-2, Mdr, TGase, or superoxide dismutase (SOD). The SOD1 (Cu/Zn SOD), the most abundant and ubiquitous isoform, has great physiological significance and therapeutical potential in several neurodegerative disorders and it is a NF-κB-dependent gene product (Rojo et. al., 2004). This SOD did not allow further increase in ROI generation by doxorubicin treatments in Dox-resistant cells. Hence, understanding the molecular mechanisms of doxorubicin-mediated apoptosis and a comparative study on doxorubicin sensitive and resistant tumors will help to improve the potency of cytotoxicity and decrease the resistance of tumor and adopt combination therapy for effective treatment of tumor.

Results

In this study, we examined the effect of doxorubicin in MCF-7 (designed as Wild), doxorubicin-resistant (Dox-resistant) and revertant (Dox-revertant) MCF-7 cells. Dox-resistant cells were cultured in presence of 2 μM doxorubicin. The doxorubicin, BAY, and P_3-25 were used as a solution in DMSO at 10 mM concentration. Further dilution was carried out in cell culture medium.

Dox-resistant cells show constitutive activation of NF-κB.

MCF-7 cells (Wild, Dox-resistant, and Dox-revertant) were treated with 1 μM doxorubicin for different times; nuclear extracts were prepared, and 8μg nuclear extract were analyzed in 6.6% native page to detect NF-κB by gel shift assay. As shown in Fig.1A, Dox-resistant cells showed high basal NF-κB activity, whereas the Wild type or Dox-revertant cells showed transient induction of NF-κB. Oct1 DNA binding activity did not alter in those samples as shown by gel shift assay (Fig.1B).

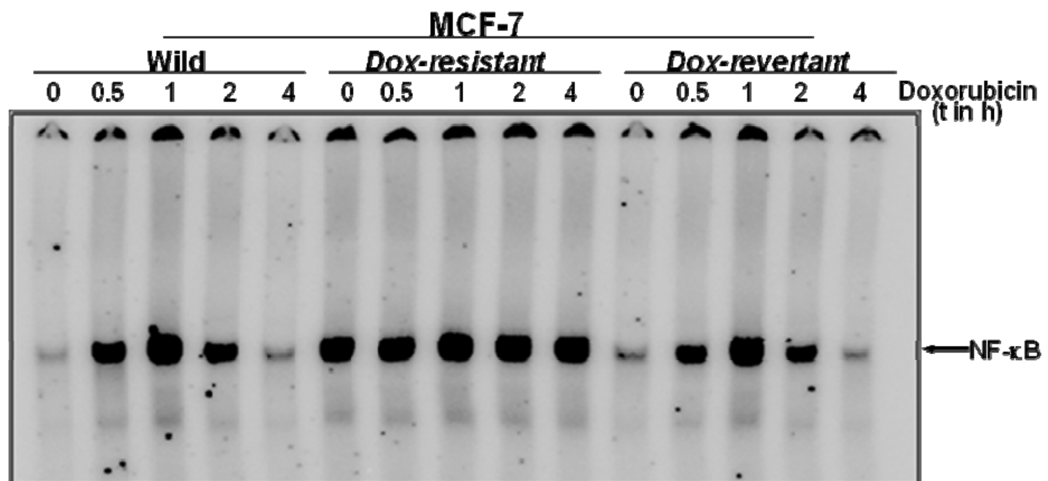


Fig 1A. Effect of doxorubicin on NF-κB activation in wild type, Dox-resistant and Dox-revertant cells. MCF-7 (Wild type, Dox-resistant and Dox-revertant) cells were treated with 1 μM doxorubicin for different times. After these treatments, cytoplasmic (CE) and nuclear extracts (NE) were prepared and NE was assayed for NF-κB by gel shift assay.

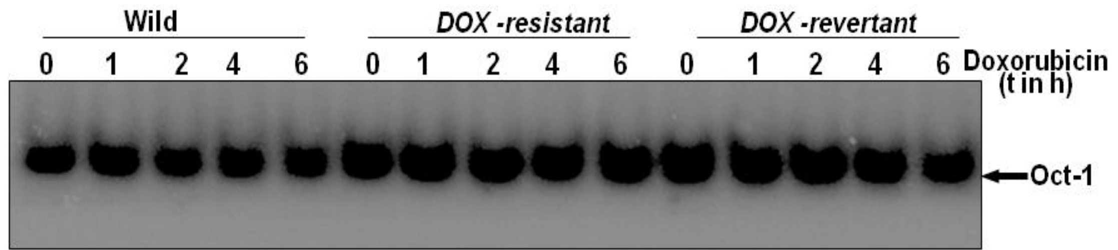


Fig 1B. Effect of doxorubicin on Oct-1 activation in wild type, Dox-resistant and Dox-revertant cells. MCF-7 (Wild type, Dox-resistant and Dox-revertant) cells were treated with 1 μM doxorubicin for different times. After these treatments, cytoplasmic (CE) and nuclear extracts (NE) were prepared and NE was assayed for Oct-1 by gel shift assay.

Various combinations of Rel/ NF-κB proteins can constitute an active NF-κB heterodimer that binds to specific sequences in DNA. Nuclear extracts from doxorubicin-treated MCF-7 cells were incubated with antibodies (Abs) to p50 and p65 alone or in combination, and then conducted gel shift assay to study the specificity and composition of the retarded band. Abs to either subunit of NF-κB shifted the band to a higher molecular weight (Fig.1C), thus suggesting that the retarded complex consisted of both p50 and p65 subunits. Neither preimmune serum nor irrelevant Ab such as anti-c-Rel had any effect on the mobility of NF-κB. This complex completely disappeared in the presence of 50-fold molar excess of cold NF-κB and was unable to bind with mutant oligonucleotides, indicating its specificity.

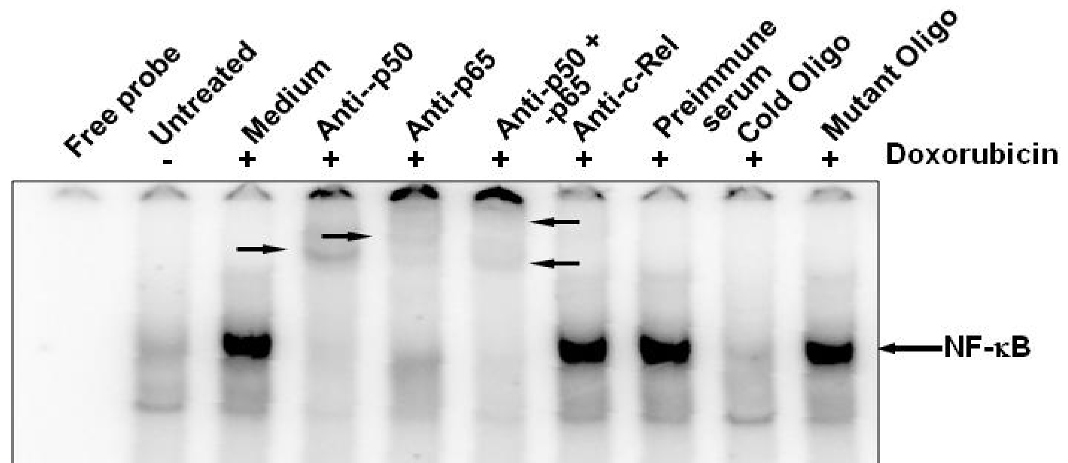


Fig 1C. Supershift of NF-κB band. NE was prepared from untreated or Dox-treated wild type cells, incubated for 15 min with different Abs and unlabeled and mutated NF-κB oligonucleotides (50-fold), and then assayed for NF-κB.

Dox-resistant cells show constitutive activation of NF-κB dependent genes.

To determine the effect of doxorubicin on NF-κB dependent reporter gene expression, we transiently transfected MCF-7, Dox-resistant, and Dox-revertant cells with the NF-κB SEAP reporter construct then treated with different concentrations of doxorubicin for 6 h. Dox-resistant cells had high basal activity of SEAP. This SEAP activity was not altered significantly by treatment with increasing concentrations of doxorubicin. In wild type and Dox-revertant cells, the SEAP activity increased with the increased concentrations of doxorubicin (Fig.1D).

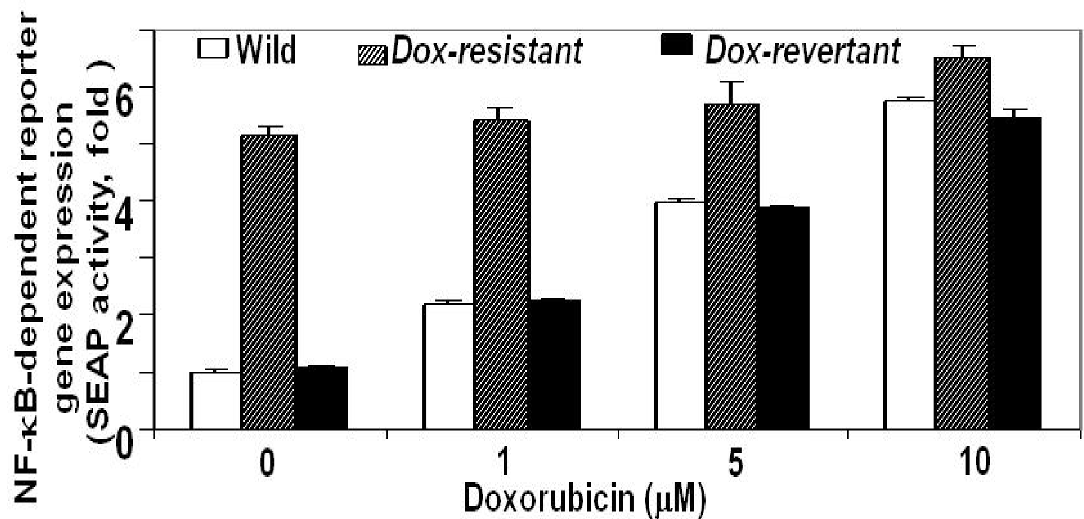


Fig 1D. Effect of doxorubicin on NF-κB activation in wild type, Dox-resistant and Dox-revertant cells. Wild type, Dox-resistant and Dox-revertant cells were transiently transfected with *NF-κB-SEAP* construct for 3 h, cultured for 12 h, then treated with different concentrations of doxorubicin for 6 h. Cell culture supernatant was assayed for secreted alkaline phosphatase (SEAP) activity and mean SEAP activity was indicated as fold of activation above vector-transfected cells.

Dox-resistant cells show high basal expression of Cox-2 and ICAM1 and Cox-2-dependent luciferase.

Several genes such as cytokines, cyclooxygenase-2, metalloproteases, urinary plasminogen activator, and cell surface adhesion molecules which are involved in inflammation and tumor promotion, are regulated by NF-κB. As Dox-resistant cells

show constitutive activation of NF-κB we checked different biological responses, the expression of Cox-2 and adhesion molecule, ICAM-1 was analyzed. Then 100μg cell extract proteins were analyzed in 9% SDS-PAGE for Cox-2 and ICAM-1 using western blot technique. In MCF-7 cells (Wild type), doxorubicin increased the amounts of Cox-2 and ICAM1 at 3 h and 6 h and reduced partially at 12 h as shown by Western blot. In Dox-resistant cells, the high basal amount of Cox-2 was observed and this amount did not alter with the increasing time of doxorubicin treatments (Fig.2A). MCF-7 cells (Wild type), transiently transfected with the Cox-2-luciferase and GFP constructs for 3 h were cultured for 12 h. Cells were then treated with 1 μM doxorubicin for different times. The induction of Cox-2 dependent luciferase activity was transient in wild type (Fig.2B). In Dox-resistant cells showed constitutive activation of luciferase activity (Fig 2B). These results demonstrate Dox-resistant cells show constitutive expression of Cox-2 and ICAM1; whereas the expression in wild type is transient.

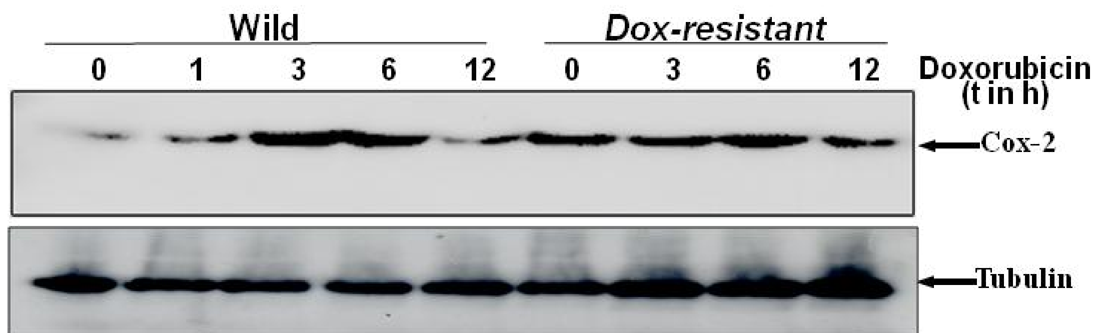


Fig 2A. Effect of doxorubicin on Cox-2 expression. Wild type and Dox-resistant cells were treated with 1 μM doxorubicin for varying time periods. The Cox-2 protein expression was detected from whole cell extract by Western blot The blot was reprobed with anti-tubulin antibody as loading control.

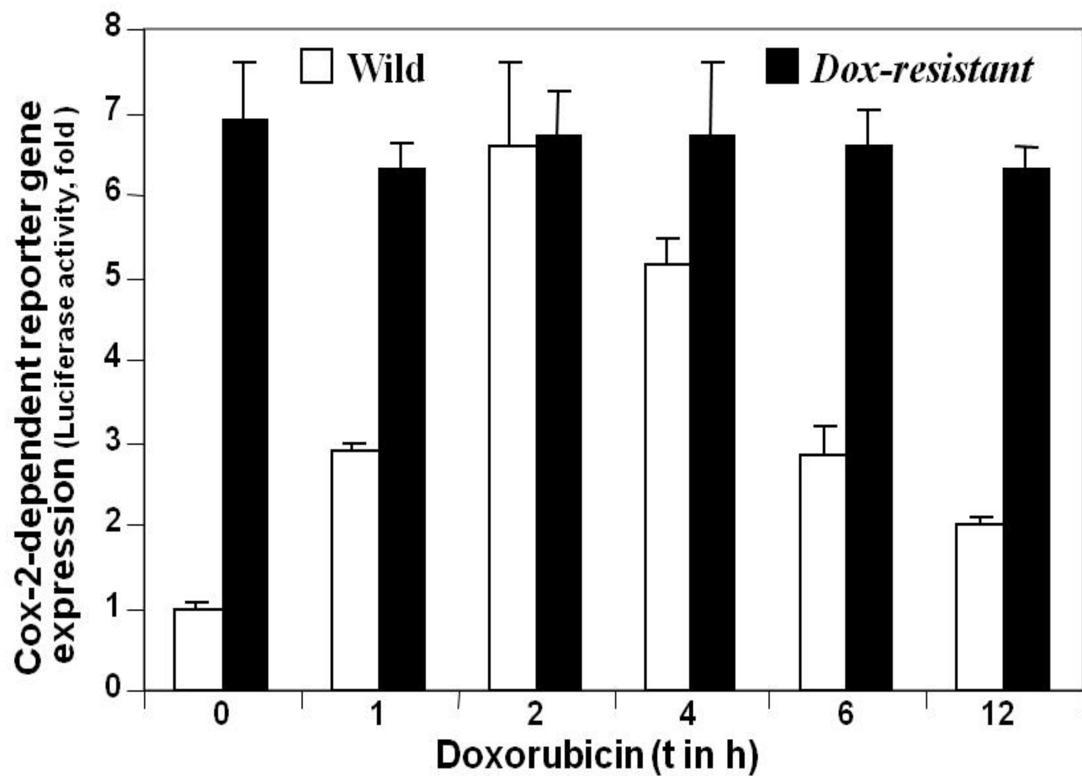


Fig 2B. Effect of doxorubicin on Cox-2-dependent luciferase expression. Wild type and Dox–resistant cells were transfected with *Cox-2-luciferase* expression vector and *β-galactosidase* gene for 3 h, culture for 12 h, then treated with 1 μM doxorubicin for different times. The luciferase and β-galactosidase enzymes activity was measured from whole cell extracts as described in Materials and Methods.

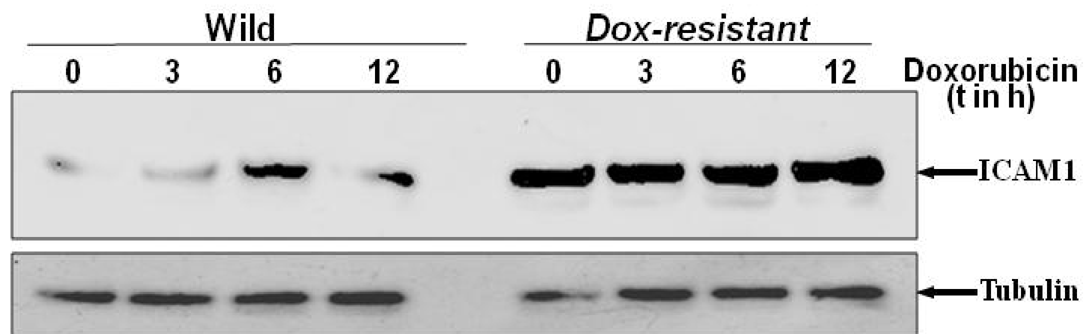


Fig 2C. Effect of doxorubicin on ICAM1 expression. Wild type and Dox-resistant cells were treated with 1 μM doxorubicin for varying time periods. The ICAM1 protein expression was detected from whole cell extract by Western blot. The blot was reprobed with anti-tubulin antibody as loading control.

Dox-resistant cells show high basal levels of IκBα, phospho- IκBα, and p65.

The activity of NF-κB is tightly regulated by its interaction with inhibitory IκB proteins. When the NF-κB signaling cascade is activated, it leads to the phosphorylation and ubiquitination followed by proteasomal degradation of IκB. This allows the translocation of unmasked NF-κB from the cytoplasm to the nucleus where it binds to NF-κB response elements in target genes and regulates their transcription. Doxorubicin treated cells showed degradation of IκBα after 1 h in wild-type and Dox-revertant cells. Phospho- IκBα was observed at 0.5 and 1 h of doxorubicin treatment (Fig.3A).

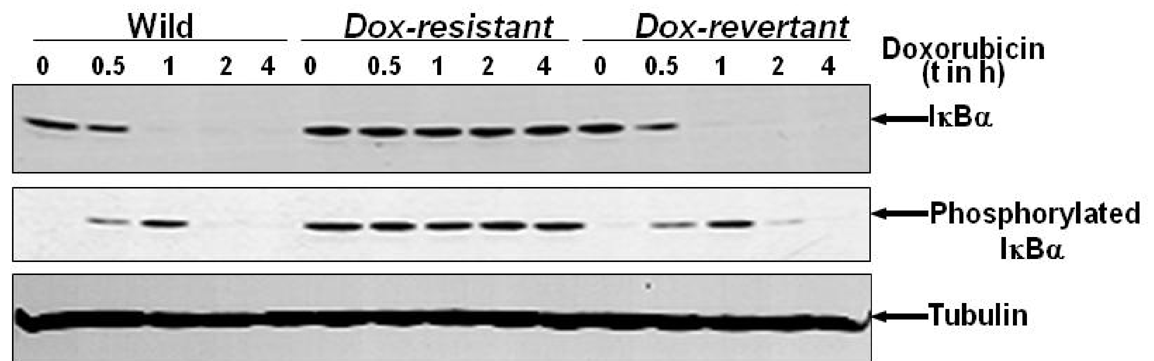


Fig 3A. **Effect of doxorubicin on the amounts of IκBα and phospho- IκBα in wild type, Dox-resistant, and Dox-revertant cells.** Wild type, Dox-resistant and Dox-revertant cells were treated with 1 μM doxorubicin for varying time periods. CE and NE were prepared. CE was assayed for IκBα and phospho-IκBα by Western blot. The blot was reprobed for anti-tubulin antibody.

High basal expression of IκBα and phospho-IκBα were observed in Dox-resistant cells and these were not altered on doxorubicin treatment. The amount of p65 was decreased with increasing time of doxorubicin treatment in wild-type and Dox-revertant cells in cytoplasmic extracts. The amount of p65 in nuclear extracts was increased till 1 h of doxorubicin treatment and decreased thereafter. In Dox-resistant cells high basal level of p65 was observed both in cytoplasmic and nuclear extracts and this level did not alter due to doxorubicin treatment (Fig.3B). The p65 concentration was increased at 3 h in nucleus and decreased at 6 h both in

cytoplasm and nucleus in doxorubicin-treated wild type cells. The p65 level did not alter upon doxorubicin treatment in Dox-resistant cells (Fig.3C).

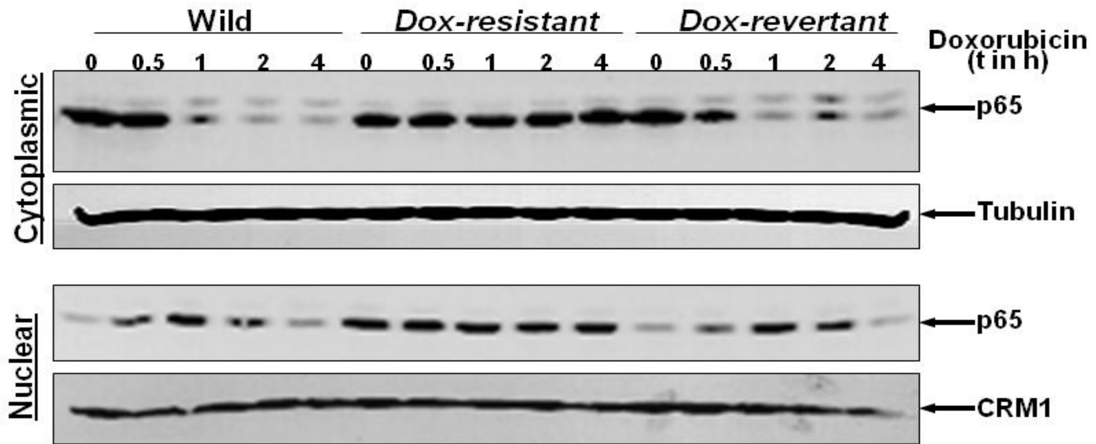


Fig 3B. **Effect of doxorubicin on the amounts of p65 in wild type, Dox-resistant, and Dox-revertant cells.** Wild type, Dox-resistant and Dox-revertant cells were treated with 1 μM doxorubicin for varying time periods. CE and NE were prepared and the p65 was detected from both by Western blot and blots were reprobed with anti-tubulin and anti CRM1 antibodies respectively.

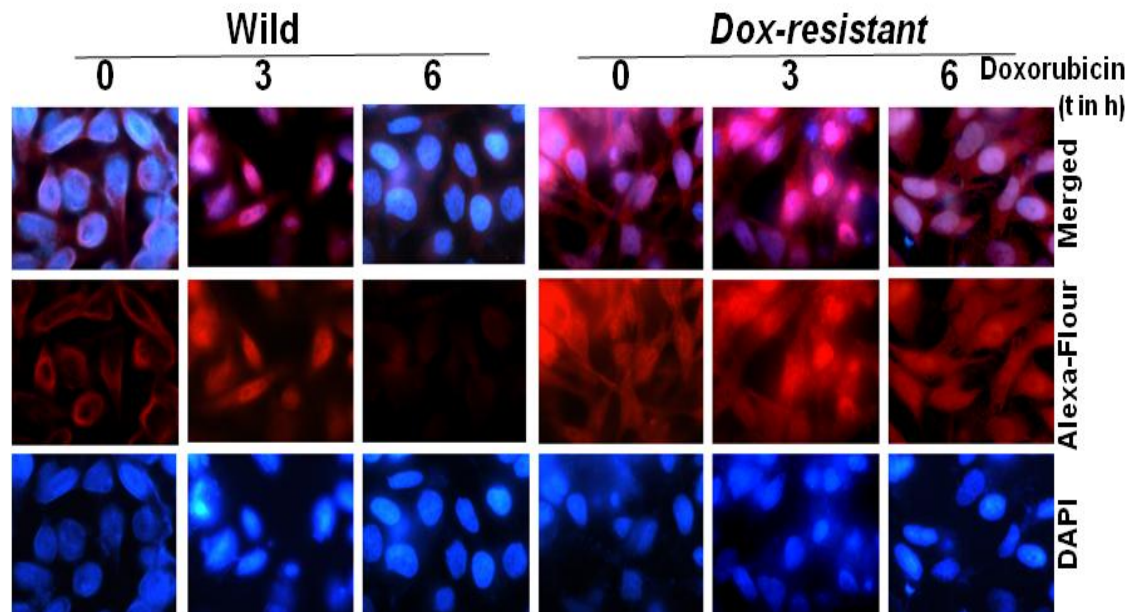


Fig 3C. **Effect of doxorubicin on the amounts of p65 in wild type, Dox-resistant, and Dox-revertant cells.** Wild type, Dox-resistant and Dox-revertant cells were treated with 1 μM doxorubicin for varying time periods. The level of p65 was examined by the immunofluorescence using anti-p65 antibody followed by goat-anti-rabbit IgG-Alexa-Fluor antibody. Cells were then mounted on a slide and visualized under fluorescence microscope.

Dox-resistant cells do not show apoptosis.

Analysis of resistant cells revealed a high NF-κB level and its dependent genes like Cox-2 and ICAM which are known to play a role in tumor progression. NF-κB dependent genes play key roles in the regulation of apoptosis and may contribute to cancer development and resistance to chemotherapy. Hence we studied doxorubicin-mediated apoptosis in Dox-resistant cells. Cytotoxicity as measured by MTT assay was increased in wild type and Dox-revertant, but not Dox-resistant cells with increasing concentrations of doxorubicin (Fig.4A).

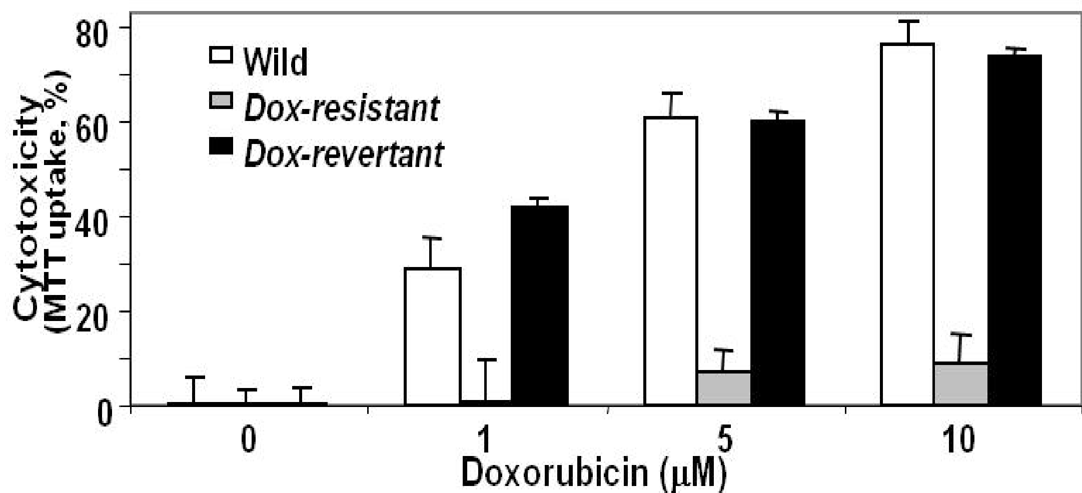


Fig 4A. **Effect of doxorubicin on apoptosis in wild type, Dox-resistant and Dox-revertant MCF-7 cells.** Cells were incubated with varying concentration of doxorubicin for 72 h. Then cytotoxicity was assayed by MTT assay. Results are represented as inhibition of cell viability in percentage.

Proteolytic cleavage of PARP by caspases is a hallmark of apoptosis. To investigate Dox-resistant cells undergo apoptosis, cells were treated with doxorubicin and looked for activated PARP. As shown in Fig.4B, doxorubicin induced cleavage of PARP with increasing time of incubation in wild type and Dox-revertant cells, but not in Dox-resistant cells. Bcl-2, an antiapoptotic protein, prevents release of cytochrome c from mitochondria. Therefore, the effect of doxorubicin on the concentration of Bcl-2 was assessed. As shown in Fig. 4C, treatment of cells with doxorubicin increased the concentration of Bcl-2 at 6 h and then decreased at 12 and 24 h. A high basal level of Bcl-2 was detected in Dox-resistant cells and this was not altered at any time of doxorubicin treatment.

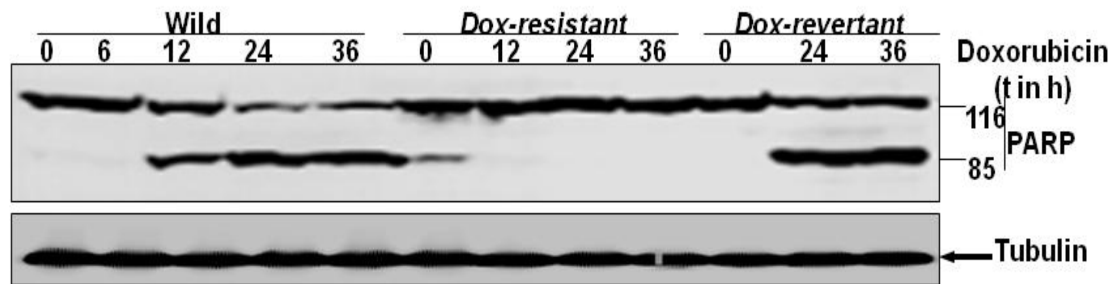


Fig 4B. **Effect of doxorubicin on cleavage of PARP in wild type, Dox-resistant and Dox-revertant MCF-7 cells.** Wild type, Dox-resistant, and Dox-revertant MCF-7cells were treated with 1 μM doxorubicin for various time intervals. Then whole cell extracts were prepared and 100 μg proteins were analyzed by 7.5% SDS–PAGE to detect PARP by Western blot using anti-PARP Ab. The blot was then reprobed for tubulin.

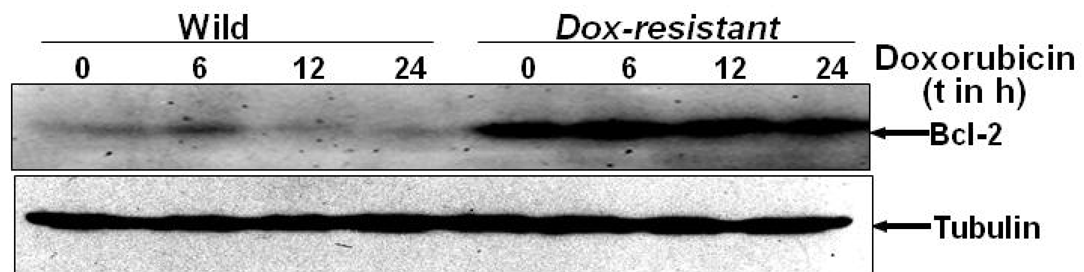


Fig 4C. **Effect of doxorubicin on Bcl-2, an antiapoptotic protein in wild type, Dox-resistant MCF-7 cells.** Wild type and Dox-resistant MCF-7cells were treated with 1 μM doxorubicin for various time intervals. Then whole cell extracts were prepared and 100 μg proteins were analyzed by 10% SDS–PAGE to detect Bcl-2 by Western blot using anti-Bcl-2 Ab. The blot was then reprobed for tubulin.

Dox-resistant cells show high basal level of ROI, but not lipid peroxidation.

The transcription factor NF-κB is well-known for its roles in preventing apoptotic cell death. Both *in vitro* and *in vivo* experiments have demonstrated that ROS can lead to activation of NF-κB. However, this is highly cell type and stimulus specific (Hayakawa, M. et.al 2003).Reactive oxygen species (ROS) play an important role in apoptosis induction under both physiologic and pathologic conditions. The effect of doxorubicin on ROS in Dox-resistant cells was examined by flow cytometry. Doxorubicin induced ROI in a dose-dependent manner in wild type and Dox-revertant, but not in Dox-resistant cells (Fig 4D).

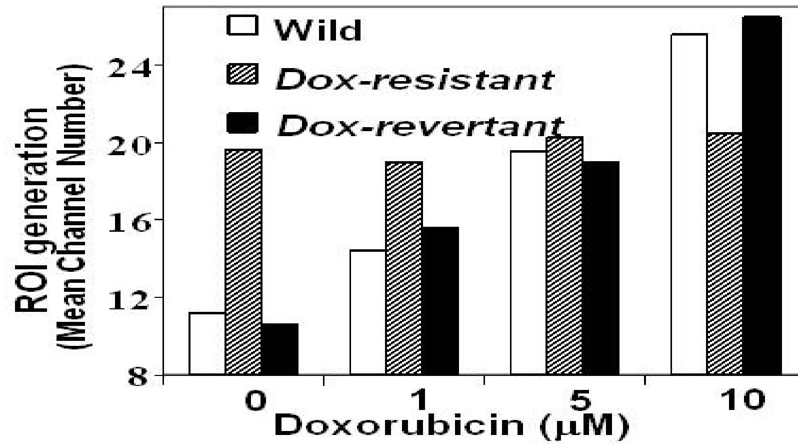


Fig 4D: Effect of doxorubicin on reactive oxygen intermediates generation. Wild type, Dox-resistant, and Dox-revertant cells were treated with varying concentration of doxorubicin for 6 h. ROI was then measured in flow cytometer. The results shown are representative of two independent experiments.

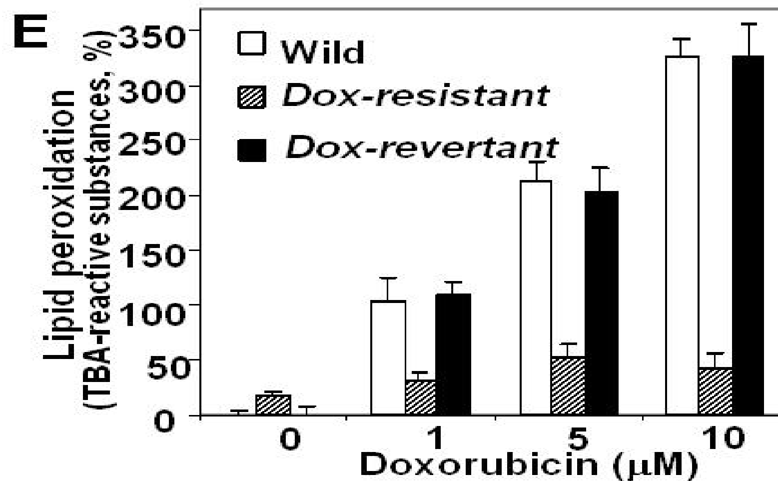


Fig 4E: Effect of doxorubicin on lipid peroxidation. Wild type, Dox-resistant and Dox-revertant cells were treated with varying concentration of doxorubicin for 6 h. Cell pellets were extracted by 3 times freeze thaw method with addition of 200 μl water and 500 μg protein sample was used to measure malondialdehyde by TBA-SDS buffer as described in Materials and Methods. The results shown are representative of two independent experiments. Untreated cells showed 0.524 ± 0.076 nmol of MDA equivalents/mg protein.

Dox-resistant cells showed high basal level of ROI and this level did not alter at any concentrations of doxorubicin. Doxorubicin at 10 μM concentration induced ROI generation in wild type and Dox-revertant cells beyond the basal of Dox-resistant cells. Doxorubicin induced lipid peroxidation in a dose-dependent manner in wild

type and Dox-revertant, but not in Dox-resistant cells (Fig.4E). These results further suggest that Dox-resistant cells are also resistant to doxorubicin-induced apoptosis.

Dox-resistant cells show basal expression of superoxide dismutase (SOD) 1.

Dox-resistant cells showed high basal level of ROS which was not further increased upon doxorubicin treatment. Evidences indicate that doxorubicin-mediated cardiotoxicity may be caused by increased generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2), or hydroxyl radicals through redox-activation of doxorubicin. To combat the damage caused due to ROS production, body has developed endogenous anti-oxidant defense system. We have checked the amount of ROI detoxifying factors like SOD1. The amount of SOD1 was increased in Dox-resistant cells than wild type or Dox-revertant MCF-7 cells (Fig.4F). Doxorubicin treatment did not alter the basal amount of SOD1 in these cells. These results suggest that doxorubicin-induced ROI generation was not observed in Dox-resistant cells may be due to high basal expression of SOD1.

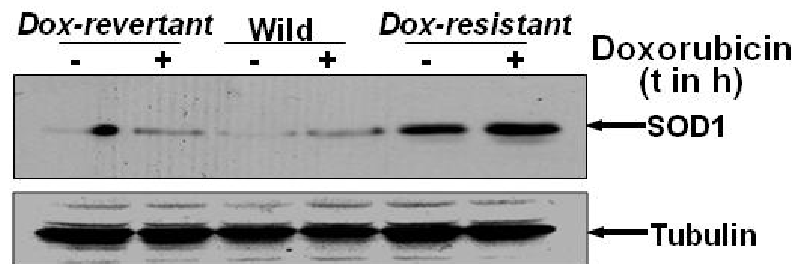


Fig 4F: Effect of doxorubicin on expression of SOD-1. Wild type, Dox-resistant, and Dox-reveretant MCF-7 cells were treated with doxorubicin (5 μM) for 6 h. Then whole cell extracts were prepared and 100 μg proteins were analyzed by 10% SDS–PAGE to detect SOD-1 by Western blot using anti-SOD-1 Ab. The same blot was reprobed with anti-tubulin antibody.

Inhibition of NF-κB sensitizes the Dox-resistant cells to apoptosis.

The role of NF-κB in maintaining the delicate balance between cell proliferation and apoptosis is well known and studies also reveal NF-κB as a novel target for developing cancer drugs. NF-κB inhibition will help in halt tumor progression and eliminates tumors thereby making it an ideal target for drugs to be used at later stages of cancers. In our study, NF-κB was downregulated in Dox-resistant cells by multiple ways - i) transfecting with *IκBα-DN* plasmid; ii) treating

with 5 μM BAY for 6 h; and iii) treating with 100 nM P_3-25 for 6 h. Dox-resistant cells showed high basal activity of NF-κB. *IκBα-DN* transfected or BAY-treated, but not P_3-25-treated cells showed decrease in NF-κB DNA binding (Fig.5A). Expression of NF-κB -dependent reporter gene, SEAP, was decreased in *IκBα-DN* transfected, BAY-treated, or P_3-25-treated Dox-resistant cells (Fig.5B).

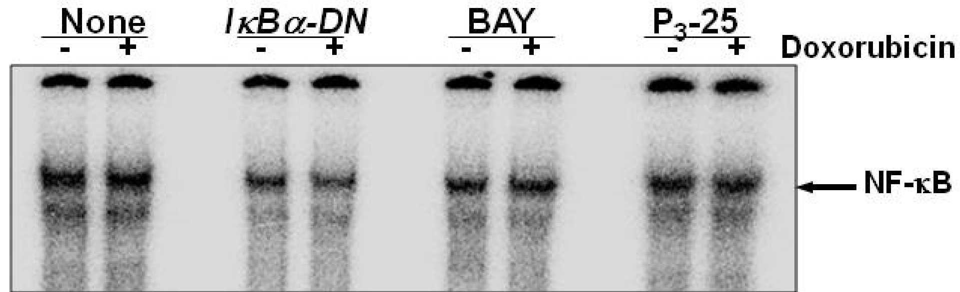


Fig 5A. Downregulation of NF-κB in Dox-resistant cells. Dox-resistant cells were pretreated with BAY (5 μM for 6 h); P_3-25 (100 nM for 6 h); or transiently co-transfected with *IκBα-DN* and *GFP* constructs. Cells, in these conditions were treated with 1 μM doxorubicin for 12 h. NF-κB DNA-binding activity was assayed from nuclear extracts.

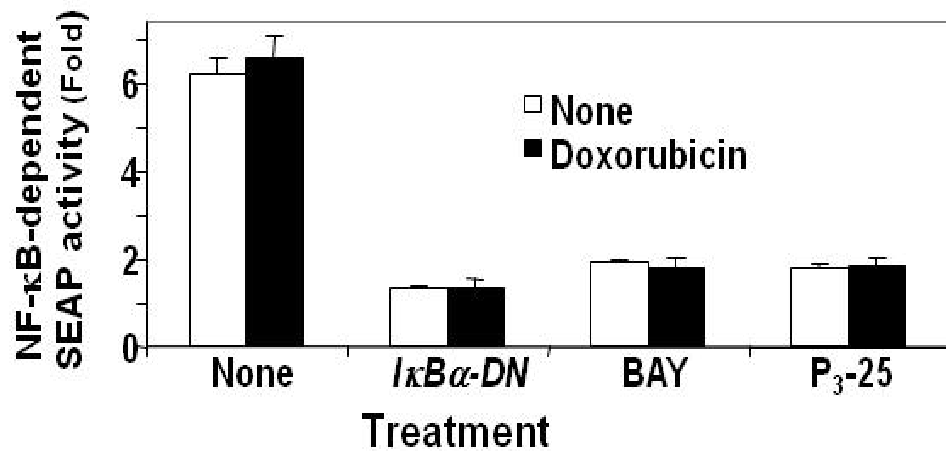


Fig 5B. Downregulation of NF-κB in Dox-resistant cells. Dox-resistant cells were transfected with *NF-κB-SEAP* alone or together with *NF-κB-SEAP* and *IκBα-DN* constructs for 3 h and cultured for 12 h. *NF-κB-SEAP* transfected cells were treated with BAY or P_3-25 last 6 h while culturing. All these cells were treated with 1 μM doxorubicin for 12 h. SEAP activity was assayed from culture supernatant and indicated as fold activation.

Doxorubicin-treated cells did not alter NF-κB DNA binding or NF-κB-dependent SEAP activities. Doxorubicin induced cytotoxicity in these NF-κB downregulated Dox-resistant cells in a concentration dependent manner (Fig.5C). They showed 20-25% cell death due to NF-κB downregulation. These results suggest that inhibition of NF-κB activity can induce cell death in Dox-resistant cells. The results are in accordance with the suppression of colorectal and gastric tumors by several anti-inflammatory drugs like aspirin, curcumin, or resveratrol which also inhibit NF-κB activation. Further studies are necessary to substantiate this hypothesis.

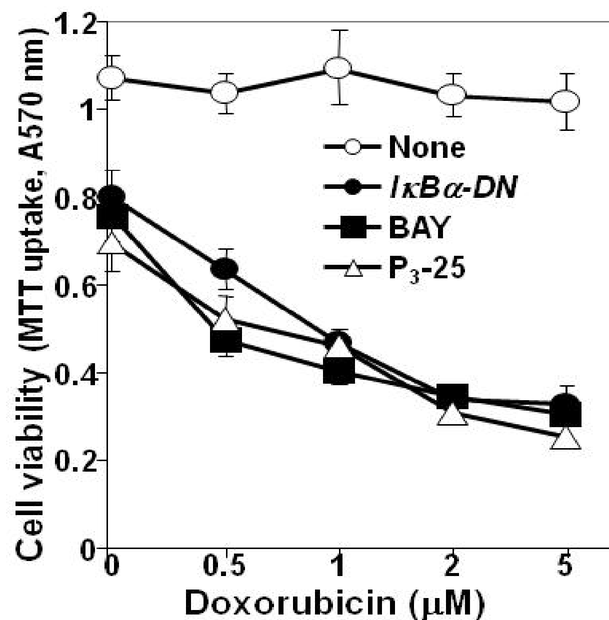


Fig 5C. Effect of doxorubicin on cell viability in NF-κB downregulated Dox-resistant cells*.* Dox-resistant cells were pretreated with BAY or P_3-25, or transiently transfected with *IκBα-DN*. Cells, in these conditions were treated with different concentrations of doxorubicin for 72 h. Cell viability was assayed by MTT dye uptake and indicated in mean absorbance from triplicate samples.

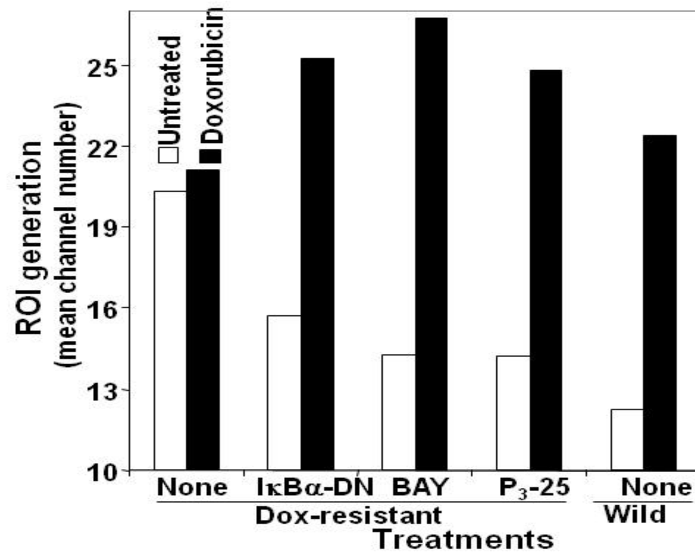


Fig 5D. Effect of doxorubicin on ROI generation in NF-κB downregulated Dox-resistant cells. ROI generation was measured in Dox-resistant cells (pretreated with BAY or P_3-25, or transiently transfected with *IκBα-DN*) and wild type cells, treated with 1 μM doxorubicin for 6 h using Dihydrorhodamine fluorescent dye in FACS.

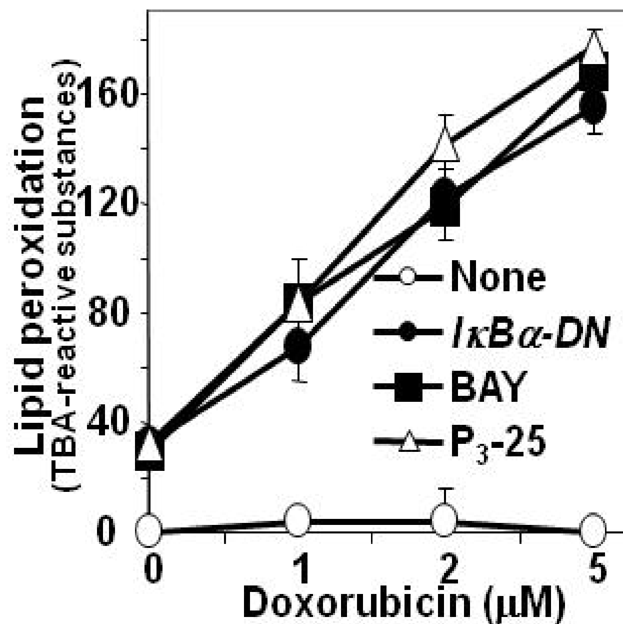


Fig 5E. Effect of doxorubicin on lipid peroxidation in NF-κB downregulated Dox-resistant cells. Dox-resistant cells (pretreated with BAY or P_3-25, or transiently transfected with *IκBα-DN*), treated with different concentrations of doxorubicin for 12 h. Cell extracts were prepared by the freeze–thaw method and 500 μg proteins were used for TBA-SDS reactive MDA assay as described in Materials and Methods. The results are indicated as MDA production in percentage above control.

Low level of ROI was observed in NF-κB downregulated cells than untreated Dox-resistant cells. Doxorubicin induced ROI generation in NF-κB-inhibited cells were more than the high basal level of ROI in Dox-resistant cells (Fig. 5D). Doxorubicin alone did not induce lipid peroxidation in these cells. When NF-κB was downregulated, cells showed increase in lipid peroxidation by doxorubicin treatment in a concentration dependent manner (Fig.5E). The high basal expression of SOD1 was decreased in NF-κB -downregulated Dox-resistant cells (Fig.5F). These results support that the decrease in the amount of SOD1 by inhibiting NF-κB may induce ROI generation by doxorubicin in Dox-resistant cells as shown in Fig.5D. As shown in Fig. 4C, Dox-resistant cells showed high basal level of Bcl-2, an antiapoptotic protein. NF-κB downregulation decreased the level of Bcl-2 and this level did not alter due to doxorubicin treatment in these cells (Fig.5F). These data suggest that Dox-resistant cells become sensitive to doxorubicin when NF-κB is downregulated or in otherwords high NF-κB confers resistance to these cells.

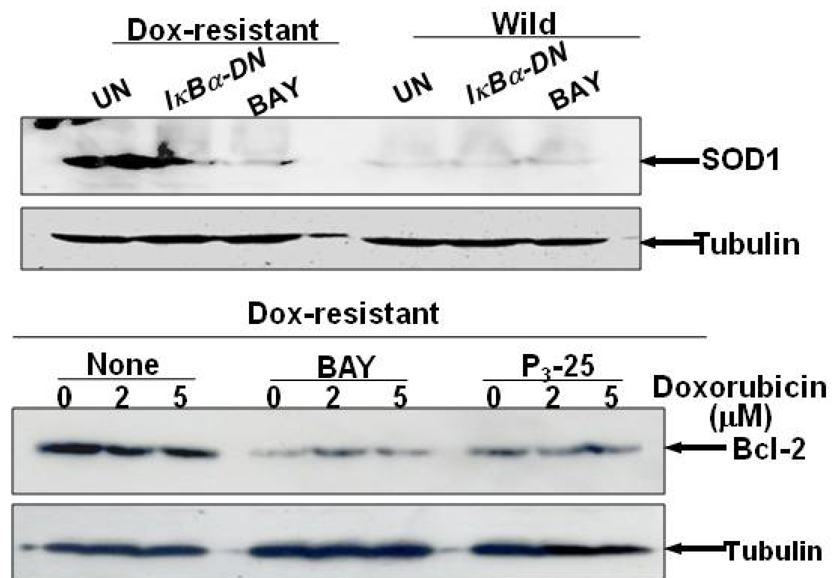


Fig 5F. Effect of doxorubicin on SOD-1 and Bcl-2 in NF-κB downregulated Dox-resistant cells. Dox-resistant and wild type cells (pretreated with BAY or transiently transfected with *IκBα-DN*) were cultured for 12 h and the SOD1 was measured by Western blot. Dox-resistant cells (pretreated with BAY or P_3-25 for 6 h), treated with 1 μM doxorubicin for 12 h. Cell extracts were prepared and 100 μg proteins were used to detect Bcl-2 by Western blot. The same blots were reprobed for tubulin.

Inhibition of NF-κB decreases basal expression of Mdr and TGase in Dox-resistant cells.

The development of cellular resistance mechanism to doxorubicin includes P-glycoprotein/multidrug resistance proteins and Bcl-2 over expression, altered topoisomerase II activity, loss of p53 function etc. Also increased expression of transglutaminase (TGase) in cancer cells has been implicated in conferring resistance to chemotherapeutic drugs and promotes metastatic phenotype and cell survival functions in breast, melanoma and pancreatic cancer cancer cells (Mehta et. al., 2004). Hence we examined the levels of two important proteins Mdr and TGase involved in resistance in Dox-resistant cells. In wild type and Dox-revertant cells, the amount of Mdr was not detected but high basal expression of Mdr was detected in Dox-resistant cells. The amount of Mdr decreased in Dox-resistant cells when NF-κB was downregulated (by incubating cells with BAY, P_3-25, or transfecting with *IκBα-DN* construct) (Fig.6A). The high basal expression of Mdr was decreased kinetically by BAY-treated Dox-resistant cells as shown by Western blot (Fig.6A)

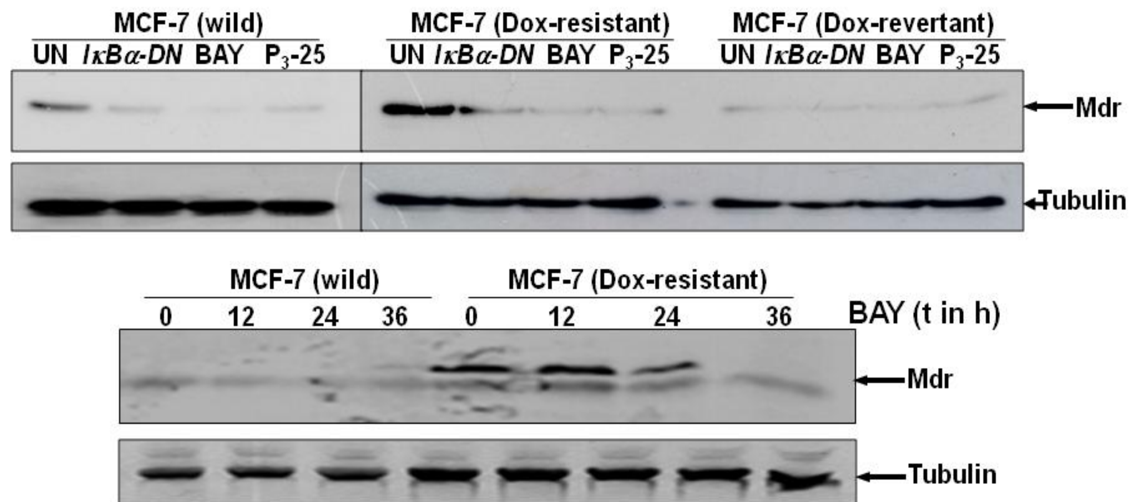


Fig 6A. Effect of BAY or P_3-25 on expression of Mdr in Dox-resistant cells. MCF-7 (wild type, Dox-resistant, and Dox-revertant) cells were either treated with BAY (5 μM), P_3-25 (100 nM), or transfected with *IκBα-DN* construct for 36 h. The amount of Mdr was measured by Western blot . MCF-7 (wild type and Dox-resistant) cells were treated with BAY (5 μM) for different times and whole cell extracts (150 μg proteins) were used to detect Mdr by Westen blot. The same blots were reprobed against tubulin.

To examine the effect of BAY or P_3-25 on expression of Mdr in Dox-resistant cells, the immunofluorescence of Mdr protein was observed by confocal microscopy, which provides a visual detection of the location of Mdr in Dox-resistant cells. In the Dox-resistant cells not treated by BAY, the expression of Mdr is predominant, which was monitored by tracking a strong fluorescence in the cytoplasm and nuclei. Treatment of Dox-resistant cells with BAY effectively decreased the expression of drug resistant Mdr protein in these cells (Fig.6B).

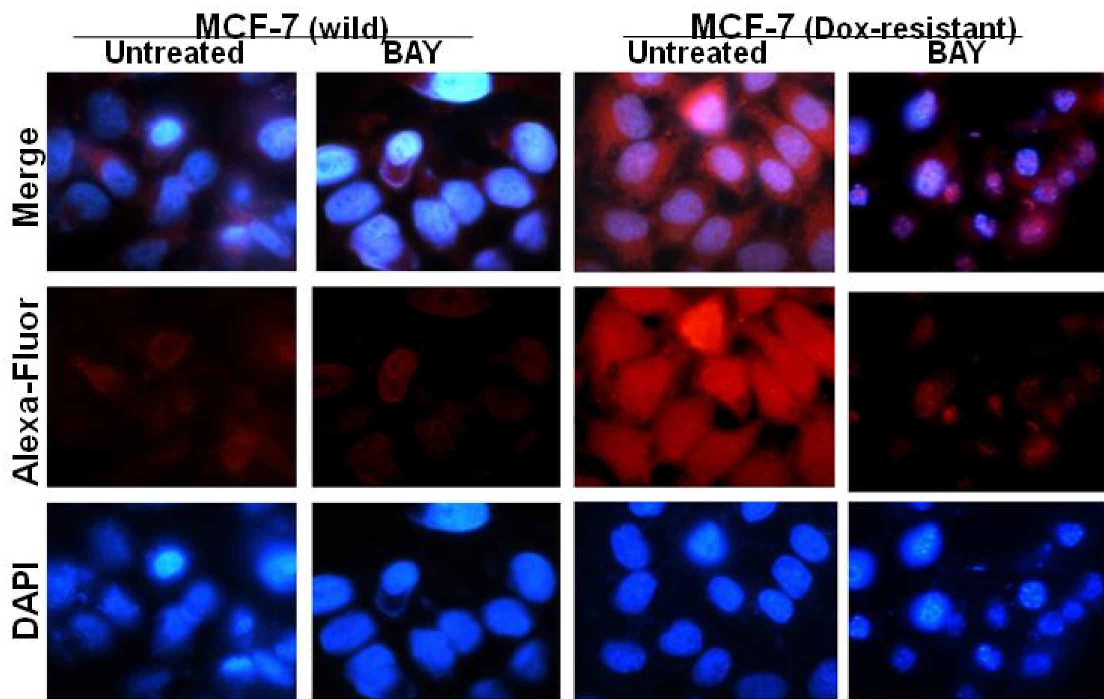


Fig 6B. Effect of BAY on expression of Mdr in Dox-resistant cells. MCF-7 (wild type and Dox-resistant) cells were treated with BAY for 36 h and Mdr was detected using anti-Mdr antibody followed by goat-anti-rabbit IgG-Alexa-Fluor antibody. Cells were then mounted on a slide and visualized through a fluorescence microscope.

Various studies have demonstrated that cancer cells and cancer cell lines selected for resistance against chemotherapeutic drugs express elevated levels of the multifunctional protein, tissue transglutaminase, that is implicated to play a role in apoptosis, wound healing, cell migration, cell attachment, cell growth and matrix assembly. Additionally, transglutaminase forms a ternary complex with IκB/p65:p50 and results in constitutive activation of the nuclear transcription

factor-κB (NF-κB). To determine the effect of TGase in Dox-resistant cells, we checked the expression level of TGase protein by western blot in these cells. In wild type and Dox-revertant cells, the amount of TGase was not observed but high basal expression was detected in Dox-resistant cells. The amount of transglutaminase (TGase) decreased in Dox-resistant cells when NF-κB was downregulated (Fig.6C). The high basal amount of TGase was decreased in Dox-resistant cells kinetically by BAY-treatment (Fig.6C).

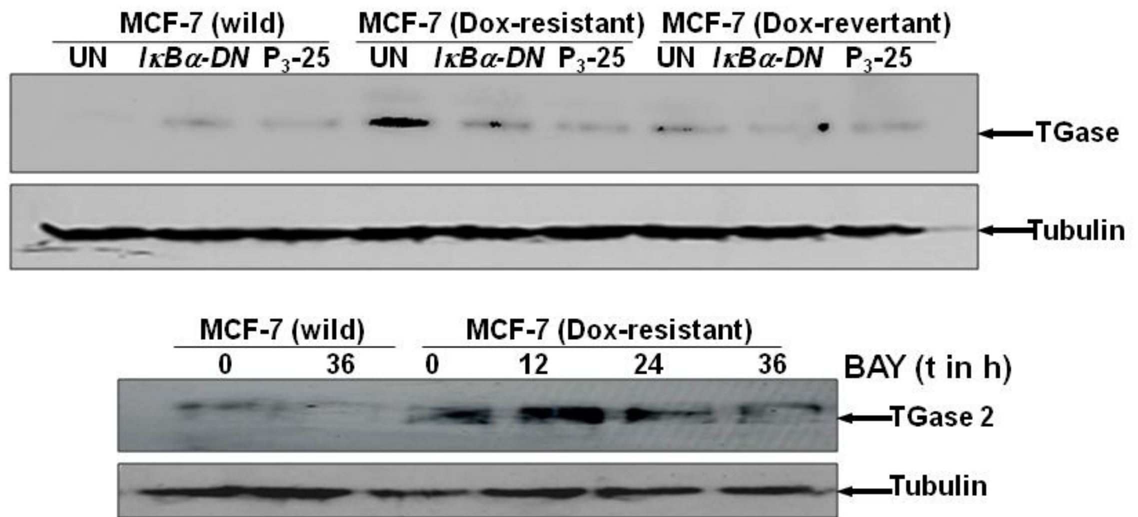


Fig 6C. Effect of BAY on expression of TGase in Dox-resistant cells. MCF-7 (wild type, Dox-resistant, and Dox-revertant) cells were either treated with P_3-25 (100 nM) or transfected with *IκBα-DN* construct for 36 h. The amount of TGase was measured by Western blot. Cells were treated with BAY for different times and amount of TGase 2 was detected by Western blot. The same blots were reprobed for tubulin.

These results collectively suggest that Dox-resistant cells show high basal expression of Mdr and TGase and by inhibiting NF-κB these expressions are reduced. Moreover inhibiting NF-κB decreases cell survival and sensitizes Dox-resistant cells to apoptosis. This may lead to the birth of therapeutic inhibitors that selectively block NF-κB activation in cancer cells to overcome resistance.

Discussion

NF-κB is an apoptotic regulator that plays an important role in regulating the response of tumor cells to chemotherapy. Previous studies have shown that NF-κB in tumor cells is activated by treatment with TNF and certain chemotherapeutic agents. Activation of NF-κB promotes cell proliferation, blocks apoptosis and induces resistance to various chemotherapeutic agents. Similarly, constitutively activated NF-κB has been associated with increased cell proliferation and survival in cancer cells, and may be linked to drug resistance in these cells. Inhibition of NF-κB activation by transfection of the dominant-negative inhibitor IκBα into malignant cells has been considered as a method to sensitize tumor cells to apoptosis induced by chemotherapeutic agents (Garg & Aggarwal, 2002). However, several studies have shown that stable inhibition of NF-κB activation in cancer cells by transfection of IκBα did not increase sensitivity to cytotoxic drugs. Therefore, this study was designed to eliminate chemoresistant pathways of tumor cells with therapeutic agents (BAY, P_3-25) to achieve optimal results.

This study provides evidence in support of the concept that high levels of NF-κB and expression of genes dependent on it confer resistance to doxorubicin in breast tumor cell lines. Doxorubicin induced NF-κB transiently may in some way be responsible for some of the cells becoming resistance to cell death. In the laboratory conditions, a few populations of MCF-7 breast tumor cell lines attain resistance, which later on grow happily in presence of doxorubicin even at higher concentration (10 μM) (Rojo et. al., 2004). How these cells attain resistance still remains elusive. While culturing these resistant cells in absence of doxorubicin for 6 months, a few populations showed cell death against doxorubicin. These populations were isolated and considered as Dox-revertant cells. These cells showed low expression of NF-κB, SOD1, Mdr, and TGase. Considering doxorubicin's usefulness in treating different tumors alone or in combination generating resistance against it caused much distress to these patients. Understanding the mechanism of this resistance against doxorubicin is of value. How doxorubicin induces cell death has not yet been fully established. To address these issues, we used doxorubicin resistant and revertant

MCF-7 cells to compare the signaling mechanism in these cells targeting for better and effective treatment of tumor.

We found that NF-κB DNA binding activity was initially induced and later on decreased in doxorubicin treated wild type and Dox-revertant cells. Dox-resistant cells, however, showed high basal NF-κB activity. Doxorubicin induced NF-κB DNA binding activity till 2 h of treatment on wild type or Dox-revertant cells and then this level decreased significantly with increasing time. It induced cell death from 24 h onwards. So, decrease in NF-κB DNA binding is not due to reduction in the cell number. It is also observed that the Oct1 DNA binding activity did not alter at any time of doxorubicin treatment, though decrease in NF-κB binding. These data support the genuine downregulation of NF-κB activity in wild type or Dox-revertant cells. High basal activity of NF-κB DNA binding was detected in nuclear extracts of Dox-resistant cells. Though 4 h onwards NF-κB DNA binding activity was decreased, but 6 h of incubation with different concentrations of doxorubicin showed gradual increase in reporter gene expression. As SEAP is secreted from cells into the culture supernatant, the decrease in NF-κB DNA binding at later time points with doxorubicin was not reflected in the results. The concentrations of Cox-2 and ICAM1 were reflected with the NF-κB activity in all these three types of cells as both are NF-κB-dependent genes. Doxorubicin-mediated NF-κB activation is correlated with phospho-IκBα and IκBα levels in wild type and Dox-revertant cells. In Dox-resistant cells, high basal levels of phospho-IκBα and IκBα were observed. IκBα is an NF-κB -dependent gene product. Constitutive activity of NF-κB might lead to expression of high IκBα and rapid turnover of NF-κB in the nucleus may be required for constitutive level of phospho-IκBα in Dox-resistant cells. The p65 subunit of NF-κB is known to be vital for its function by heterodimerizing with p50 subunit. Though p65 level is decreasing from cytoplasm with increasing time of doxorubicin treatment, it is translocating to nucleus at early time of doxorubicin treatment. Surprisingly, at later time points, nuclear p65 levels decreased without shuttling back to cytoplasm. How the amount of p65 decreases from cells due to doxorubicin treatment in wild type and Dox-revertant cells needs to be studied. Involvement of ubiquitination for degradation of p65 needs to be addressed.

Doxorubicin induced cell death in wild type and Dox-revertant, but not Dox-resistant cells. Doxorubicin-mediated cell death was observed at 12 h onwards with doxorubicin treatment which correlates with downregulation of NF-κB. Dox-resistant cells with constitutive active NF-κB are fully resistant to cell death even at 10 μM concentration of doxorubicin, indicating its possible role in proliferating tumor cells and also aiding them to abate apoptosis. Bcl-2, an NF-κB -dependent gene product (Christman et. al., 1998; Sarkar et. al., 2004), also transiently increased by doxorubicin treatment in wild type cells and constitutive expression was shown in Dox-resistant cells. In Dox-resistant cells, doxorubicin was unable to induce lipid peroxidation and ROI generation, further indicating that the cells are not following the apoptotic pathway. Dox-resistant cells showed a constitutive level of ROI, which did not alter due to doxorubicin treatment. NF-κB activation is redox-sensitive (Anddrassy et. al., 2002; Shimokawa, 2004; Fischer et. al., 2006). Certain amounts of ROI are required for NF-κB activation. The basal amounts of ROI, detected in Dox-resistant cells, may be threshold to attain resistance against doxorubicin, which is also important to keep its high basal NF-κB activity. Induced generation of ROI above this level might have deleterious effect such as lipid peroxidation and other oxidative stress that leads to apoptosis. We did not see any lipid peroxidation in Dox-resistant cells. Our data suggest that the basal expression of SOD1 in Dox-resistant cells might have role in inhibiting doxorubicin-induced ROI generation in these cells. High basal expression of NF-κB induced the expression of SOD1 as downregulation of NF-κB decreased the expression of SOD1. As SOD1 expression is dependent of NF-κB, the basal SOD1 expression was shown in high basal NF-κB containing cells like Dox-resistant cells. Though several reports suggest that high SOD can inhibit redox-sensitive NF-κB activation, how Dox-resistant cells have both high NF-κB and SOD1 needs further study. The high level of ROI in Dox-resistant cells might induce other antioxidant enzymes such as peroxidase, gamma glutamyl synthetase etc (Manna et. al., 1999b; Sarkar et. al., 2004) which neutralize the toxic effect of high ROI level.

As NF-κB is an ideal target for anticancer drug development, switching off aberrant NF-κB activity could have a major therapeutic benefit. Hence we

investigated whether inhibition of NF-κB can sensitize the Dox-resistant cells to apoptosis. In these NF-κB downregulated cells doxorubicin induced apoptosis, increased ROI generation over its basal level, induced lipid peroxidation, and decreased Bcl-2 and SOD1 expressions, further indicating that the cells are following the apoptotic pathway by doxorubicin treatment. Inhibition of NF-κB decreases high basal expression of Mdr and TGase. Mdr is a NF-κB -dependent gene product (Deng et. al., 2001; Kuo et. al., 2002). Possibly, multi-drug resistant proteins are inhibiting doxorubicin's effect in the drug-resistant cells and decreasing these levels, cells are becoming sensitive again to doxorubicin. Downregulation of p65, an important subunit of NF-κB in wild type or Dox-revertant cells, potentiates cell death, which is an important observation. Dox-resistant cells showed constitutive activation of NF-κB whose inhibition resulted in sensitization to cell death by the causal agent, doxorubicin.

The resistance, inherent or acquired, primarily results from the dysregulation of apoptotic pathways in the tumor cells and thus rendering them refractory to apoptotic stimuli including chemotherapy and radiation. As NF-κB is currently being used as a target for cancer therapy, it is important to understand the possible interplay of NF-κB and drug resistance. Overall, our results suggest that doxorubicin-resistant cells show high basal NF-κB activity and bypasses apoptosis. The high NF-κB activity confers resistance to doxorubicin in Dox-resistant cells and down regulation of NF-κB in these cells potentiates apoptosis. This study will help to understand doxorubicin mediated chemoresistance and further designing for combination chemotherapy in intervene several tumors involving doxorubicin. Our results open a new avenue and challenge the current paradigm for the prevention and/or treatment of breast cancer.

Chapter-V

Decrease in Rel-A Phosphorylation by Inhibiting Protein Kinase A induces Cell Death in NF-κB Expressing and Drug-Resistant Tumor Cells.

Introduction

NF-κB, discovered in the laboratory of David Baltimore in 1986, plays an important role in regulating inflammatory and autoimmune responses, cell proliferation, viral replication, asthma and allergic diseases, and apoptosis by regulating the expression of genes encoding inflammatory cytokines, cell adhesion molecules, and cyclooxygenase-2 (Cox-2). NF-κB is known to be activated by over 450 different activators, including cytokines, oxidative stress, mitogens, bacteria, viral proteins and mediators of apoptosis (Baeuerle et. al., 1997; Karin et. al., 2005). The activity of NF-κB is tightly regulated by its interaction with inhibitory IκB proteins. In most resting cells, NF-κB is sequestered in the cytoplasm in an inactive form associated with inhibitory molecules such as IκBα, IκBβ, IκBε, p105, and p100. This interaction blocks the ability of NF-κB to translocate to the nucleus and bind to DNA. Following exposure to pro-inflammatory cytokines, UV light, reactive oxygen species, or bacterial and viral toxins, the NF-κB signaling cascade is activated, leading to the proteasomal degradation of IκB. This allows the translocation of unmasked NF-κB from the cytoplasm to the nucleus where it binds to NF-κB response elements in target genes and regulates their transcription (Baichwal et. al., 1997). Besides the phosphorylation and subsequent degradation of inhibitory molecules, protein kinases are also required for optimal NF-κB activation by targeting functional domains of NF-κB proteins themselves. Numerous other studies have reported the ability of various kinases to phosphorylate p65. PKA is a cAMP-dependent protein kinase and is involved in several cellular functions like regulating cell cycle along with cyclins. Forskolin, an activator of adenylate cyclase activates PKA through generation of cAMP (Lee et. al., 2004). Stimulation of PKA by cAMP is also known to activate another transcription factor, cyclic AMP-responsive element binding-protein (CREB) directly or via p38 MAPK and MSK1 pathway (Delghandi et. al., 2005). The p65 phosphorylation events occur in the cytoplasm or in the nucleus and are stimuli-specific and, probably, cell-type specific. In the cytoplasm, the Protein Kinase A catalytic subunit (PKAc) is maintained in an inactive form by binding to IκBα. After stimulus-induced IκBα-degradation, activated PKAc

phosphorylates p65 on Ser^{276} (Zhong et. al., 1997; Zhong et. al., 1998). This phosphorylation of p65 enhances its ability to recruit histone acetyl transferases such as cAMP response element-binding (CREB)-binding protein (CBP) and p300 (Zhong et. al., 1997; Zhong et. al., 1998) and to displace p50–histone deacetylase (HDAC)-1 complexes from DNA (Zhong et. al., 2002). Therefore, PKAc-mediated phosphorylation positively regulates the transactivation potential of p65 (Zhong et. al., 2002). Ser^{276} of p65 is also phosphorylated by the mitogen- and stress-activated protein kinase-1 (MSK1) in the nucleus. MSK1 is activated by both ERK and p38 MAPK, and phosphorylation of p65 by MSK1 is required for an optimal TNFα-mediated NF-κB activation (Vermeulen et. al., 2003; Viatour et. al., 2005). Aberrant kinase activity is implicated in a variety of human diseases, in particular those involving inflammatory or proliferative responses, such as cancer, rheumatoid arthritis, cardiovascular and neurological disorders, asthma and psoriasis. The ability to modulate kinase activity therefore represents an attractive therapeutic strategy for the treatment of several human illnesses.

Unlike normal cells, in most cancer cells NF-κB is constitutively active and resides in the nucleus. Several key processes, such as self-sufficiency in growth signals, insensitivity to growth inhibitors, evading apoptosis, unlimited replicative potential, tissue invasion, and sustained angiogenesis, are all enhanced following NF-κB activation. Constitutive NF-κB activation can be caused by genetic alterations affecting the genes encoding NF-κB or IκB, or by unrestrained IKK stimulation (Rayet & Gelinas, 1999). Chromosomal aberrations involving the genes encoding REL, RELA, NF-κB1 and NF-κB2 are found in many haematopoietic and solid tumours. Constitutively high levels of nuclear NF-κB activity have also been described in many types of cancer cell, as a result of the constitutive activation of upstream kinases or mutations inactivating IκBs (Rayet & Gelinas, 1999). Constitutive IKK activity is also observed in Hodgkin's disease and childhood acute lymphoblastic leukaemia (Kochetkova et. al., 1997; Nakshatri et. al., 1997; Rayet & Gelinas, 1999; Izban et. al., 2000). Constitutively activated NF-κB might be crucial in the development of drug resistance in cancer cells (Patel et. al., 2000; Arlt et. al., 2001; Arlt et. al., 2003; Kikuchi et. al., 2003; Mabuchi et. al., 2004). For example,

the basal levels of phosphorylation of IκB and activity of NF-κB in cisplatin-resistant ovarian cancer cells (Caov-3 cells) were significantly higher than those in cisplatin-sensitive cells (A2780 cells). Hence downregulation of constitutively expressed NF-κB is an important target for cancer therapy. *In vivo* models of ovarian cancer, colorectal cancer and pancreatic cancer have shown that NF-κB inhibition increases the efficacy of anticancer drugs (Cusack et. al., 2001; Mabuchi et. al., 2004). Inspite of the growing evidence of the important role of NF-κB in tumorigenesis and its resistance to chemotherapy, only few attempts have been made to understand the mechanisms of the constitutive activity of NF-κB in tumor cells and its regulation for successful therapy.

The thiazolidones and thiadiazolie have drawn considerable attention for their anti-bacterial (Abdel-Halin et. al., 1994), anti-fungal (Abdel-Halin et. al., 1994), and anti-inflammatory (Geard et. al., 2003) activities. Anti-fungal activity of some of N and S containing 1,2,4-thiadiazolidines and 3-oxo-1,2,4-thiadiazolidines (Choubey et. al., 1998) prompted us to synthesize newer 1,2,4-thiadiazolidines by employing oxidative debenzylation technique (Manna et. al., 2004) and study their biological activities. Previously we found that these derivatives inhibited TNF-induced NF-κB activation through IKK inactivation (Manna et. al., 2005). As constitutively expressed NF-κB has shown resistance to cell death by different inducers of apoptosis and p65 subunit has been shown to transactivate NF-κB-dependent genes involved in cell progression and differentiation, we are interested in understanding the role of thiadiazolidine derivatives on the p65 level in NF-κB-expressing (HuT-78 or *p65* transfected U-937) and doxorubicin-resistant cells. In laboratory conditions serum upon incubation with lipopolysaccharide (LPS) forms LPS-LBP (LPS binding protein) complex (Hailman et. al., 1994). Cells, stimulated with serum activated LPS (SA-LPS) prolong NF-κB DNA binding activity and are resistant to TNF-induced apoptosis (Manna & Aggarwal, 1999a). Tumor cells, such as HuT-78, HT-29, PC3, have constitutive activity of NF-κB (Sreenivasan et. al., 2003; Huang et. al., 2005). Certain population of MCF-7 cells, when cultured with doxorubicin attained resistance to it (Devarajan et. al., 2002).

In this report we provide data that 5-(4-methoxyarylimino)2-N-(3,4-dichlorophenyl)-3-Oxo-1,2,4-thiadiazolidine (designated as P_3-25 hereafter) is a potent

inhibitor of protein kinase A (PKA) involved in phosphorylation of p65 and thereby NF-κB-dependent genes transcription. The P_3-25 blocks PKA activity without altering cAMP level or adenylate cyclase activity. P_3-25 potently induces cell death in NF-κB-expressing and doxorubicin-resistant cells. For the first time, we provide data that P_3-25 blocks phosphorylation of p65 by inhibiting activity of PKA and thereby blocking expression of NF-κB-dependent genes in NF-κB-expressing cells. This observation may be helpful to design P_3-25 as a novel anti-inflammatory and/or anti-tumor drug in case of drug-resistant tumor cells where NF-κB-dependent genes expression is believed to be an important determinant.

Results

In this study, we examined the effect of 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-Oxo-1,2,4-thiadiazolidine (designated as P_3-25), which was chemically synthesized in the laboratory (Manna et. al., 2005). All these derivatives were dissolved in DMSO at 10 mM concentration. Further dilution was carried out in medium. The concentrations and times used for P_3-25 for this study had no effect in cytolysis as detected by lactate dehydrogenase (LDH) assay. Wherever statistical values (p value) mentioned was calculated from five or more sets of data and analyzed in t test.

P_3-25 induces cell death in NF-κB-expressing cells

Expression of high levels of NF-κB contributes to tumor growth and angiogenesis because it lowers the apoptotic threshold of the cancer cells. Hence to test the apoptotic potential of P_3-25 we used cells that express high levels of NF-κB. U-937 (transfected with vector alone or *p65* constructs) and HuT-78 (constitutively expressed NF-κB) cells were incubated with different concentrations of P_3-25 for 24 h and then cell viability was assayed by MTT assay. P_3-25 induced cell death as shown by decrease in absorbance in a dose-dependent manner in U-937 (vector- or *p65*-transfected) and HuT-78 cells (Fig.1A). Tumor necrosis factor (TNF) is known to induce cytotoxicity in a wide variety of tumor cells and cell lines. But activation of NF-κB and subsequent induction of antiapoptotic genes or protective proteins can protect against TNF-induced apoptosis and hence are involved in TNF-induced cell survival (Rae et. al., 2007). We also found that TNF was unable to induce cell death in p65-tranfected and HuT-78, but not vector-transfected cells (Fig.1A). The level of p65 in U-937 (vector- or *p65*-overexpressed) and HuT-78 cells treated with different concentrations of P_3-25 for 24 h did not change. P_3-25 induced cell death as detected by 'Live & Dead' assay equally in vector- or *p65*-transfected cells, but not in peripheral blood mononuclear cells (PBMC) isolated from fresh human blood (Fig.1B). P_3-25 induced cell death 54% ($p<0.005$) in vector transfected U-937 cells, 52% ($p<0.005$) in p65-transfected cells, 46% ($p<0.005$) in HuT-78 cells, and 4% ($p<0.01$) in PBMC (Fig.1B). Apoptosis can also be assayed by the degradation of internucleosomal DNA using the DNA binding fluorescent dye

propidium iodide. P_3-25 induced nuclear fragmentation equally in U-937 vector- or p65-transfected cells (Fig.1Ca). DNA laddering analysis for the detection of fragmentation of DNA into multiples of the 180 bp nucleosomal unit, known as DNA laddering, where the small fragments of oligonucleosomal DNA is extracted selectively from the cells. P_3-25 induced DNA fragmentation in HuT-78 in a concentration-dependent manner (Fig.1Cb).These results suggest that P_3-25 mediates cell death in NF-κB-expressing cells without decreasing the level of p65.

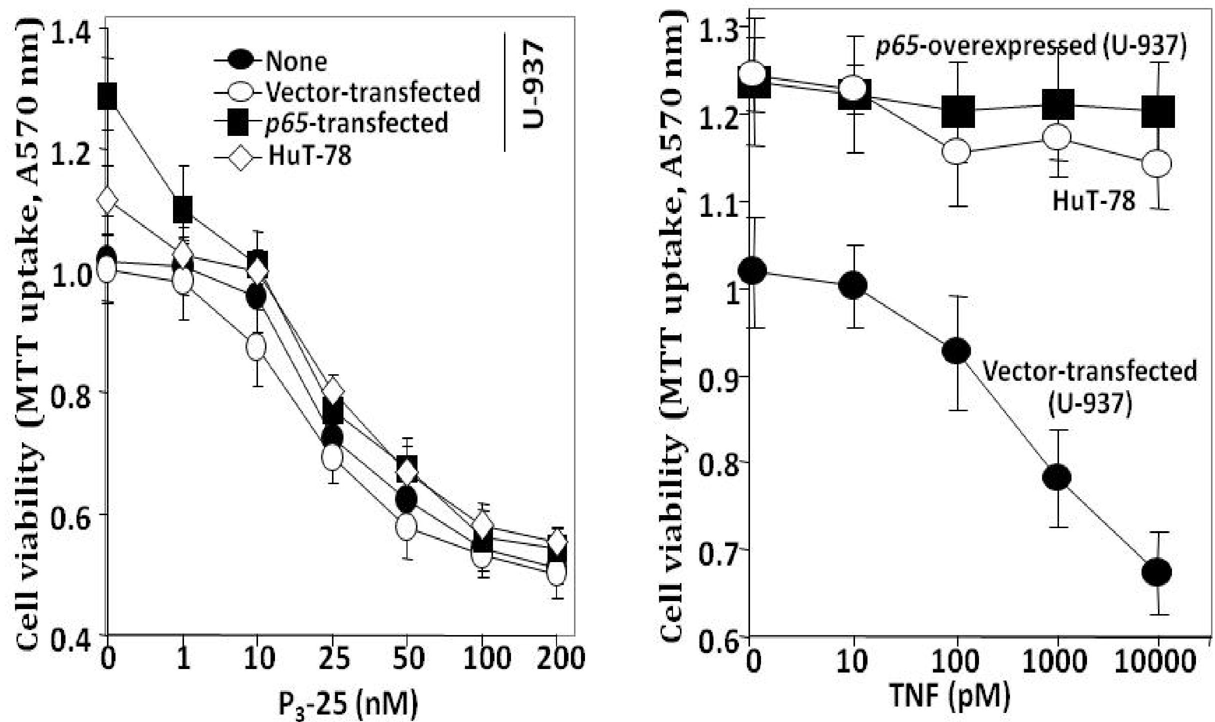


Fig 1A: Effect of P_3-25 on cell viability in NF-κB-expressing cells. U-937 cells were transfected with vector or *p65-NF-κB* DNA with GFP constructs for 3 h. Cells were cultured and GFP positive cells were counted under fluorescence microscope. Transfected and HuT-78 cells were treated with different concentrations of P_3-25 or TNF for 24 h. Cell viability was assayed by MTT method. Mean absorbance of triplicate samples were indicated in the Figure.

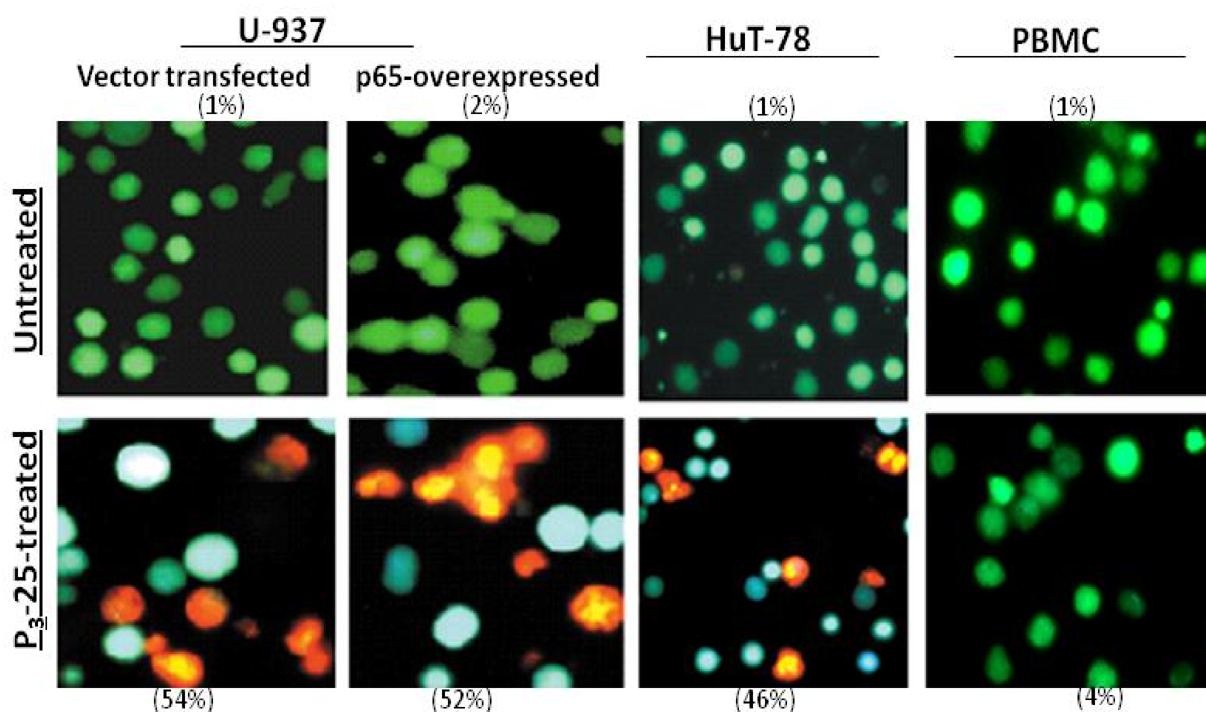


Fig 1B. Effect of P_3-25 on cell viability in NF-κB-expressing cells. Transfected (Vector- or *p65* construct) U-937, HuT-78 cells, and PBMC were treated with 100 nM of P_3-25 for 24 h and then cell viability assayed by Live & Dead cell assay reagent. Dead cells were red and viable cells were blue in color. The percentage of dead cells is indicated in brackets.

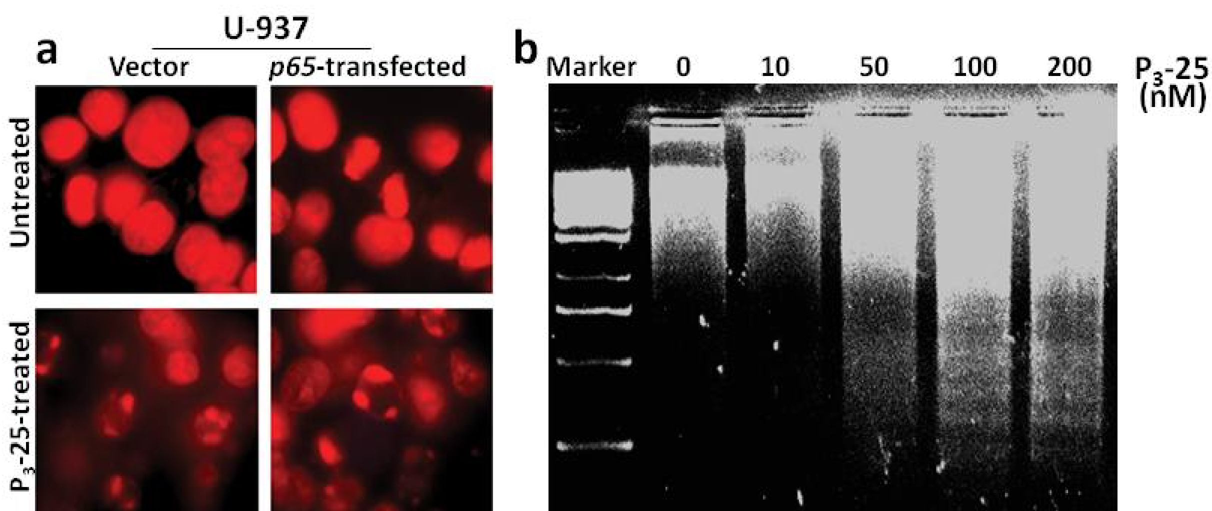


Fig 1C. Effect of P_3-25 on cell viability in NF-κB-expressing cells. Transfected (Vector- or *p65* construct) U-937 cells were treated with 100 nM of P_3-25 for 24 h. Then cells were incubated with propidium iodide (PI) stain, washed, fixed with methanol and then viewed under fluorescent microscope (**a**). U-937 cells were treated with 100 nM P_3-25 for 24 h at 37^0C. Then DNA was extracted and analyzed in 1.5% agarose gel (**b**).

P_3-25 potentiates chemotherapeutic drugs-mediated cell death

Different chemotherapeutic agents (adriamycin, cis-platin, doxorubicin, etoposide, taxol, or vincristine) induced cell death about 50-60% ($p<0.01$) and P_3-25 pre-treated cells showed 80-90% ($p<0.005$) cell death (Fig.2A) without increasing the levels of LDH in these treatments. The data suggest that P_3-25 potentiates cell death in presence of chemotherapeutic agents.

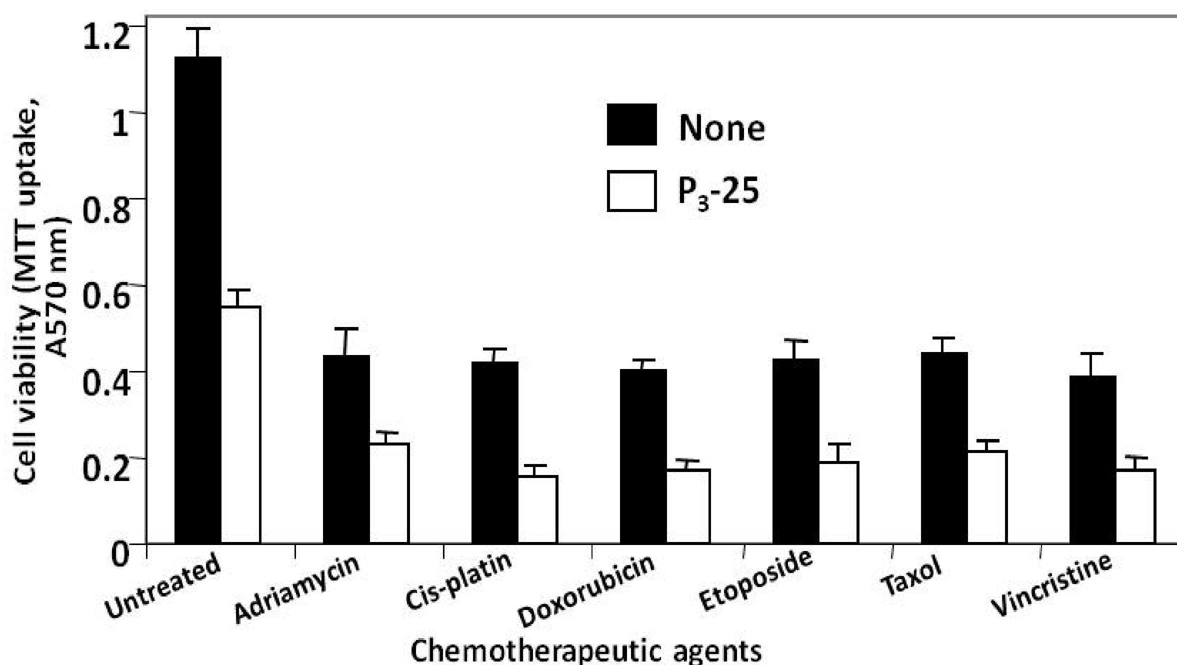


Fig 2A. Effect of chemotherapeutic drugs or P_3-25 on cell viability of U937 cells. U-937 cells, treated without or with 100 nM P_3-25 for 4 h were incubated with 10 μM each of adriamycin, cis-platin, doxorubicin, etoposide, taxol, or vincristine for 24 h. The cell viability was assayed by MTT method. The mean absorbance of triplicate samples was presented in the Figure.

P_3-25 induces cell death in doxorubicin-resistant MCF-7 cells

P_3-25 decreased cell viability in a dose-dependent manner in Dox-resistant or Dox-revertant cells like wild type MCF-7 cells (Fig.2Ba). P_3-25 induced cell death 6.32, 12.56, 26.82, 42.24, and 48.62% at 1, 10, 25, 50, and 100 nM concentrations respectively in Dox-revertant cells as shown by MTT assay. P_3-25 potentiated

doxorubicin-mediated cell death in Dox-revertant cells. Doxorubicin induced cell death 44.62% alone. In presence of doxorubicin, P_3-25 induced cell death 48.26, 54.44, 68.62, 82.28, and 86.72% at 1, 10, 25, 50, and 100 nM concentrations respectively (Fig.2Bb). The results suggest that P_3-25 shows an additive effect for cell death with doxorubicin.

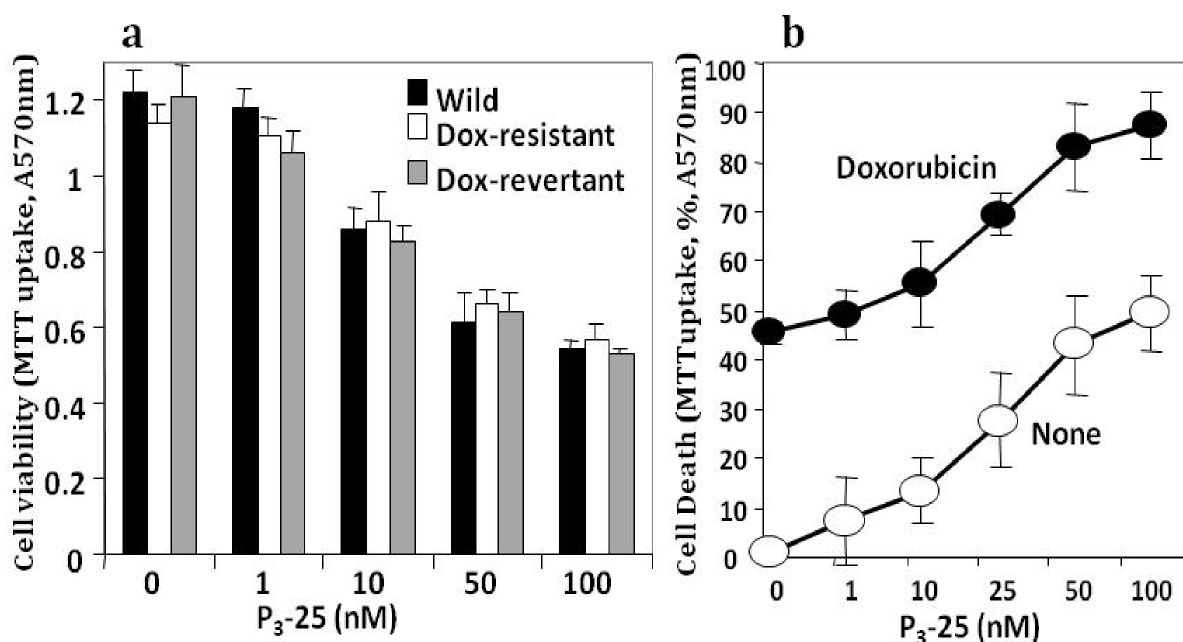


Fig 2B. Effect of P_3-25 on cell viability in MCF-7 cells, Dox- resistant and Dox-revertant cells. MCF-7 (wild), doxorubicin-resistant (Dox-resistant), and doxorubicin-revertant (Dox-revertant) cells were treated with different concentrations of P_3-25 for 24 h and cell viability was assayed by MTT dye uptake. Results represented as mean absorbance of triplicate samples and figure represented one out of three independent experiments. **(a).** Dox-revertant MCF-7 cells were treated with different concentrations of P_3-25 for 4 h followed by incubation without or with 10 μM doxorubicin for 24 h. Cell viability was assayed by MTT method. Results represented as cell death considering 100% absorbance for un-stimulated cells **(b).**

P_3-25 decreases amounts of Bcl-2, Bcl-xL, IAP 1, survivin, ICAM-1, and Cyclin D1 in Dox-resistant MCF-7 cells

Doxorubicin-resistant MCF-7 cells differ from the wild type cells in having altered expression of caspase 3 and tissue transglutaminase (Devarajan et. al., 2002). We tested the sensitivity of these cells to P_3-25 with respect to the properties of expression of NF-κB-dependent genes. Dox-resistant cells showed high basal NF-κB

DNA binding activity and that was marginally decreased upon P_3-25 treatment (Fig.3A). High basal levels of Bcl-2, Bcl-xL, IAP 1, survivin, ICAM-1, and Cyclin D1 were decreased with increasing time of P_3-25 treatment in Dox-resistant cells (Fig.3A).

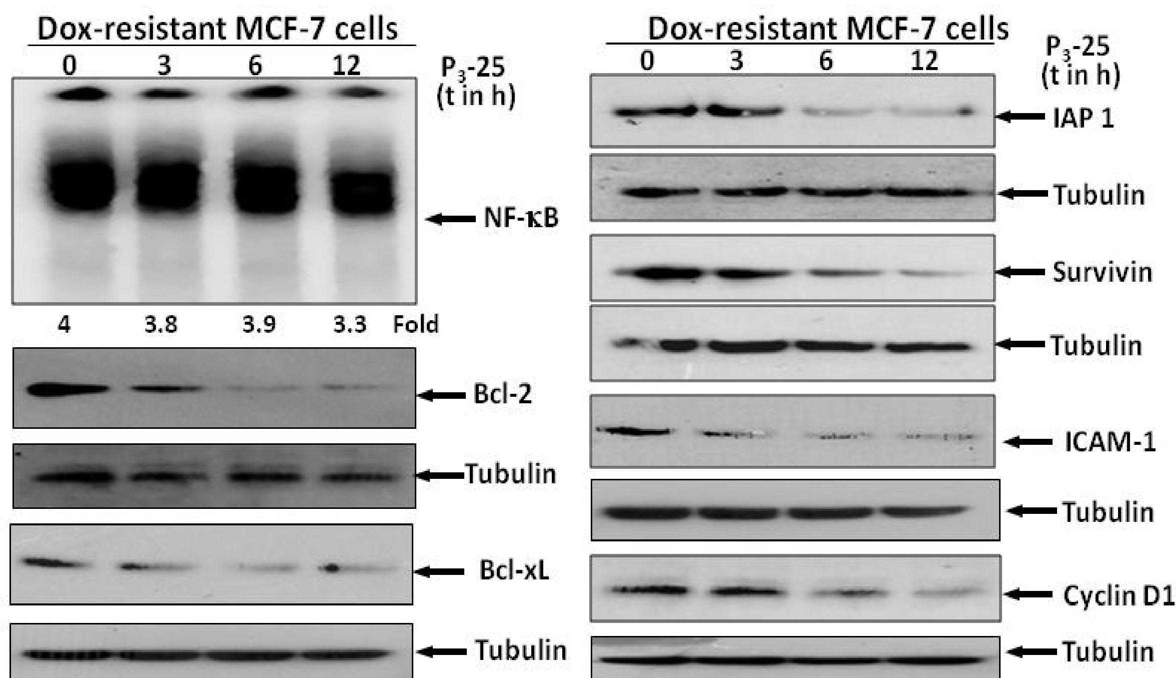


Fig 3A. Effect of P_3-25 on NF-κB DNA binding and NF-κB-dependent genes expression. Dox-resistant cells were treated with 100 nM P_3-25 for different times. NF-κB was assayed from nuclear extract. Expression of NF-κB dependent genes like Bcl-2, Bcl-xL, IAP 1, survivin, ICAM-1, and Cyclin D1 were detected from whole cell extracts by Western blot and all these blots were reprobed with anti-tubulin antibody.

P_3-25 inhibits NF-κB-DNA binding and dependent SEAP activity in NF-κB-expressing cells

P_3-25 inhibited NF-κB DNA binding activity about 20-30% ($p<0.01$) at 100 nM concentration and 24 h of treatment in NF-κB-expressing cells (*p65*-overexpressed, and HuT-78) (Fig.3B). Sp1 DNA binding activity did not alter in those samples as shown by gel shift assay (Fig.3B). Since P_3-25 inhibited NF-κB DNA binding, we looked for NF-κB dependent gene expression by promoter assay. SEAP activity was inhibited kinetically

and almost complete inhibition was achieved at 24 h of P_3-25 treatment in these cells (Fig.3B).

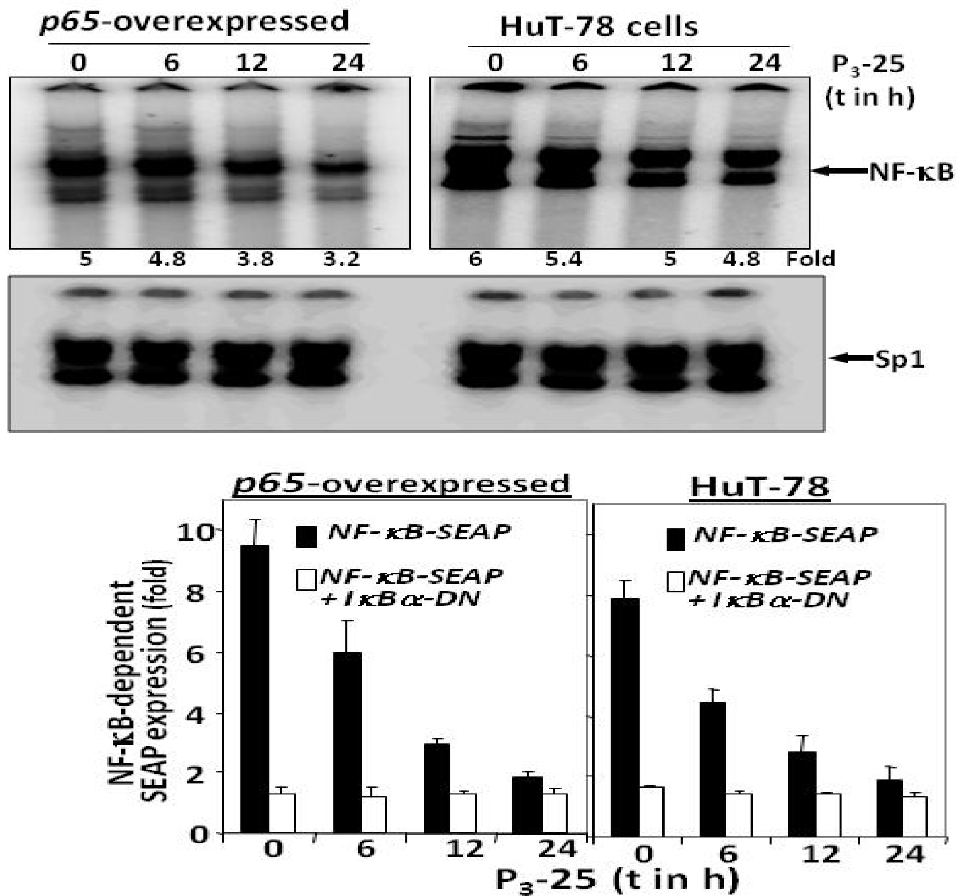


Fig 3B. Effect of P_3-25 on NF-κB, NF-κB-dependent SEAP activity, in NF-κB-expressing cells. NF-κB-expressing cells (U-937 cells, transfected with *p65-NF-κB* DNA and HuT-78 cells) were co-transfected with GFP and SEAP reporter genes for 3 h. Cells were cultured for 12 h and GFP positive cells were counted under fluorescence microscope. Cells were treated with 100 nM P_3-25 for different times. NF-κB DNA binding activity was assayed from nuclear extract and SEAP activity was assayed from cell culture supernatant.

P_3-25 inhibits NF-κB-dependent Cox-2 expression in NF-κB-expressing cells

Cox-2 is an NF-κB regulated gene involved in tumorigenesis, hence we assayed the expression of RNA and protein level of Cox-2 by western blotting and RT-PCR. Cells did not show any toxic effect due to P_3-25 treatment till 24 h as shown by LDH release assay. The level of Cox-2, an NF-κB-dependent gene product, decreased kinetically with

the treatment of P_3-25 in NF-κB -expressing cells as shown by Western blot (Fig.3C) and RT-PCR using Cox-2 specific primer (Fig.3C).

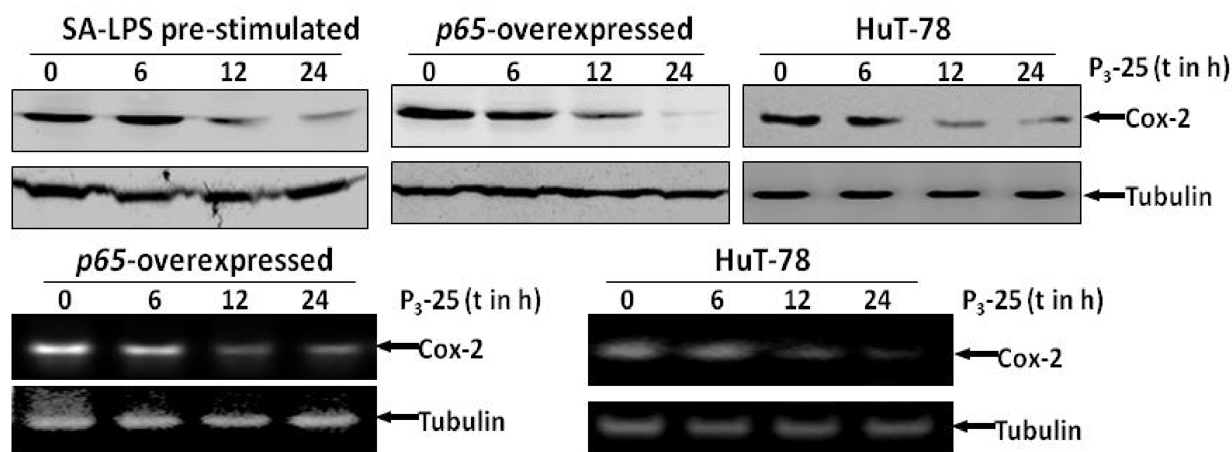


Fig 3C. Effect of P_3-25 on NF-κB-dependent Cox-2 level in NF-κB-expressing cells. NF-κB-expressing cells (U-937 cells, transfected with *p65-NF-κB* DNA and HuT-78 cells) were co-transfected with GFP for 3 h. Cells were cultured for 12 h and GFP positive cells were counted under fluorescence microscope. Cells were treated with 100 nM P_3-25 for different times. Cox-2 levels were detected from cell extracts by Western blot**.** Total RNA was isolated. Cox-2 and actin were detected by RT-PCR using Cox-2 and actin specific primers at 305 and 496 bp bands, respectively**.**

P_3-25 inhibits activity of PKAα, but not IKK or MSK1 in NF-κB-expressing cells

In the nucleus, recruitment of NF-κB to its target genes and regulation of NF-κB-mediated transcriptional activation are mediated mainly by phosphorylation and acetylation of NF-κB, which enhance its DNA binding activity. Several protein kinases, including PKA, MSK1, PKC, and casein kinase II directly phosphorylate p65 (at Ser276, Ser311, and Ser529, respectively). To detect role of upstream kinases involved in NF-κB-dependent gene activation, the MSK1 activity was measured in P_3-25-treated HuT-78 cells and PKAα activity was measured in P_3-25-treated NF-κB-expressing cells (SA-LPS-stimulated or *p65*-overexpressed). P_3-25 treatment did not induce MSK1 activity in HuT-78 cells (Fig.4C). P_3-25 decreased PKAα activity kinetically in SA-LPS-stimulated or

p65-overexpressed cells, whereas partial inhibition of IKK activity was observed at 6 h of P_3-25 treatment (Fig.3D).

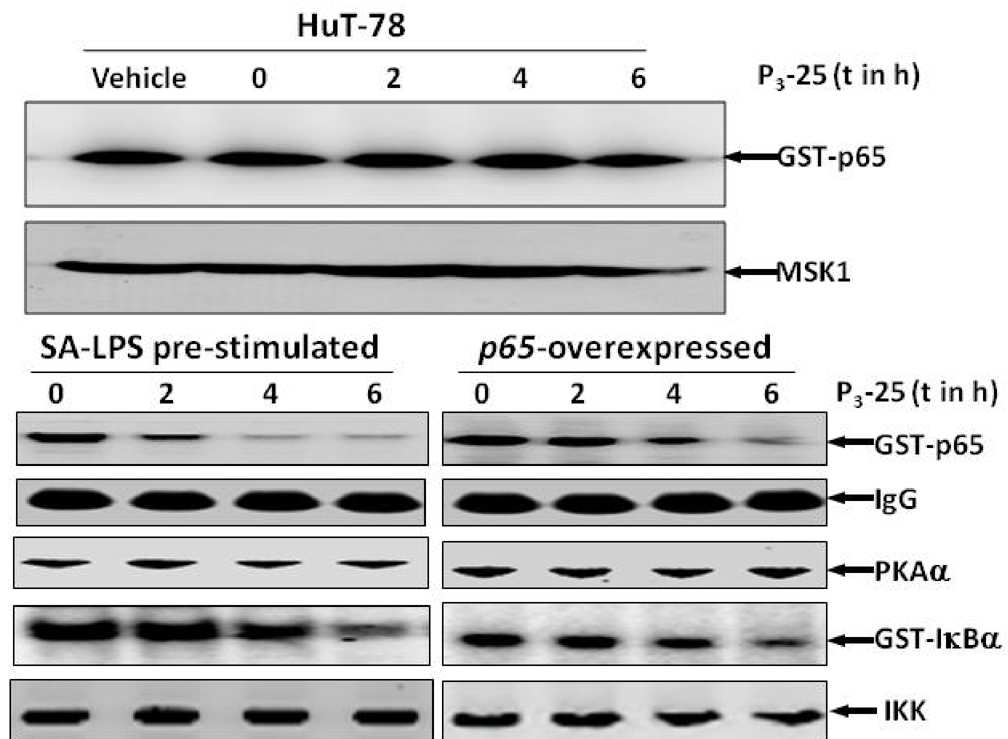


Fig 3D. Effect of P_3-25 on MSK1, PKA and IKK activities in NF-κB-expressing cells. HuT-78 cells were treated with P_3-25 for different times. MSK1 activity was detected using GST-p65 as substrate. U-937 cells, SA-LPS-stimulated or transfected with *p65-NF-κB* DNA with GFP constructs for 3 h were treated with P_3-25 for different times. PKA and IKK activities were detected using GST-p65 and GST-IκBα substrate respectively. The amounts of MSK1, PKA and IKK were detected from same extract proteins by Western blot.

P_3-25 decreases phospho-p65 level in Dox-resistant cells

As we have shown the activity of PKA decreased by P_3-25 treatment in NF-κB -expressing cells, the level of phospho-p65 was detected in MCF-7 and Dox-resistant MCF-7 cells. Basal level of phospho-p65 was decreased in both MCF-7 and Dox-resistant MCF-7 cells (Fig.3E). High basal level of phospho-p65 was observed in Dox-resistant MCF-7 cells.

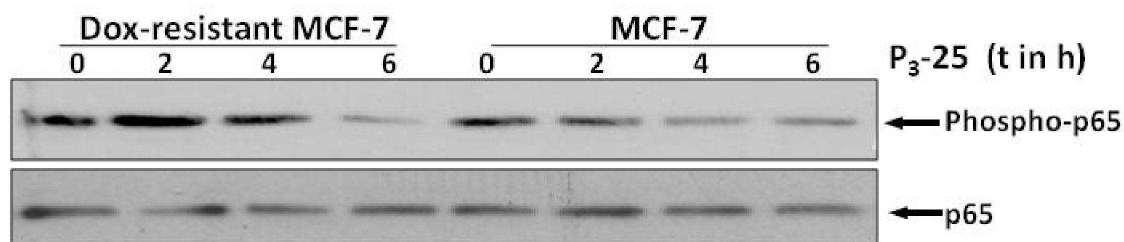


Fig 3E. Effect of P_3-25 on p65 phosphorylation. MCF-7 (wild type and Dox-resistant) cells were treated with P_3-25 for different times and amounts of phospho-p65 were detected from whole cell extracts by Western blot. The same blot was reprobed for p65.

P_3-25 inhibits PKAα activation induced by dibutyryl cAMP

Cyclic AMP (cAMP) has shown to activate PKAα. To detect the role of P_3-25 on PKA activity, cells were pre-treated with P_3-25 for 6 h and then incubated with different concentrations of dibutyryl cAMP. The activity of PKAα was detected by using GST-p65 as substrate. Dibutyryl cAMP increased PKAα activity in a dose-dependent manner and this activity was completely inhibited by P_3-25 upon incubation with cells (Fig.4A).

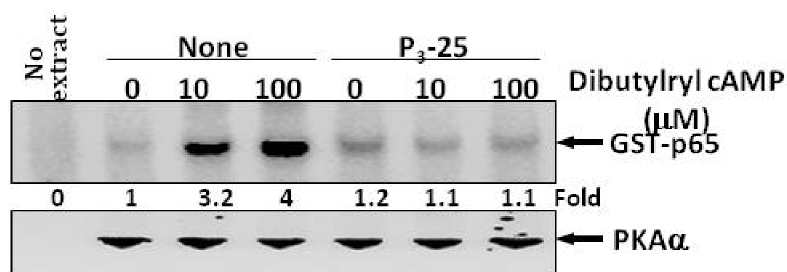


Fig 4A. Effect of P_3-25 on PKAα activation. U-937 cells, pre-treated with 100 nM P_3-25 for 6 h were incubated with different concentrations of dibutyryl cAMP for 2 h. The PKAα was assayed from cell extracts using GST-p65 as substrate.

P_3-25 inhibits activity of PKAα induced by forskolin

Forskolin is commonly used to raise levels of cyclic AMP (cAMP) in this study. Forskolin resensitizes cell receptors by activating the enzyme adenylate cyclase and

increasing the intracellular levels of cAMP and hence increased PKAα activity. Cells were pre-treated with P_3-25 (100 nM) for 6 h and then stimulated with 25 μM of forskolin for 2 h. The PKAα activity assayed using GST-p65 as substrate. The forskolin induced the PKAα activity and P_3-25 completely inhibited the activity as shown by the intensity of radioactive bands (Fig.4B). The data suggest that P_3-25 possibly blocks adenylate cyclase.

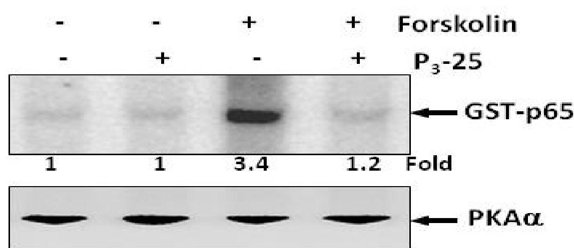


Fig 4B. Effect of P_3-25 and Forskolin on PKAα activation. U937 cells, pre-treated with 100 nM P_3-25 for 4 h were incubated with forskolin (25 μM) for 2 h. The cell extracts were then assayed for PKAα using GST-p65. The level of PKAα was detected from cell extracts by Western blot.

P_3-25 does not decrease level of cAMP induced by forskolin

Forskolin, a direct adenylate cyclase-stimulating agent, has been reported to increase cAMP levels in U937 cells. U-937 cells were treated with P_3-25 (100 nM) for 6 h and then stimulated with forskolin (25 μM) for 2 h. The cAMP level was detected. The forskolin increased the level of cAMP, but P_3-25 pre-treated cells did not block the increased level of cAMP induced by forskolin (Fig.4C). The results suggest that P_3-25 does not affect adenylate cyclase.

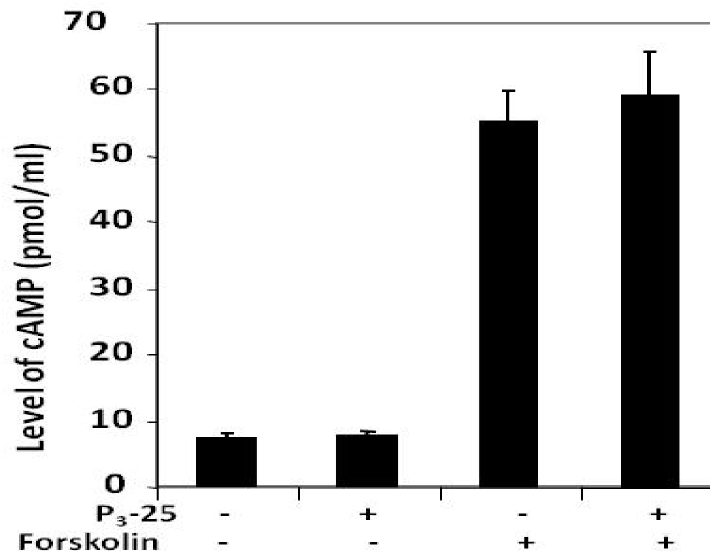


Fig 4C. Effect of P_3-25 and Forskolin on cyclic AMP generation. U937 cells, pre-treated with 100 nM P_3-25 for 6 h were incubated with 25 μM forskolin for 2 h and then extracted with 0.1 N HCl in liquid nitrogen and cAMP was assayed. Results were expressed in pmol/ml as detected from standard curve with known concentrations of cAMP**.** The results are representative of one of three independent experiments.

P_3-25 or Rp-cAMPS (adenosine cyclic 3', 5'-phosphorothioate triethylammonium salt) inhibits activity of PKA induced by SA-LPS

Cells were incubated with Rp-cAMPS, a known inhibitor of PKA or P_3-25 for 6 h followed by stimulated with SA-LPS and then PKA activity was assayed. The PKA activity was inhibited by P_3-25 and Rp-cAMPS kinetically (Fig.4D), indicating P_3-25 as potent inhibitor of PKA activity and thereby NF-κB -dependent gene expression.

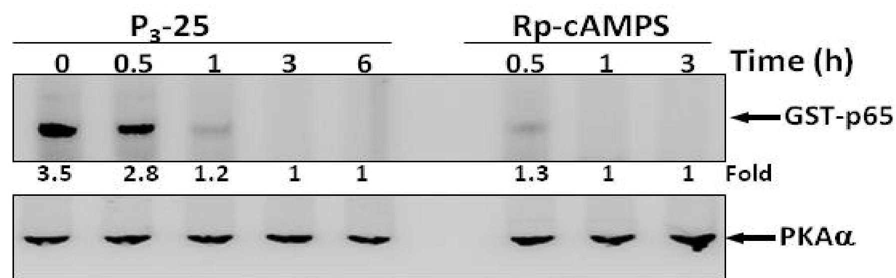


Fig 4D. Effect of P_3-25 and Rp-cAMP on PKAα activation. Cells, treated with 100 nM of P_3-25 or 100 μM of Rp-cAMPS for different times were stimulated with SA-LPS for 1 h. Whole cell extracts were prepared and 300 μg proteins were immunoprecipitated with anti-PKA antibody (1 μg) and assayed for PKA using GST-p65 as substrate.

P_3-25 and PKA inhibitors inhibit NF-κB-dependent gene, but not NF-κB DNA binding activity

HuT-78 cells treated with H-7 and H-8 (5 μM each) in combination with P_3-25 (100 nM) showed no alteration of constitutively expressed NF-κB DNA binding activity by gel shift assay (Fig.4E, upper panel). H-7 or H-8 partially inhibited NF-κB -dependent SEAP activity in NF-κB promoter driven SEAP reporter gene transfected HuT-78 cells and P_3-25 alone or in combination of H-7 or H-8 completely inhibited SEAP activity (Fig.4E, side panel). The inhibition of SEAP activity reflected in the PKA activity in different treatments (Fig.4E, lower panel). These results suggest that inhibition of PKA activity reflects NF-κB-dependent gene expression.

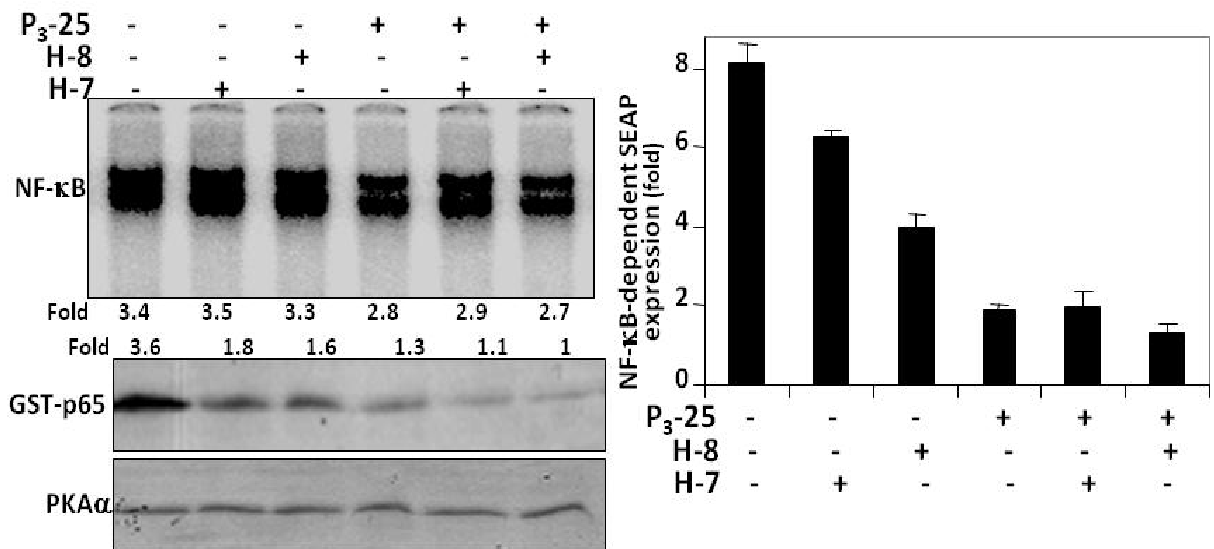


Fig 4E. Effect of P_3-25, H-8, H-7 on NF-κB, NF-κB dependent gene expression and PKAα activity. HuT-78 cells, transfected with NF-κB -containing plasmid linked to the *SEAP* gene and *GFP* constructs were treated with H-7 and H-8 (5 μM each) for 4 h and then treated with P_3-25 for 12 h. NF-κB was assayed from nuclear extracts; SEAP activity was assayed from culture supernatant; and PKA activity was assayed from whole cell extracts.

P_3-25 inhibits activity of CREB DNA binding induced by dibutyryl cAMP

As P_3-25 inhibits PKA, another transcription factor CREB, which is induced by cAMP-mediated PKA activation was assayed by gel shift assay. The cAMP increased CREB DNA binding activity in a dose-dependent manner and P_3-25 completely inhibited this binding activity (Fig.4F). Cold CREB oligonucleotide almost completely suppressed labeled oligonucleotide binding suggesting specificity of assay. This result suggests that P_3-25 inhibits CREB DNA binding activity.

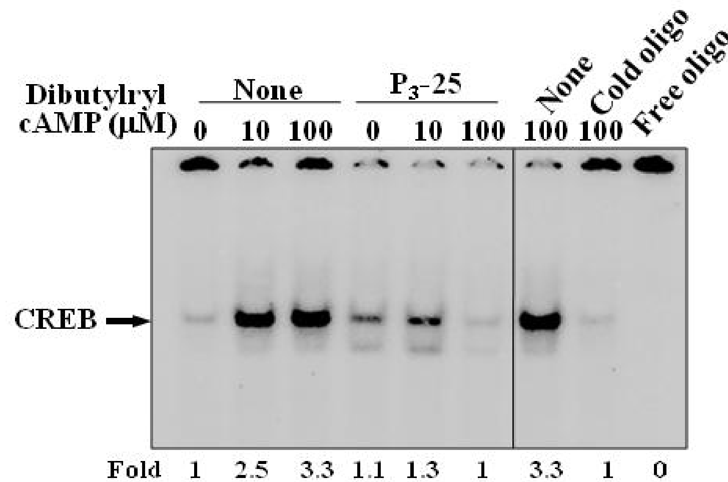


Fig 4F. Effect of P_3-25 on CREB DNA binding. Cells, treated without or with 100 nM P_3-25 were stimulated with 10 and 100 μM of dibutyryl cAMP for 2 h. CREB DNA binding was assayed from nuclear extracts by gel shift assay, keeping one lane with 100 fold unlabeled CREB oligonucleotide.

P_3-25 inhibits activity of PKAα catalytic subunit *in vitro*

To detect the role of P_3-25 on the activity of PKAα, cell extracts from SA-LPS treated cells was immunoprecipitated with anti-PKAα antibody. The immune complexes were treated with 100 nM of P_3-25 for different times and PKAα activity assayed using GST-p65 protein (Fig.5a) or kemptide (a phosphate acceptor peptide with sequence of Leu-Arg-Arg-Ala-Ser-Leu-Gly) (Fig.5a, lower panel) as substrates. P_3-25 kinetically inhibited PKAα activity indicating its ability to inhibit PKAα *in vitro*. P_3-25 (100 nM) also inhibited on the recombinant PKAα (catalytic subunit) activity kinetically

as detected by using GST-p65 protein (Fig.5b) or kemptide (Fig.5b, lower panel) as substrates. The IC50 (50% inhibitory concentration) of P_3-25 was 10.5 nM for PKAα as detected by measuring the incorporated ^{32}P to kemptide by recombinant PKAα treated with P_3-25 (Fig.5C).

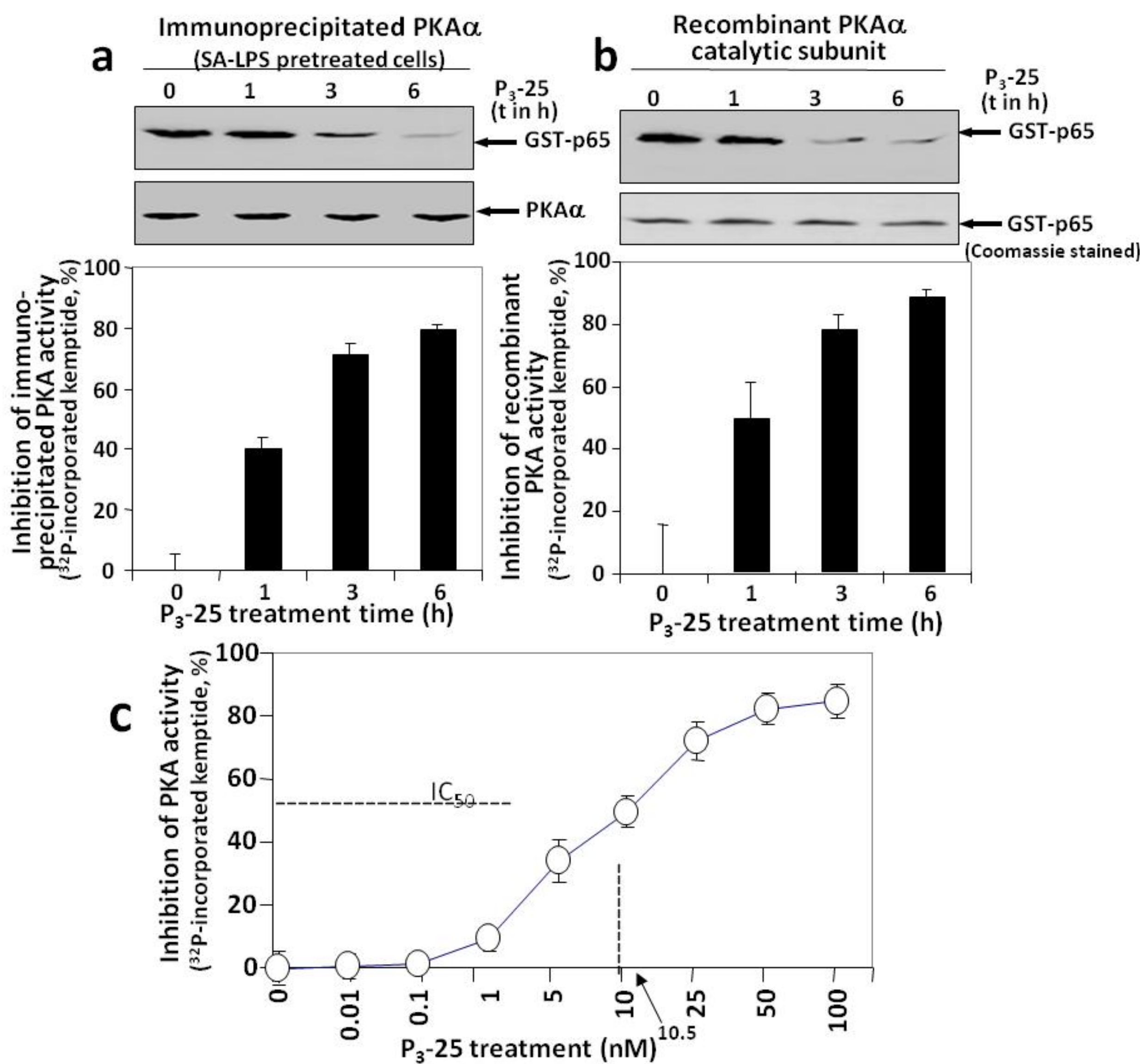


Fig 5A. Effect of P_3-25 on PKAα activity *in vitro*. PKAα, immunoprecipitated from SA-LPS-treated cell extract was incubated with P_3-25 (100 nM) for different times and then assayed for PKAα using GST-p65 protein **(a)** or kemptide **(a lower panel)** as substrates. The levels of PKAα were detected from the respective treatments. Thirty units of PKAα catalytic subunit were incubated with 100 nM P_3-25 for different times and then PKAα activity assayed using GST-p65 protein **(b)** or kemptide **(b lower panel**) as substrates and IC50 of P_3-25 for recombinant PKA was calculated from diagram **(c).** Data represented one out of three independent experiments.

P_3-25 does not compete H-7, H-8, or Rp-cAMPS binding site into PKAα

Whether P_3-25 competes with other PKAα inhibitors for PKAα activity, HuT-78 cell extracts were immunoprecipitated with anti-PKAα antibody. Immune complexes were treated with H-7, H-8, or Rp-cAMPS in presence of different concentrations of P_3-25 for 2 h at 37^{0}C. PKAα activity assayed using GST-p65 (full-length) substrate protein. The level of phosphorylated GST-p65 band decreased with increasing concentrations of P_3-25. H-7 or H-8 pretreated cells did not alter P_3-25-mediated decrease in the GST-p65 band, whereas Rp-cAMPS potentiated the decrease in GST-p65 band upon P_3-25 treatment (Fig.5B). The concentrations of phospho-p65 were measured in whole cell extracts using anti-phospho-p65 (Ser^{276}) antibody by Western blot upon same treatments and similar results obtained as shown by in vitro PKAα activity assayed in the upper panel (Fig.5B). Upon re-probing those blots with anti-p65 antibody the amounts of p65 protein were found to be unchanged. These results suggest that Rp-cAMPS may potentiate P_3-25-mediated inhibition of p65 phosphorylation.

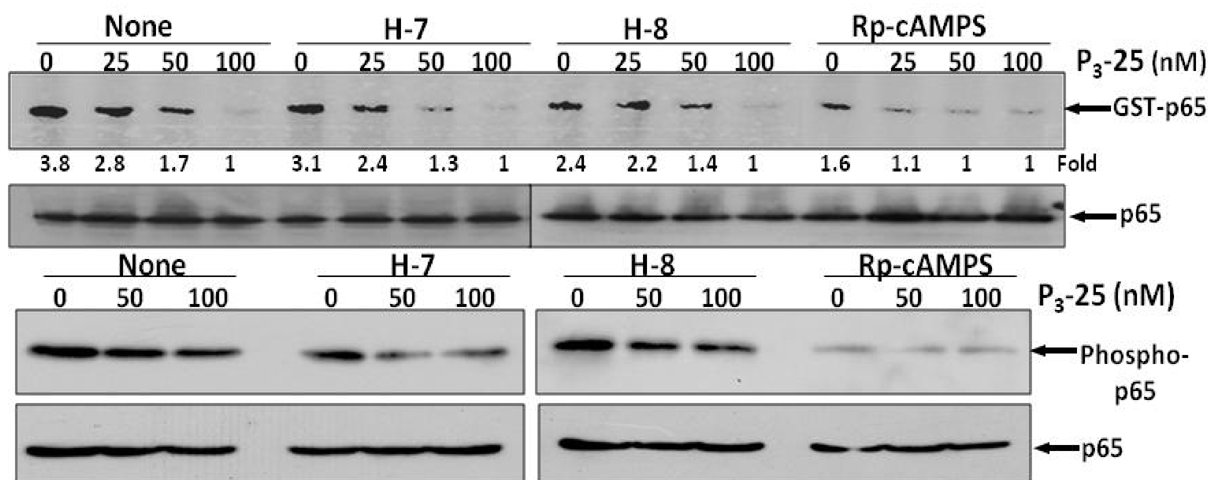


Fig 5B. Effect of PKA inhibitors on P_3-25-mediated inhibition of PKAα. HuT-78 cell extracts were immunoprecipitated with anti-PKAα antibody. Immune complex was treated with H-7 (2 μM), H-8 (2 μM), and Rp-cAMPS (50 μM) for 2 h followed by P_3-25 (100 nM) for 2 h. Then PKAα activity was assayed using GST-p65 substrate protein. 50 μg of extract proteins were used to detect p65 level by Western blot. Hut-78 cells, treated with H-7 (5 μM), H-8 (2 μM), and Rp-cAMPS (50 μM) for 2 h were treated with different concentrations of P_3-25 for 4 h. Whole cell extracts were used to detect phospho-p65 using anti-phospho-p65 antibody(**D**, lower panel). The same blot was reprobed with p65.

Abrogation of PKAα activity inhibits NF-κB gene expression and induces cell death

To detect the specific role of PKAα in NF-κB-dependent gene expression, we abrogated the PKAα expression by ShRNA transfection. HuT-78 cells were cotransfected with *ShRNA*, *NF-κB-SEAP* and *GFP* and then the amounts of PKAα was determined by Western blot. The amount of PKAα was not altered in vehicle (ShRNA transfection reagent and transfection buffer provided in ShRNA transfection kit and *pCMV* vector) treatment along with 100 nM P_3-25 or 25 μM forskolin for 24 h. Complete inhibition of amount of PKAα was observed in ShRNA-transfected cells (Fig.6A).

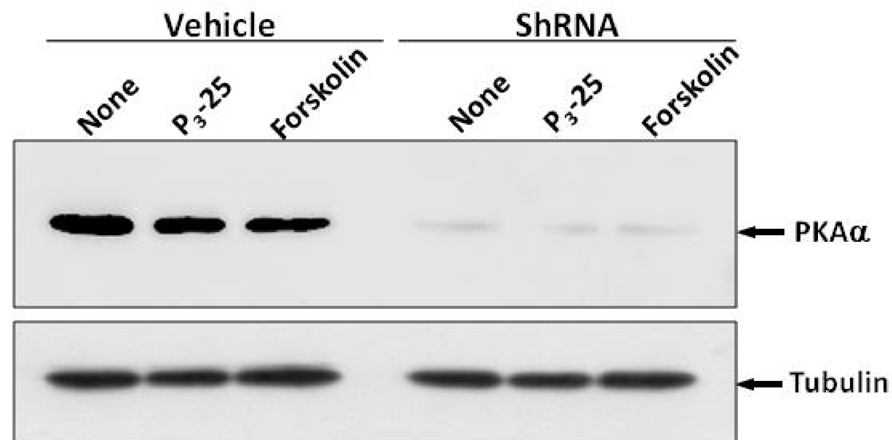


Fig 6A. **Abrogation of PKAα by ShRNA.** HuT-78 cells were co-transfected with *ShRNA* plasmid DNA of PKAα, and *GFP* constructs for 6 h and then cultured for 12 h. Cells were then untreated or treated with (10 μl/ml), 100 nM P_3-25, or 25 μM forskolin for 24 h. Amount of PKAα was detected from whole cell extracts and the same blot was reprobed for tubulin.

Transfected cells, either vehicle or ShRNA treated with 1% DMSO, 100 nM P_3-25, or 25 μM forskolin for 24 h did not show any alteration of NF-κB DNA binding activity (Fig.6B). ShRNA transfected cells showed almost 75% inhibition of NF-κB-dependent SEAP activity. P_3-25 alone induced almost similar effect in ShRNA nontransfected cells (Fig.6B).

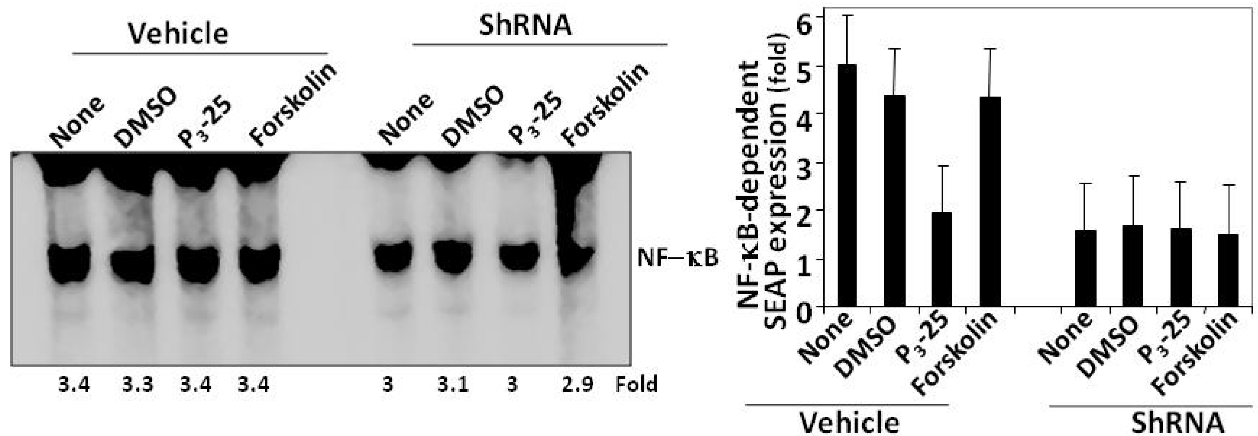


Fig 6B. Effect of ShRNA abrogated PKAα on P_3-25- and forskolin-mediated NF-κB activation. HuT-78 cells were co-transfected with *ShRNA* plasmid DNA of PKAα, NF-κB-containing plasmid linked to the *SEAP* gene, and *GFP* constructs for 6 h and then cultured for 12 h. Cells were then untreated or treated with DMSO (10 μl/ml), 100 nM P_3-25, or 25 μM forskolin for 24 h. NF-κB DNA binding was assayed from nuclear extracts and SEAP activity was assayed from culture supernatant.

Almost 50% cell death was observed in PKAα inhibited cells, which was almost similar with P_3-25 alone treated HuT-78 cells as shown in MTT assay (Fig6C). Almost similar results were obtained from Live/Dead assay (Fig.6C). All these data support that PKAα inhibited cells are prone to cell death equally with P_3-25 alone treated cells.

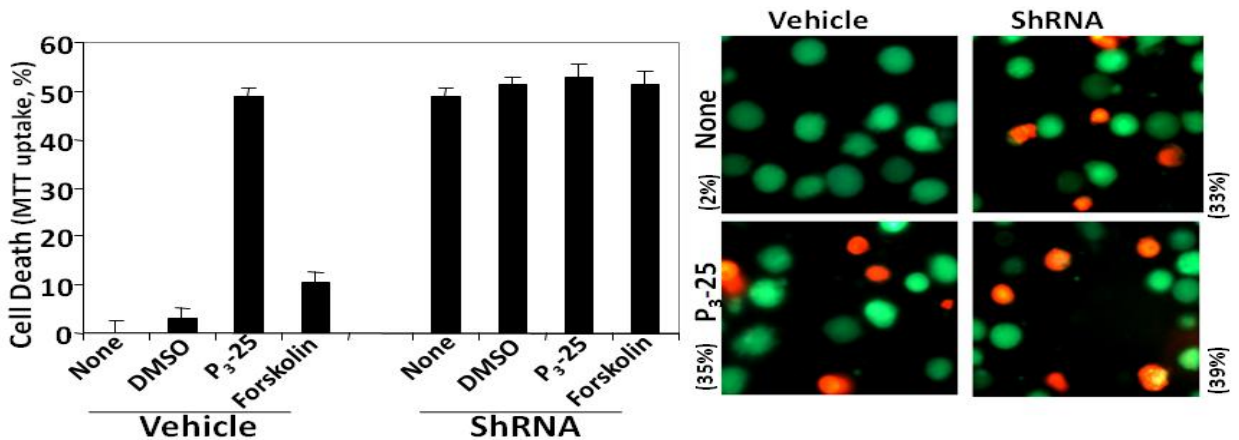


Fig 6C. Effect of ShRNA abrogated PKAα on P_3-25- and forskolin-mediated cell death. HuT-78 cells were co-transfected with *ShRNA* plasmid DNA of PKAα, and *GFP* constructs for 6 h and then cultured for 12 h. Cells were then treated with DMSO (10 μl/ml), 100 nM P_3-25, or 25 μM forskolin for 24 h. Cell viability was detected by MTT assay from duplicate samples and indicated as cell death in percentage. The number of dead cells (red in color) was counted under fluorescence microscope using 'Live/Dead' assay kit and indicated in percentage.

Overall, our results suggest that P_3-25 exerts antitumorigenic activity by inhibiting phosphorylation of p65, the transcriptional active subunit of NF-κB by inhibiting its upstream kinase PKAα, and potentiates apoptosis mediated by chemotherapeutic agents. These results suggest novel approaches for designing of anticancer drugs for combination chemotherapy.

Discussion

A role for NF-κB in cancer is supported from numerous reports showing that NF-κB is activated (i.e. nuclear) in a number of tumors. Thus, NF-κB (RelA) activation has been detected in a variety of solid tumors, including prostate tumors, breast tumors, melanoma, pancreatic cancer, and lung adenocarcinoma (Baldwin, 2001; Yamamoto et. al., 2001; Karin et. al., 2002). Negative regulation of NF-κB is controlled primarily through interactions with the IκB family of proteins, which prevent DNA binding and promote cytoplasmic accumulation of the interacting dimeric complex. The positive regulation of NF-κB is controlled through the activity of the IκB kinase (IKK) complex which phosphorylates IκB proteins leading to their ubiquitination and subsequent proteasomal degradation, leading to nuclear accumulation of the released NF-κB complex. Evidences suggest that, a second level of regulation of NF-κB activity independent of IκB, which relies in the activation of the transcriptional activity of p65, is also found (Schmitz et. al., 1995; Schmitz & Baeuerle, 1995; Lee et. al., 2002). Thus, the catalytic subunit of PKA was shown to be bound to inactive NF-κB complexes and upon IκB degradation this catalytic subunit phosphorylated p65, resulting in an enhanced transcription promoting activity (Zhong et .al., 1998). Moreover, TNFα treatment of cells results in phosphorylation of Ser 529 in the transactivation domain of p65, resulting in the activation of the transcriptional activity of the protein (Lee et. al., 2002). Hence high basal activities of kinases like PKA, CKII, and IKK result in the constitutive expression of NF-κB and other genes involved in tumorigenesis. In the present report, we demonstrated that dichlorophenyl form of 1,2,4-thiadiazolidine (P_3-25) is a potent inhibitor of p65 phosphorylation. As p65 phosphorylation is required for

NF-κB-dependent genes expression involved in inflammatory, allergic, and tumorigenic responses, we used NF-κB-expressing cells to detect the role of the synthesized derivative for various cellular responses. The removal of IκBα is required for the activation of IKK complex. The IKKα is a rate-limiting enzyme for IKK complex for IκBα phosphorylation. Activation of IKKα results in degradation of IκBα and release of NF-κB complex. Surprisingly, in NF-κB-expressing cells P_3-25 partially inhibited activities of IKK and NF-κB DNA binding, but completely inhibited NF-κB-dependent SEAP reporter gene expression and Cox-2 expression (Fig.3B and 3C). Phosphorylation of p65 is mediated by PKA. P_3-25 blocks PKA and abrogates NF-κB-dependent genes expression. P_3-25 equally decreased NF-κB-dependent genes and induced cell death in PKAα inhibited cells by using pools SiRNA against PKAα regulatory subunit (Fig.6). P_3-25 specifically blocks PKAα catalytic subunit without interfering upstream molecules such as adenylate cyclase or cAMP generation that further evidenced by P_3-25-mediated inhibition of CREB DNA binding activity. The effect of P_3-25 completely lies on tumor cells, but not primary cells, like PBMC isolated from fresh human blood (Fig.1B). How P_3-25 did not affect primary cells need to be studied further. The uptake of nutrients and drugs by PBMC might be less than aggressive tumor cells.

As most of the cells, involved in asthmatic and tumorigenic responses, have basal activity of NF-κB, the downregulation of the NF-κB-dependent genes with this derivative might help to design it as potential drug for asthma or tumor therapy. Modification of different side group(s) of known compound(s) to make it more efficient drug would be viable strategy in the medical science considering the genesis of in-effective anti-inflammatory and chemotherapeutic drugs. Previously, we reported TNF-induced NF-κB activation is inhibited by P_3-25 through inhibition of IKK complex and thereby inhibiting translocation of p65 to the nucleus (Manna et. al., 2005). High basal activity of IKK, MSK1, and PKA was observed in NF-κB-expressing and Doxorubicin resistant cells. High basal concentration of NF-κB-dependent genes such as Bcl-2, Bcl-xL, IAP 1, survivin, ICAM-1, and Cyclin D1 were observed in Dox-resistant cells.

Antiapoptotic genes expression shown by Dox-resistant cells, may be important for the generation of resistance against widely used chemotherapeutic drug, doxorubicin. These cells show high basal activity of PKAα, required for p65 phosphorylation and NF-κB-dependent genes expression. The partial inhibition of IKK might correlate with the partial inhibition of NF-κB DNA binding activity on P_3-25 treatment in NF-κB-expressing cells. Complete inhibition of NF-κB-dependent genes expression clearly suggests that P_3-25's effect lies other than IKK. P_3-25 completely inhibited p65 phosphorylation by inhibiting activity of PKAα, but not MSK1 in cells and *in vitro* (recombinant PKAα catalytic subunit) suggesting P_3-25's effect lies on the catalytic site of PKAα. Although H-7 and H-8, PKA inhibitors did not completely block PKA activity and thereby NF-κB-dependent gene expression but P_3-25 completely inhibited both. These data suggest that P_3-25 might abrogate other pathways than PKA inhibition. In H-7 or H-8 pretreated cells, P_3-25 did not show any additive effect for PKAα activity. In Rp-cAMPS pretreated cells, P_3-25 showed an additive effect to inhibit PKAα activity. P_3-25 might interact at H-7 or H-8 binding site of PKAα but not in Rp-cAMPS interacting site. All these possibilities need to be studied further.

Several genes such as cytokines, cyclooxygenase-2, metalloproteases, urinary plasminogen activator, and cell surface adhesion molecules are involved in tumor promotion and are also regulated by NF-κB. P_3-25 was found to be a potent inhibitor of Cox-2 activation and it blocked Cox-2 protein in time-dependent manner in NF-κB-expressing cells. Hence it is now partially conceivable that blockade of specific subsets of the NF-κB/Rel family by P_3-25 could be used to inhibit inflammatory disease. As P_3-25 blocked NF-κB-dependent reporter gene expression, it may play a critical role in carcinogenesis and inflammation exhibiting anticarcinogenic and anti-inflammatory effects. Therefore, suppressive ability of P_3-25 for NF-κB-mediated active gene transcription could be exploited by combination with chemotherapy. As aspirin is beneficial to prevent colon cancer by inhibiting Cox-2 through NF-κB (Wunsch, 1998; Sreenivasan et. al., 2006) the search for novel inhibitor(s) might be important to

regulate Cox-2-mediated immunotherapy as well as cancer therapy. As most of the tumor cells and bronchial epithelial cells involved in asthma and allergic diseases have basal activity of NF-κB (Romieu-Mourez et. al., 2001; Wright et. al., 2003; Suh et. al., 2004; Manna et. al., 2006). P_3-25 may be beneficial for different cancers, inflammatory and allergic diseases. P_3-25's ability to suppress p65 phosphorylation by inhibiting protein kinase A in NF-κB-expressing cells and thereby inhibition of NF-κB-dependent genes involved in several biological responses may provide the molecular basis for the anti-carcinogenic properties of P_3-25. Induction of cell death in NF-κB-expressing cells, mediated by P_3-25 but not by TNF, indicates its potential as a drug. P_3-25 potentiates cell death mediated by chemotherapeutic agents. It sensitizes cell death in doxorubicin-resistant cells and shows an additive cell death effect with doxorubicin. All these data indicate P_3-25's potent cytotoxic effect in chemo-resistant cells.

Since it is now apparent that the effects of cytotoxic drugs on NF-κB function are quite diverse and may differ between cell and tumor types or even the different stages of tumor development, it is necessary to understand the different roles and regulations of NF-κB. Moreover, selective targeting of specific NF-κB subunits, IκB proteins, or kinases that have a degree of tissue specificity or by identifying individual component as key to a particular disease one might attain therapeutic efficacy and minimize systemic toxicity. In order to use NF-κB inhibitors in the clinic, thorough approaches must be initiated to determine the molecular mechanisms that the drug dictates on the complexity of oncologic and therapeutic outcomes. Here we report for the first time that dichlorophenyl derivative of 1,2,4 thiadiazolidine (P_3-25) potentially inhibits NF-κB-dependent several genes involved in inflammation and tumorigenesis by inhibiting PKA thereby p65 phosphorylation without interefering NF-κB DNA binding. Thus, P_3-25 induces cell death in NF-κB-expressing and Dox-resistant breast tumor cells. Our results suggest that P_3-25 may also have application(s) for various other diseases including inflammation and arthritis, where constitutive level of NF-κB activation has been shown to mediate pathogenesis.

SUMMARY

It is the nature of cells to divide and increase their number in a process called mitosis. Normal cells divide to replace those lost, or to repair injuries only, then stop dividing. Cancer is an abnormal growth of cells (usually derived from a single cell). The cells have lost normal control mechanisms and thus are able to expand continuously, invade adjacent tissues, migrate to distant parts of the body, and promote the growth of new blood vessels from which the cells derive nutrients. Cancer remains the second-leading cause of death in the industrialized world and worldwide. The disease accounted for 7.9 million deaths (or around 13% of all deaths worldwide) in 2007. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030. Incidences of breast cancer in India are also on rise: it is reported that one in 22 women in India is likely to suffer from breast cancer during her lifetime. Unfortunately, effective therapeutic agents to combat cancer continue to be lacking. Further, many of the available agents act systemically and therefore have side effects that range from uncomfortable to life threatening. Another cause of failure in the treatment of cancer is the development of drug resistance by the cancer cells. The life span after getting the disease is also becoming shorter as the drugs become ineffective. Novel therapeutic approaches might be an urgent need today for better life expectancy. Attempts to overcome resistance mainly involve the use of combination drug therapy using different classes of drugs with minimally overlapping toxicities.

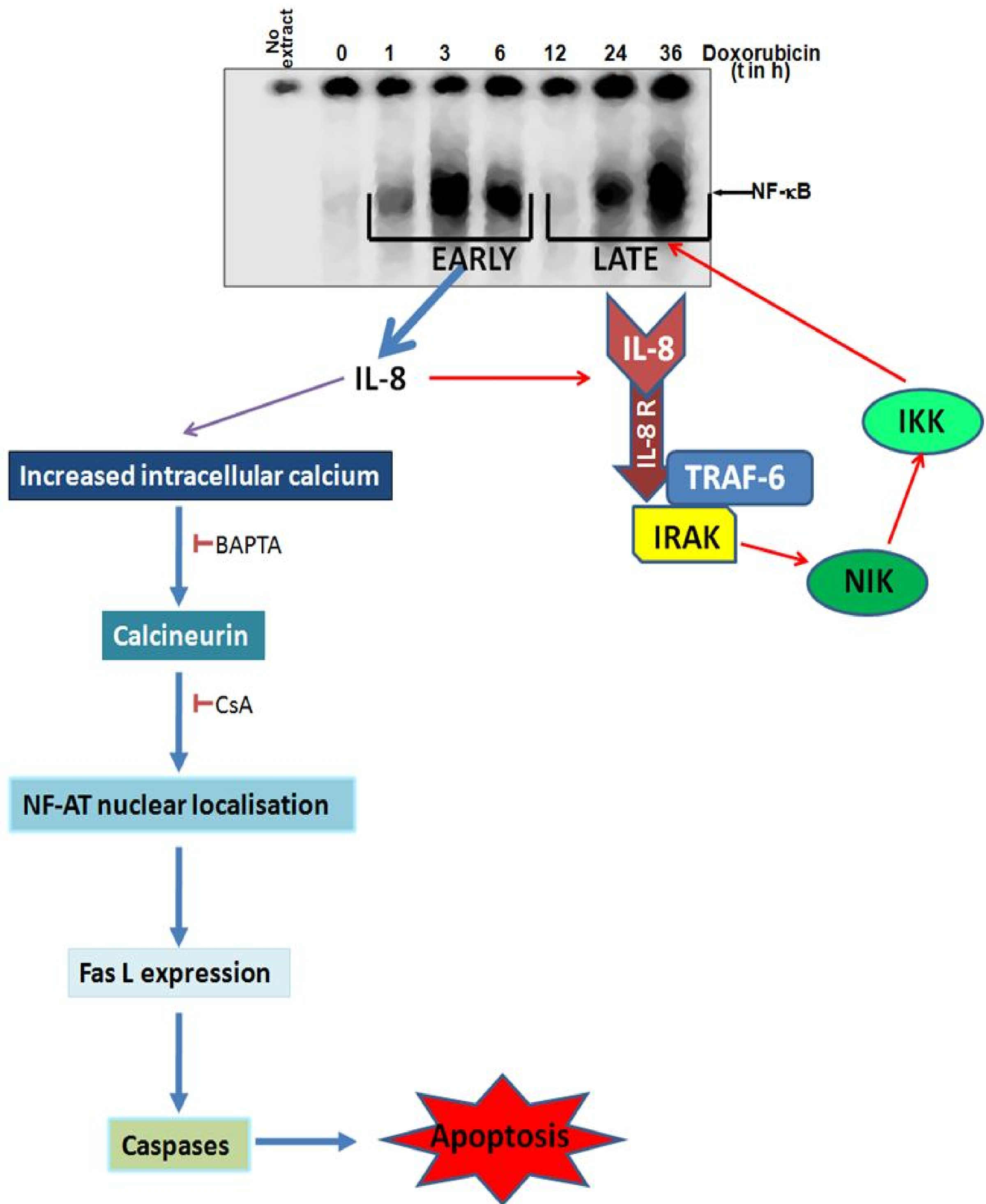
The nuclear factor κB (NF-κB) comprises a family of transcription factors involved in the regulation of a wide variety of biological responses. NF-κB plays a well-known function in the regulation of immune responses and inflammation, but growing evidences support a major role in oncogenesis. NF-κB regulates the expression of genes involved in many processes that play a key role in the development and progression of cancer such as proliferation, migration and apoptosis. Aberrant or constitutive NF-κB activation has been detected in many human malignancies. The activation of NF-κB not

only enables malignant transformation and tumor progression, but also provides a mechanism by which tumor cells escape immune surveillance and resist therapy. In recent years, numerous studies have focused in elucidating the functional consequences of NF-κB activation as well as its signaling mechanisms and have turned out to be an interesting therapeutic target for treatment of cancer.

Doxorubicin is an anthracycline that has been widely used in clinical oncology for the treatment of acute leukemia, Hodgkin's or non-Hodgkin's lymphomas and also for some solid tumors like breast ovarian and endometrial cancers. It was previously shown as a cell cycle dependent cytotoxic drug as it inhibits cell proliferation and triggers apoptosis. Its intracellular effects include DNA intercalation, generation of reactive oxygen species (ROS), and direct inhibition of topoisomerase II. How such properties account for the cytotoxic effect of doxorubicin is not well understood. However its use as a drug is restricted by the development of resistance to apoptosis, the mechanism of which is not clear.

Since nuclear transcription factor kappa B (NF-κB) has been shown to both block apoptosis and promotes cell proliferation, it has been considered as an important target for anticancer drug development. In terms of cell signaling, doxorubicin increases DNA binding activity of NF-κB. However, most of the effects of NF-κB are cell proliferative. We analysed the pattern of NF-κB induction upon doxorubicin treatment. Interestingly, doxorubicin-induced NF-κB DNA-binding activity was biphasic with its strongest peak at 3 to 6 h post treatment and a reproducible second increase in DNA-binding noted at 24 to 36 h. Doxorubicin is known to increase reactive oxygen species by reacting with cellular iron and this may cause the initial activation of NF-κB through activation of IKK (IκB kinase) cascade. Late phase activation of NF-κB is very much surprising as doxorubicin induces cell death at this time of incubation. We found that doxorubicin induced the production of IL-8 and activated caspases. Increased amount of IL-8 induced apoptosis via increase in intracellular Ca^{2+}, activation of calcineurin, nuclear

translocation of nuclear factor activated T cell (NF-AT), NF-AT-dependent FasL expression and subsequent activation of caspases.

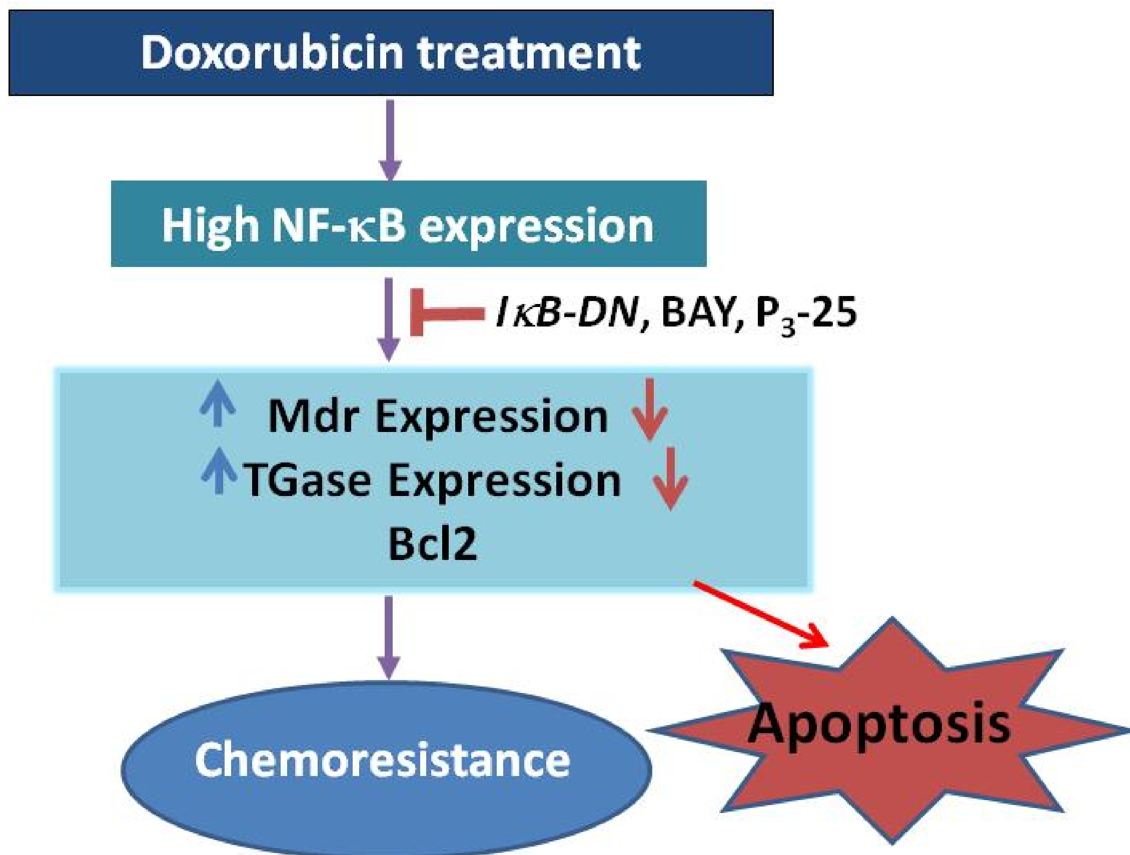


As NF-κB is currently being used as a target for cancer therapy, we investigated the possible interplay of NF-κB and doxorubicin resistance. In the study, we demonstrate that doxorubicin induces NF-κB in wild type and Dox-revertant, but not in Dox-resistant MCF-7 cells. Dox-resistant cells show high basal NF-κB activity and expression of the genes dependent on NF-κB. We also demonstrate that high NF-κB activity confers resistance to Dox-resistant cells and down regulation of NF-κB in these cells sensitizes the cells to apoptosis. High basal NF-κB activity in doxorubicin-resistant cells confers basal expression of Bcl-2, superoxide dismutase (SOD), multi-drug resistance gene (Mdr), and transglutaminase (TGase). Basal amounts of reactive oxygen intermediates (ROI) might maintain high basal activity of NF-κB in Dox-resistant cells that leads to expression of its dependent genes like Bcl-2, Mdr, TGase, or superoxide dismutase (SOD). Inhibition of NF-κB in doxorubicin resistant cells decreased high basal expression of Mdr and TGase, also induced apoptosis by increasing ROI generation over its basal level, and induced lipid peroxidation, and decreased Bcl-2 and SOD1 expressions. In conclusion, high NF-κB activity confers resistance to doxorubicin in Dox-resistant cells and down regulation of NF-κB in these cells potentiated apoptosis further suggested the role of NF-κB in chemoresistance.

A Comparative study of doxorubicin sensitive & resistant tumors

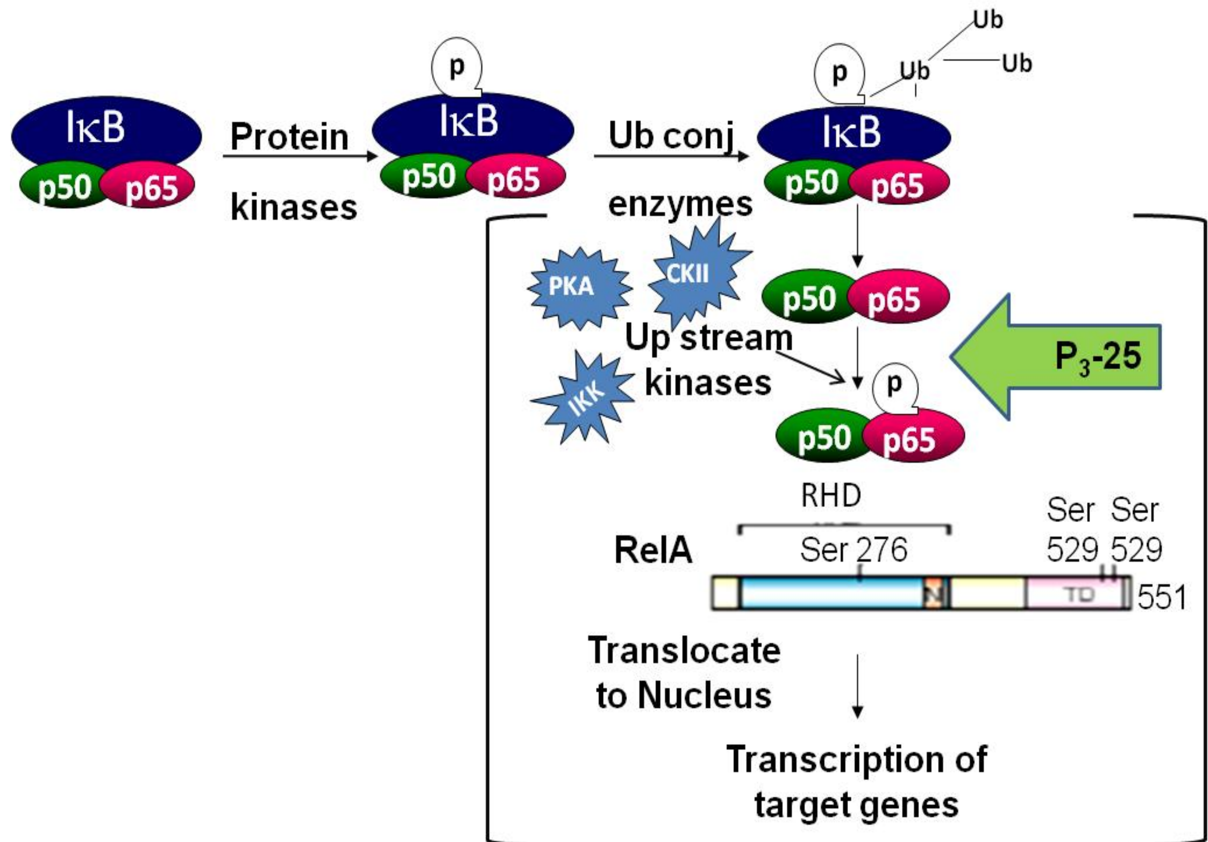
	Wild	**Dox-Resistant**	**Dox-revertant**
NF-κB DNA binding activity	Transient	Constitutive	Transient
NF-κB dependent genes expression	Low	High	Low
Cell Death	High	Low	High

Possible strategy to overcome doxorubicin resistance



NF-κB is a heterodimer of two subunits p50 (NF-κB1) and p65 (RelA). The RelA (p65) subunit of NF-κB actively participates in expression of NF-κB-dependent genes involved in inflammation and tumorigenesis. It is normally present in the cytoplasm in an inactive state in complex with an inhibitory subunit of kappa B (IκBα). Upon phosphorylation and subsequent degradation of IκBα, a nuclear localization signal on the p50-p65 heterodimer is exposed, leading to nuclear translocation of NF-κB. The p50–p65 heterodimer binds with a specific sequence in DNA, which in turn results in gene transcription. NF-κB may be inhibited by targeting either the apical signaling proteins responsible for its activation in specific types of cancer, the downstream kinases (IκB kinase and casein kinase 2) at which NF-κB-activating signaling pathways converge, the proteasome-mediated degradation of the inhibitor of κB (IκB) proteins, or the transcriptional activity of Rel proteins. Phosphorylation of p65 of NF-κB (RelA) is required for effective NF-κB-dependent gene transcription. Hence, we hypothesized the-regulation of p65 subunit by a chemically synthesised compound P_3-25 should control those responses. We show that P_3-25 inhibits protein kinase A (PKA) activity, leading to decreased phosphorylation of p65. The resulting reduction in transcriptional activity of NF-κB leads to decrease in the expression of antiapoptotic proteins further leading to apoptosis in dox-resistant cells and NF-κB-expressing cells.

P_3-25, a possible potential drug in combination therapy interrupts NF-κB signal transduction pathway



Overall, the present study helps to understand the role of various signaling pathways mediated by doxorubicin to counter or (and) sensitize the survival of a cancer cell. This study may further help in formulation of combination chemotherapy to intervene several tumors involving doxorubicin.

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List of Publications and Presentations

PUBLICATIONS:

1. **Charitha Gangadharan,** Sunil Kumar Manna., Decrease in RelA phosphorylation by inhibiting protein kinase A induces cell death in NF-κB-expressing and drug-resistant tumor cells. **Molecular Immunology**. (**46: 1340-1350, 2009**)

2. **Charitha Gangadharan,** Maikho Thoh, and Sunil Kumar Manna., Inhibition of constitutive activity of nuclear transcription factor kappaB sensitizes doxorubicin-resistant cells to apoptosis. **Journal of Cellular Biochemistry**. (**107: 203-213, 2009**)

3. **Charitha Gangadharan,** Maikho Thoh, and Sunil Kumar Manna., Late phase activation of nuclear transcription factor kappaB by doxorubicin is mediated by interleukin-8 and induction of apoptosis via FasL. **Breast Cancer Ressearch and Treatment** DOI 10.1007/s10549-009-0493-z (**Manuscript in press 2010**)

PRESENTATIONS (POSTERS):

1. Constitutive activity of nuclear factor kappa B confers resistance to doxorubicin-mediated apoptosis. **Charitha Gangadharan**, M. Thoh, PB Raghavendra, Abira Sarkar and Sunil K Manna at "Third Indo-Australian Conference on Biotechnology", March 2006, Hyderabad, India.

2. Decrease in Rel A phosphorylation by inhibiting protein kinase A induces cell death in NF-κB-expressing and drug resistant tumor cells. **Charitha Gangadharan**, M. Thoh, N. Raviprakash and Sunil K Manna at "The International Conference on Perspective of Cell Signaling and Molecular Medicine" at Bose Institute, November 2008, Kolkata, India.

3. Late phase activation of nuclear transcription factor kappa B by doxorubicin is mediated by interleukin-8 and induction of apoptosis via fasL. **Charitha Gangadharan**, Maikho Thoh and Sunil kumar Manna at eighth "CELL DEATH" meeting held at Cold Spring Harbor Laboratory, New York, USA, Oct6th to Oct 10th 2009.