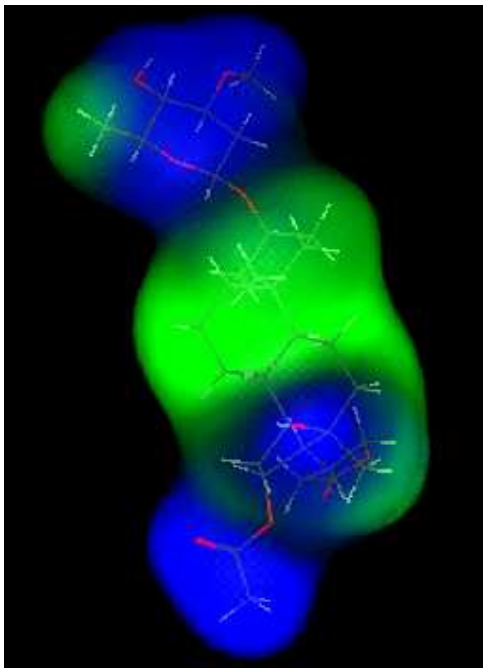
Studies on the signal transduction pathway(s) mediated by cardiac glycoside in relation to its anti-tumor activities

Thesis submitted to
University of Hyderabad
for the Degree of Doctor of Philosophy



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2005

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Hyderabad
August 2005

Dedicated to

my parents
& teachers

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Declaration

The research work embodied in this thesis entitled, "**Studies on the signal transduction pathway(s) of apoptosis mediated by cardiac glycoside in relation to its anti-tumor activities**", has been carried out by me at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad, under the guidance of Dr. Seyed E. Hasnain. I hereby declare that 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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Certificate

This is to certify that this thesis entitled, "**Studies on the signal transduction pathway(s) of apoptosis mediated by cardiac glycoside in relation to its anti-tumor activities**", submitted by Mr. Yashin Sreenivasan for the degree of Doctor of Philosophy to the University of Hyderabad is based on the work carried out by him at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad. This work is original and has not been submitted for any diploma or degree of any other university or institution.

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Chapter Two
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List of Publications and Presentations

Publications :

Yashin Sreenivasan, Abira Sarkar and Sunil K. Manna. Oleandrin potentially induces apoptosis in tumor cells, but not in primary cells (communicated).

Sunil K Manna*, **Yashin Sreenivasan*** and Abira Sarkar. Cardiac glycoside inhibits IL-8 induced biological responses by downregulating IL-8 receptors through altering membrane fluidity (communicated).

Sunil K Manna*, **Yashin Sreenivasan*** and Abira Sarkar. Cardiac glycoside-induced downregulation of p65 (RelA) is mediated by proteasome, but not by caspases (communicated).

Abira Sarkar, **Yashin Sreenivasan**, Govindarajan T. Ramesh, and Sunil K. Manna.(2004) **J. Biol. Chem**., 279(32):33768-81. Beta-D-glucoside suppresses TNF-induced activation of nuclear transcription factor kappaB but potentiates apoptosis.

Sreenivasan, Y., Sarkar, A., and Manna, S. K. (2003): Oleandrin suppresses activation of nuclear transcription factor-kappaB and activator protein-1 and potentiates apoptosis induced by ceramide. ***Biochem. Pharmacol***. 66(11): 2223-39.

Sarkar, A., Sreenivasan, Y., and Manna, S. K. (2003): Alpha-Melanocyte-stimulating hormone inhibits lipopolysaccharide-induced biological responses by downregulating CD14 from macrophages ***FEBS Letters.*** 553, 286-294.

Sarkar, A., **Sreenivasan, Y**., and Manna, S. K. (2003): alpha-Melanocyte Stimulating Hormone induces cell death in mast cells: Involvement of NF-kB. ***FEBS Letter*** .549, 87-93.

Sreenivasan, Y., Sarkar, A., and Manna, S. K. (2003): Mechanism of Cytosine arabinoside mediated apoptosis: Role of Rel A (p65) dephosphorylation. ***Oncogene*** 22(28), 4356-4369.

Meetings and Symposiums :

Yashin Sreenivasan, Abira Sarkar and Sunil. K. Manna. Mechanism of Cytosine arabinoside mediated apoptosis: Role of Rel A (p65) dephosphorylation. ***Cold Spring Harbor Meeting titled 'Programmed Cell Death' : Cold Spring Harbor Laboratories***, ***New York. USA***. 17th -21st September, 2003.

Yashin Sreenivasan Nair, Abira Sarkar and Sunil K Manna. Oleandrin suppresses ceramide-induced activation of Nuclear transcription factors NF-kB and AP-1 but potentiates apoptosis. ***XXV All India Cell Biology Conference***. ***Indian Institute of Science. Bangalore,India.***
November 1-3,2001.

*** both the autors have equally contributed**

ABBREVIATIONS

Abbreviations for standard (SI) units of measurement, and chemical formulae are not included in the list below :

ALLN --- N-acetylleucylleucylnorleucinal
AP-1 --- activator protein-1
CD95 --- Fas receptor
CE --- cytoplasmic extract
Ceramide --- N-acetyl-D-sphingosine
Cox2 --- cyclooxygenase 2
DOC --- deoxycholate
Dox --- doxorubicin
DPH --- diphenyl hexatriene
DSS --- disuccinimidyl suberate
DTT --- dithiothreitol
EMSA --- electrophoretic mobility shift assay
FasL --- fas ligand
FBS --- fetal bovine serum
FKHR --- forkhead transcription factor
FMLP --- formyl methionyl leucyl phenylalanine
ICAM --- intracellular adhesion molecule
IκBα — inhibitory subunit of kappa B
IκBα-DN --- IκBα dominant negative
IL --- interleukin
KSCN --- potassium thiocyanate
LMP7 --- low molecular-mass protein 7
MDC --- monodansyl cadaverine
MGSA --- melanocyte growth stimulatory activity
Mn-SOD --- manganese superoxide dismutase
MTT --- 3-(4,5-Dimethyl-2-thiozolyl)-2,5-diphenyl-2H-tetrazolium bromide
NBT --- nitroblue tetrazolium
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
Oleandrin --- trans-3,4',5-trihydroxystilbene
PARP --- poly(ADP-ribose) polymerase
PBMC --- peripheral blood mononuclear cells
PIS --- pre-immune serum
PMA --- phorbol 12-myristate 13-acetate
PMN --- polymorphonuclear neutrophils
PMSF --- phenyl methyl sulphonyl fluoride
Rb --- retinoblastoma
ROI --- reactive oxygen intermediate

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
TRAF --- TNF receptor associated factor
z-VAD-fmk --- z-Val-Ala-Asp fluoromethyl ketone

Preface.

Cancer arises from the step wise accumulation of genetic changes that confer upon an incipient neoplastic cell the properties of unlimited, self sufficient growth and resistance to normal homeostatic regulatory mechanisms, one among them being apoptosis. Cancer is a growing health problem around the world with more than 10 million new cases are diagnosed each year and from a total of 10.8 million cases in 2002 it is estimated to reach to about 16.5 million cases by 2020. Rapid advances since a quarter century; cancer research has generated a rich and complex body of knowledge, revealing cancer to be a disease involving dynamic changes in the genome. Cancer research is developing into a logical science where the complexities of the disease, described in the laboratory and clinic will become understandable in terms of a small number of underlying principles that govern the transformation of normal cell into malignant cancer. 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.

The use of cardiac glycosides has been reported to have beneficial side effects in cancer patients. Also pre-clinical studies have demonstrated that oleandrin, a cardiac glycoside derived from the leaves of *Nerium oleander* has excellent activity against a variety of human solid tumor cell lines. However, a thorough understanding of the antineoplastic activities of oleandrin has not been addressed. Interestingly, the study in this thesis was undertaken to decipher the signal transduction pathway(s) mediated by oleandrin in relation to its anti tumor activities.

Chapter 1 reviews the current understanding of cancer accumulated over the years, describing in detail the signal transduction pathways mediated which are denoted to be the life lines of a cancer cell. The importance of certain transcription factors and signaling intermediates involved in tumorigenesis is vividly documented. Also mentioned is the role and means of drug resistance in cancer, reiterating the need to decipher signaling pathway(s) mediated by chemotherapeutic agents and a need for more new and potent agents for countering the resistance phenomenon. Chapter 2 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. Furthermore constitutive activation of NF-κB in cancer cells is known to confer resistance to tumors against chemotherapeutics. Hence, the effect of oleandrin on NF-κB activation was investigated as described in Chapter 3. Here ceramide, a major secondary signaling intermediate was used as an inducer of NF-κB activation. It is seen that oleandrin inhibits ceramide induced NF-κB activation but potentiats apoptosis mediated by ceramide. However oleandrin is unable to inhibit NF-κB activation in primary cells (isolated PBMC and neutrophils). This work is published as **Sreenivasan *et al*.,** (2003). ***Biochem Pharmacol***. 66, 2223-2239.

It is reported that classical inducers of apoptosis like TNF-α and ceramide fail to trigger apoptosis in NF-κB expressing cells. Hence in chapter 4 documents answers to the question asked whether oleandrin is able to mediate cell death in NF-κB expressing cells, if so how. The results from studies show that oleandrin is able to mediate inhibition of NF-κB activation, degradation of p65 via the activation of the proteasome and also induction of apoptosis in NF-κB expressing cells.

Interleukin (IL)-8 a neutrophill chemotactic factor belongs to the CXC family of cytokines and functions by interactions with IL-8 receptors, which are G protein, coupled

receptors with seven transmembrane domains. IL-8 elicits multiple effects on tumor growth, angiogenesis and metastasis. Oncogenic Ras, PI3K, NF-κB and MAPK activities in tumor cells promote the expression of IL-8. It was seen that oleandrin blocked IL-8, EGF or NGF but not TNF or IL-1 mediated ligand binding and NF-κB activation as described in chapter 5. The usage of lipid molecules like lecithin and sphingosine rescued the inhibition of IL-8 binding to its receptor mediated by oleandrin. Also oleandrin induces reduction in membrane fluidity thereby possibly inhibiting IL-8 mediated signaling.

Akt is a serine/threonine kinase, which is activated upon phosphorylation by growth receptor signaling, is reported to be over expressed in tumor cells and is implicated in giving the tumor cells a strong proliferative drive. Oleandrin inhibits Akt phosphorylation leading to the nuclear translocation of forkhead transcription factor thereby inducing fas ligand expression and apoptosis. Also described in chapter 6 are the activation of JNK, Erk2 kinases and nuclear localization of transcription factor NFAT mediated by oleandrin. NFAT, JNK and Erk2 are reported to induce the expression of fas ligand.

Hence the study presented in this thesis for the first time reports the role of various signaling pathways mediated by the cardiac glycoside, oleandrin which has the ability to counter the life lines of a cancer cell.

Apart from the studies on oleandrin the mechanism by which cytosine arabinoside (AraC) mediates apoptosis in NF-κB expressing cells was deciphered. AraC induces Dephosphorylation of Rel A (p65) by activation of the protein phosphatases PP2A and PP2B-A thereby inhibiting NF-κB signaling leading to apoptosis. This work is described in appendix and has been published as **Sreenivasan *et al***., (2003). ***Oncogene***. 22, 4356-4369.

Chapter One

Introduction and Review of Literature

Cells are the structural units of all living things, which make it possible to carry out all kinds of functions of life. The most fundamental characteristic of cells is their ability to reproduce themselves simply by dividing. The division of normal and healthy cells occurs in a regulated and systematic fashion. In most parts of the body, the cells continually divide and form new cells to supply the material for growth or to replace worn-out or injured cells. In contrast, some cells divide rapidly in a haphazard manner. The result is that they typically pile up into a non-structured mass or tumor. Tumors become cancerous when they spread to other parts of the body from their originating tissue and start new growth. Hence cancer could be defined as a group of diseases characterized by uncontrolled growth and spread of abnormal cells.

In 2003, more than 7 million people died from cancer. More than 10 million new cancer cases are diagnosed each year. Cancer claimed twice as many lives as AIDS in 2004. 12.6% of all deaths each year are caused by cancer. 43% of cancer deaths are due to tobacco, diet and infection. The number of new cases annually is estimated to rise from 10.8 million in 2002 to more than 16.5 million by 2020. In developing countries, 80% to 90% of cancer patients suffer from advanced and incurable cancers at time of diagnosis. Lung cancer kills more people than any other cancer. By 2020, 60% of all new cancer cases will occur in the developing world. There will be more than 10 million cancer deaths per year by 2020, if current trends continue. 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 The principles underlying transformation of normal cells into tumor

Rapid advance since a quarter century, cancer research has generated a rich and complex body of knowledge, revealing cancer to be a disease involving dynamic changes in the genome. The foundation has been set in the discovery of mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function.

Both classes of genes have been identified through their alteration in human and animal cancer cells and elicitation of cancer phenotypes in experimental models (Hanahan and Weinberg, 2000).

However, the search for the origin and treatment of this disease will continue over the next quarter century in much the same manner in the past adding further layers of complexity to a scientific literature that is already complex. On the contrary cancer research is developing into a logical science where the complexities of the disease, described in the laboratory and clinic, will become understandable in terms of a small number of underlying principles. According to Hanahan and Weinberg there are sets of rules that govern the transformation of normal human cells into malignant cancer.

Many types of cancers are diagnosed in the human population with an age dependent incidence implicating four to seven rate limiting stochastic events (Renan, 1993). Pathological analysis of a number of organ sites reveal lesions that appear to represent the intermediate steps in a process through which cells evolve progressively from normalcy via a series of premalignant states into invasive cancers (Foulds, 1954). Observations of human cancers and animal models argue that tumor development proceeds via a process formally analogous to Darwinian evolution in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells (Nowell, 1976). 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 and Weinberg, 2000). Indeed the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth. These so called hallmarks of cancer are as follows.

1.1.1 Self-sufficiency in growth signals.

The movement of normal cells from a quiescent state into an active proliferative state is triggered by several mitogenic growth signals. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signaling molecules, diffusible

growth factors, extra cellular matrix components, and cell-to-cell adhesion/interaction molecules. Dependence on growth signaling is apparent when propagating normal cells in culture, which typically proliferate only when supplied with appropriate diffusible mitogenic factors. In contrast, tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulating from their normal tissue microenvironment. Many of the oncogenes in the cancer catalog act by mimicking normal growth signaling one way or another. 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 and 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). Some of the other strategy adopted in attaining self-sufficiency of growth factor signaling are, the cell surface receptors that transduce growth stimulatory signals into cell interiors are deregulated during tumor formation. This could be in the form of over expression of the growth factor receptors themselves enabling cancer cells to become hyper responsive to ambient levels of growth factor that normally would not trigger proliferation (Fedi *et al.,* 1997). For example, the epidermal growth factor receptors (EGF-R/erb B) upregulated in the stomach, brain and breast tumors and HER2/neu receptor in stomach and mammary carcinomas (Slamon *et al.,* 1987; Yarden and Ulrich, 1988). Cancer cells can also switch the types of extra cellular matrix receptors they express, favouring ones that transmit pro-growth signals (Lukashev and Werb, 1998; Giancotti and Ruoslahti, 1999). The ligand activated growth factor receptors and pro-growth integrins engaged to extracellular matrix components can activate the SOS-Ras-Ras associated factor-Mitogen Activated Protein kinase pathway (Aplin *et al.,* 1998; Giancotti and Ruoslahti, 1999). The most complex mechanisms of acquired growth factor autonomy derive from alterations in components of the downstream cytoplasmic circuitry that receives and processes the signals emitted by ligand activated growth factor receptors and integrins. In about 25% of human tumors, Ras proteins are present in structurally altered forms that enable them to release a flux of mitogenic signals into cells, without ongoing stimulation by their normal upstream regulators. (Medema and Bos, 1993). Heterotypic

signaling between diverse cell types within tumor may also prove to be important for growth autonomy e.g. inflammatory cells attracted to sites of neoplasia may promote cancer cells (Cordon-Cardo and Prives, 1999; Coussens *et al.,* 1999; Hudson *et al.,* 1999).

1.1.2 Insensitivity to anti-growth signals.

In order to maintain cellular quiescence and tissue homeostasis multiple anti-proliferative signals operate within a normal tissue. These signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extra cellular matrix and on the surfaces of nearby cells. These growth inhibitory signals, like their positively acting counterparts, are received by transmembrane cell surface receptors coupled to intracellular signaling circuits. Proliferation is blocked by two ways- cells may be forced out of the active proliferative cycle into the quiescent (Go) state and alternatively cells may be induced to permanently relinquish their proliferative potential by being induced to enter into post mitotic states, usually associated with acquisition of specific differentiation-associated traits. Hence for tumor cells to enjoy the uncontrolled replicative capacity these anti-proliferative mechanisms must be evaded. Much of the molecular circuitry that enables normal cells to respond to antigrowth checks is associated with the cell cycle clock, the components governing the transit of the cells through the G1 phase of its growth cycle. Cells monitor their external environment during their period and on the basis of sensed signals; decide whether to proliferate, to be quiescent, or to enter into a post mitotic state. Most of these are channeled through the retinoblastoma protein (Rb). In hypo-phosphorylated state Rb blocks proliferation by sequestering and altering the function of E2F transcription factors that control the expression of several genes essential for progression from G1 into S phase (Weinberg, 1995). TGFβ acts in ways to prevent phosphorylation of Rb, in some cells TGFβ suppresses expression of c-myc gene which regulates the G1 cell cycle machinery (Moses *et al.,* 1990), they also cause synthesis of the p15 and p21 proteins which block cyclin: CDR complexes responsible for Rb phosphorylation (Hannon and Beach, 1994; Datto *et al.,* 1997). The pRb signaling circuits as governed by TGFβ and other extrinsic factors, can be disrupted in a variety of ways in different types of human tumors (Fynan and Riess, 1993). Some loose TGFβ responsiveness through down regulation of their receptors, while others display mutant, dysfunctional receptors (Fynan and Riess, 1993; Markowitz *et al.,* 1995). In certain cervical carcinomas,

pRb function is eliminated through sequestration by viral oncoproteins, such as the E7 oncoprotein of human papilloma virus (Dyson *et al.,* 1989). Normal tissues also constrain cellular multiplication by forcing cells to enter irreversibly into post mitotic, differentiated states. Hence it is apparent that tumor cells use various strategies to avoid this terminal differentiation. Like involving the c-myc oncogenes, which encodes a transcription factor, myc whose function is evaded by the coupling of a factor, Max with group of Mad transcription factors; the Mad-Max complexes elicit differentiation-inducing signals (Foley and Eisenman, 1999). However over expression of c-myc as seen in many tumors reverses this phenomenon by shifting the balance back to favour Max-Myc complexes thereby impairing differentiation and promoting growth.

1.1.3 Evading apoptosis.

Expansion of tumor cell populations in number is not only determined by the rate of cell proliferation but also by the rate of cell attrition. Programmed cell death or apoptosis represents a major source of this cellular attrition. Research accumulated over the past decade indicates that the apoptotic programme is present in latent form in virtually all cell types throughout the body. Once triggered by a variety of physiologic signals, this programme unfolds in a precisely choreographed series of steps. Cellular membranes are disrupted, the cytoplasmic and nuclear skeletons are broken down, the cytosol is extended, the chromosomes are degraded, and nucleus is fragmented, finally the shriveled cell corpse is engulfed by nearby cells and disappears. (Willie et. al., 1980). The possibility that apoptosis serves as a barrier to cancer was first raised in 1972 when Kerr, Wyllie and Currie described rapidly growing, hormone dependent tumors following hormone withdrawal (Kerr *et al.,* 1972). The discovery of the Bcl-2 oncogene by its up regulation via chromosomal transcription in follicular lymphoma (Korsmeyer, 1992) and its recognition as having anti-apoptotic activity (Vaux *et al.,*1988) opened up the investigation of apoptosis in cancer at the molecular level. When co-expressed with myc oncogenes in transgenic mice, the Bcl-2 gene was able to promote formation of B cell lymphomas by enhancing lymphocyte survival (Strasser *et al.,* 1990). When Rb tumor suppressor was functionally inactivated in the choriod plexus in transgenic mice, slowly growing microscopic tumors arose, exhibiting high apoptotic rates, the additional inactivation of the p53 tumor suppressor protein, led to rapidly

growing tumors containing low number of apoptotic cells (Symonds *et al.,* 1994). Cancer cells through a variety of strategies can acquire resistance to apoptosis. Surely, the most commonly occurring loss of a pro-apoptotic regulator through mutation involves he p53 tumor suppressor gene. The resulting functional inactivation of its product the p53 protein, is seen in greater than 50% of human cancers and results in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade (Harris, 1996).

1.1.4 Limitless Replicative potential.

The uncoupling of a cells growth programme from signals in its environment is envisaged in the earlier discussed three acquired capabilities. The resulting deregulated proliferation programme should suffice to enable the generation of the vast cell population that constitutes macroscopic tumors. However research over the years show that this acquired cell-to-cell signaling on its own does not ensure expansive tumor growth. Many and perhaps all types of mammalian cells carry an intrinsic cell autonomous programme that limits their multiplication. This programme appears to operate independently of the cell-to-cell signaling pathways. Once cells progress through a process termed senescence (Hayflick, 1997), it can circumvent by disabling their pRb and p53 tumor suppressors and thereby enabling these cells to continue multiplying for additional generations until they enter a second state termed crisis. The crisis state is characterized by massive cell death, karyotypic disarray associated with end to end fussion of chromosomes and the occasional emergence of a variant (1 in 10^7) cell that has acquired the ability to multiply without limit, the trait termed immortalization (Wright *et al.,* 1989). The counting device for cell generations has been discovered over the past decade: telomeres, which are composed of several thousand repeats of a short 6bp, sequence element. The progressive replication eventually causes them to loose their ability to protect the ends of chromosomal DNA, thus leading to death of affected cell (Counter *et al.,*1992). Telomere maintenance is evident in virtually all types of malignant cells (Shay and Bacchetti, 1997). 85% to 90% of them succeed in doing so by up regulating expression of the telomerase enzyme, which adds hexanucleotide repeats into the ends of telomeric DNA (Bryan and Cech, 1999). Hence a critical threshold, and this in turn permits unlimited multiplication of descendant cells. Senescence, much like apoptosis reflects a protective mechanism that can be activated by shortened telomeres or conflicting growth signals that

forces aberrant cells irreversibly into a Go like state, thereby rendering them incapable of proliferation. Hence circumvention of senescence may indeed represent an essential step in tumor progression.

1.1.5 Sustained Angiogenesis.

Once a tissue is formed, the growth of new blood vessels- the process of angiogenesis is transitory and carefully regulated. The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100μm of a capillary blood vessel. During organogenesis this closeness is ensured by coordinated growth of vessels and parenchyma. The cells with aberrant proliferative lesions initially lack angiogenic ability, curtailing their capability for expansion. In order to progress to a larger size, incipient neoplasias must develop angiogenic ability (Bouck *et al.,* 1996; Folkman, 1997). Counter balancing positive and negative signals encourage or block angiogenesis. The angiogenesis-initiating signals are exemplified by vascular endothelial growth factors. A prototypical angiogenesis inhibitor is thrombospodin-1, which binds to CD36, a transmembrane receptor on endothelial cells coupled to intracellular Src-like tyrosine kinase (Bull *et al.,* 1994). The anti-VEGF antibodies were able to impair neovascularization and growth of subcutaneous tumors in mice (Kim *et al.,* 1993) as shown by a dominant-interfering version of the VEGF receptor 2 (flk-1) (Millauer *et al.,* 1994). The ability to induce and sustain angiogenesis seems to be acquired in a discrete step during tumor development via an angiogenic switch from vascular quiescence. Tumors appear to activate the angiogenic switch by changing the balance of angiogenesis inducers and countervailing inhibitors (Hanahan and Folkman, 1996). One common strategy involves altered gene transcription as evidenced in many tumors wherein an increase in expression of VEGF and or FGFs and IL-8.

1.1.6 Tissue Invasion and Metastasis.

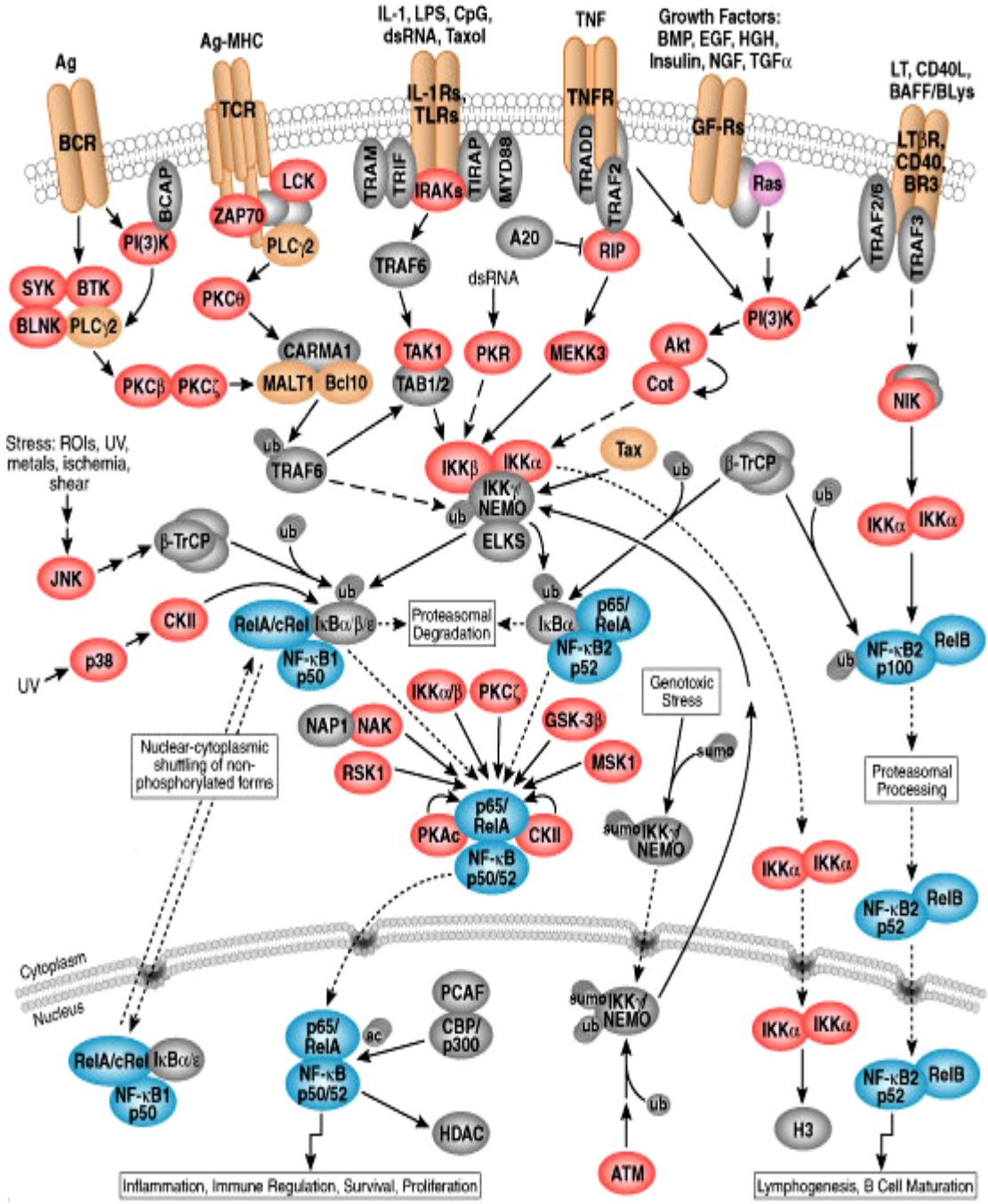
During the development of most types of human cancers, primary tumor masses spawn pioneer cells that move out, invade adjacent tissues and then travel to distant sites where they may succeed in founding new colonies. These distant settlements of tumor cells called metastasis are the cause of 90% human cancer deaths (Sporn, 1996). The capability for

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. Notably all of these adherence interactions convey regulatory signals to cells (Aplin *et al.,* 1998 A). N-CAM undergoes a switch in expression from a highly adhesive isoform to poorly adhesive (or even repulsive) forms in Wilms tumor, neuroblastoma, and small cell lung cancer (Johnson, 1991; Kaiser *et al.,* 1996) and reduction in overall expression level in invasive pancreatic and colorectal cancers (Forgar *et al.,* 1997). 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 and Cherish, 1996; Lukashev and 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 (Werb, 1997). 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.

1.2 Nuclear Transcription Factor Kappa B (NF-κB).

Nuclear factor κB (NF-κB) transcription factor are a family of structurally related eukaryotic transcription factors that promote the expression of well over 150 genes involved in a variety of cellular processes (Ghosh *et al.,* 1998; Pahl, 1999). Nuclear factor of κB is not a single protein, but a small menagerie of closely related protein dimmers that bind a common sequence motif known as the κB site (Karin *et al.,* 2002). The proteins that make up the NF-κB transcription factors essentially are placed in the Rel family of proteins. The REL proteins belong to two classes, which are distinguishable by their mode of synthesis and transactivation properties. One class consists of Rel A (also known as p65), Rel B and c-Rel-proteins that are synthesized in their mature forms. These proteins contain an amino terminal

NF-κB signaling pathways



REL homology domain (RHD) that is required for dimerization and DNA binding and transcription-modulating domains at their carboxyl terminus. The second class consists of NF- κB1 (also known as p105) and NF- κB2 (also known as p100) with an N-terminal RHD and a c-terminal series of ankyrin repeats. Ubiquitin-dependent proteolytic processing removes this c-terminal domain, resulting in production of the mature DNA-binding proteins (p50 and p52). The Rel proteins form various homo and heterodimers, where the most common active form of which is the p50/Rel A or p52/Rel A heterodimers. Dimerization of various NF- κB subunits produces complexes with different DNA binding specificities and transactivation potential (Li and Verma, 2002; Senftleben and Karin, 2002). In most cell types inactive NF- κB complexes are sequestered in the cytoplasm via their non-covalent interaction with inhibitory proteins known as IκBs. In response to multiple stimuli, including cytokines, viral and bacterial pathogens, and stress-inducing agents, the latent cytoplasmic NF- κB/IκB complexes is activated by phosphorylation on conserved serine residues in the N-terminal portion of IκB (Serine 32 and 36) (Karin and Delhase, 2000; Karin and Lin, 2002; Kumar *et al.,* 2003; Kumar and Boriek, 2003). Phosphorylation targets IkBα for ubiquitination by the SCF-ubiquitin ligase complex, which leads to the degradation of the inhibitory subunit by the 26s proteosome (Karin and Ben-Neriah, 2002; Wilkinson, 2003). This process activates NF- κB, which then translocates to the nucleus and binds to its cognate DNA-binding site (5'-GGGRNNYYCC-3') in the promoter or enhancer regions of specific genes. However, NF- κB activation represents the terminal step in a signal transduction pathway leading from the cell surface to the nucleus. A seminal event in the activation of NF- κB is the phosphorylation of IκBs, which is mediated by a multimeric complex, referred to as the IκB kinase (IKK) complex (Chen *et al.,* 1996). The IKK complex consists of two catalytic subunits (IKKα and IKKβ) (Zandi *et al.,* 1997; DiDonato *et al.,* 1997). The activated IKK complex recruits IκB proteins and phosphorylates them at serine residues. Although NF- κB inducing kinase (NIK), a serine/theronine protein kinase belonging to mitogen-activated protein kinase family has been implicated in the activation of IKK, the physiological kinase of IKK is still elusive (Ling *et al.,* 1998; Lee *et al.,* 1998). The diversity of inducers of NF- κB highlights the intriguing aspect of NF- κB regulation, namely the ability of many different signal transduction pathways originating from a wide variety of

inducing mechanisms to converge on a single target, i.e. the NF- κB/IkB complex. In certain cases such as in response of short wave UV light (Li and Karin, 1998; Bender *et al.,* 1998; Kato Jr *et al.,* 2003), pervanadate (Imbert *et al.,* 1996; Singh *et al.,* 1996; Mukhopadhyay *et al.,* 2000), H_2O_2 (Takada *et al.,* 2003; Li and Karin, 1999), hypoxia/re-oxygenation (Fan *et al.,* 2003), nerve growth factor (Bui *et al.,* 2001), erythropoietin (Digicaylioglu and Lipton, 2001), HER-2 (Pianetti, 2001) and the hepatitis B virus encoded transcription co-activator pX (Purcell, 2001) the activation of NF- κB is deviant from classical way and does not seem to involve phosphorylation of IκB by IKK or even IκB degeneration. The phosphorylation of tyrosine residues on the p65 protein transactivates it and is necessary for recruiting the transcription co-activators like p300 to the site of transcription. Several kinases like NIK (NF- κB inducing kinase), IKK have been reported to phosphorylate the p65 subunit. Gene knockout studies in mice have revealed both specific and redundant functions of each member of NF- κB family proteins in the regulation of innate and adaptive immune response and cell survival.

1.2.1 Role of NF-κB in tumorigenesis.

Earlier in this introductory chapter it was discussed that essentially six alterations to normal cell physiology necessitates its transformation. NF- κB is able to induce several of these cellular alterations and has been shown to be constitutively activated in many types of cancer cells. 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 avain REV-T oncovirus produces the constitutively active v-Rel oncoprotein, which causes rapidly progressing lymphomas and leukemias (Gilmore, 1999). The TAX oncoprotein of human T-cell leukemia virus (HTLV)-1 has been shown to directly interact with and constitutively activate the IKK complex (Xiao *et al.,* 2001). Cancer associated chromosomal translocations; deletions and mutations might also disrupt genes that encode NF- κB factors from their regulators and causing constitutive NF- κB activation. Autocrine and paracrine production of pro-inflammatory cytokines, oncogenic activation of upstream signaling molecules and chronic infections have been shown to persistently stimulate IKK activity, which leads to constitutive NF- κB activation. Constitutively activated NF- κB transcription

factors have been associated with several aspects of tumorigenesis, including promoting cancer-cell proliferation, preventing apoptosis and increasing a tumors angiogenic and metastatic potential. 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 (Guttridge *et al.,* 1999; Hinz *et al.,* 1999) 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; Nakshatri *et al.,* 1997; Cogswell *et al.,* 2000).

1.3 Programmed Cell Death-Apoptosis.

Apoptosis is a well orchestered morphological phenomenon programmed in most of the cells characterized by chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebbing, and cell shrinkage. Eventually the cells break into small membrane-surrounded bodies (apoptotic bodies), which are eaten up by phagocytes without inciting an inflammatory response in the vicinity. The release of apoptotic bodies is what inspired the term “apoptosis” from the Greek, meaning “to fall away from” and conjuring notions of the falling leaves in the autumn from deciduous trees (Kerr *et al.,* 1972).

1.3.1 Molecular mechanisms of apoptosis.

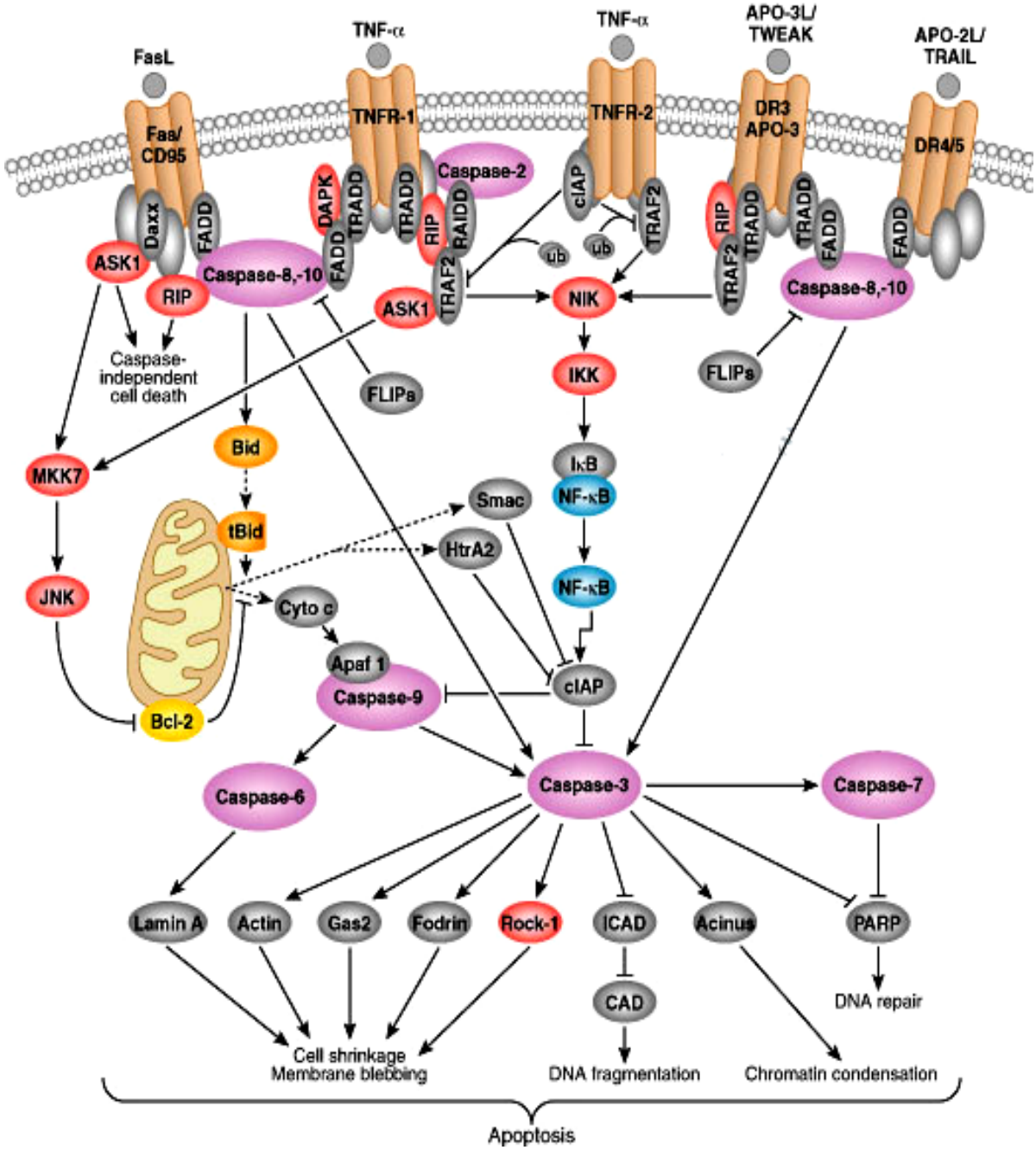
The question arises is what causes the morphological and biochemical changes associated with cells undergoing apoptosis. The answer is a class of proteases called caspases-cysteine aspartyl specific proteases (Alnemri *et al.,* 1996). These proteases are present as inactivated zymogens in essentially all animal cells, but can be triggered to assume active states,

generally involving their proteolytic processing at conserved aspartic acid residues. During activation, the zymogen pro-proteins are cleaved to generate the large and small subunits, typically liberating an N-terminal prodomain from the processed polypeptide chain. The active enzyme consists of heteromers composed of two large and two small subunits, with two active sites per molecule (Thonberry and Lazebnik, 1998). In human and mice approximately 14 caspases have been identified. They can be sub grouped according to either their amino acid sequence similarities or their protease specificities. From a functional perspective, it is useful to view the caspases as either upstream (initiator) caspases or downstream (effector) caspases (Salvesen and Dixit, 1997). The proforms of upstream initiator caspases pocess large N-terminal pro-domains, which function as protein interaction modules, allowing them to interact with various proteins that trigger caspase activation. In contrast, the proforms of down stream effector caspases contain only short N-terminal pro-domains, serving no apparent function. Downstream caspase are largely dependent on upstream caspases for their proteolytic processing and activation. The mechanisms of caspase activation has much diversity but fundamentally the biochemical mechanisms appear to be remarkably similar and can be explained by a single model, known as the induced proximity model (Salvesen and Dixit, 1999). 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. Though commonly viewed as separate pathways and capable of functioning independently, cross talk can occur between the pathways at multiple levels, depending on the repertoire of apoptosis-modulating proteins available (Reed, 2000).

1.3.1.1 Extrinsic pathway of apoptosis.

This pathway is mediated by a group of proteins called death receptors, which are members of the tumor necrosis factor (TNF) receptor super family and comprise a subfamily that is characterized by an intracellular domain-the death domain. Decoy receptors are closely related to the death receptors but lack a functional death domain (Pitti *et al.,* 1998; Yu *et al.,* 1999). Death receptors are activated by their natural ligands, when ligands bind to their respective receptors such as CD95, TRAIL-R2, TRAIL-R1, TNF-R1 & R2 the death domains attract the intracellular adaptor protein FADD, which in turn recruits the inactive

Death receptors induced pathways of apoptosis

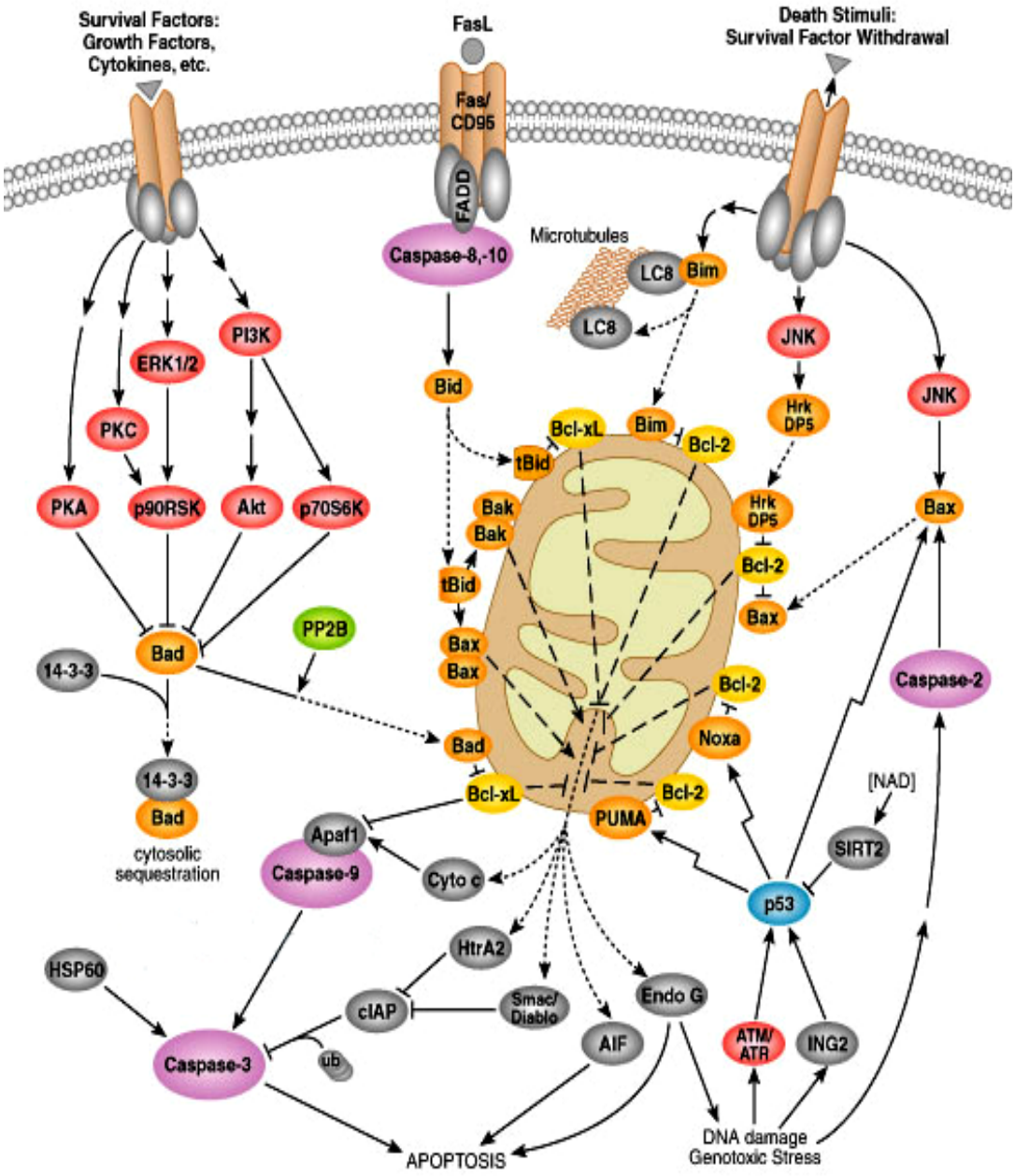


proforms of initiator caspases like caspase 8 and 10. This complex is called the death inducing signaling complex (DISC). At the DISC procasapase 8 and 10 are cleaved and yield active initiator caspase. This is explained by the induced model of caspase activation, wherein an auto catalytic process is set in the proforms of initiator caspase when they come in close proximity and thereby converting themselves to active forms F2). In some cells known as type-I cells the amount of active caspase 8 formed at the DISC is sufficient too initiate apoptosis directly, but in type-II cells, the amount is too small and the mitochondria are used as amplifiers of the apoptotic signal (Scaffidi *et al.,* 1998). Activation of mitochondria is mediated by the Bcl-2 family member BID, BID is cleaved by active caspase 8 and translocates to the mitochondria and thereby initiating the formation of mitochondrial membrane pore and subsequently the activation of caspase 9 (Igney and Krammer, 2002).

1.3.1.2 Intrinsic pathway of apoptosis.

Death at this level also called the mitochondria-dependent pathway for apoptosis governed by Bcl-2 family proteins. In humans 20 members of Bcl-2 family gene family have been described to date. These genes encode the anti-apoptotic proteins - Bcl-2, Bcl-X_L, Mcl-1, Bfl-1 (A1), Bcl-W and Boo (Diva) as well as the pro-apoptotic proteins - Bax, Bak, Bok (Mtd), Bad, Bid, Bim, Bik, Hrk, Bcl-Xs, APR (Noxa), p193, Bcl-G, Nip 3 and Nix (BNIP). Some of these proteins may display anti-apoptotic activity in some cellular backgrounds and have anti-apoptotic functions in other cellular contexts (Chen *et al.,* 1996; Middleton *et al.,* 1996; Song *et al.,* 1999; Inohara *et al.,* 1998). Gene ablation studies in mice suggest that each of the Bcl-2 family members play unique roles in controlling cell survival in-vivo, reflecting their tissue specific patterns of expression or cell context dependent requirements for these proteins (Reed, 2000). Death at mitochondria is initiated by stimulation of the mitochondrial membrane called the permeability transition pore. Subsequently there is release of cytochrome C, ATP and other apoptogenic factors from the inter-membranous space of the mitochondria (Zamzami and Kroemer, 2001; Martinou and Green, 2001). Cytochrome C and dATP/ATP binds to a protein called Apaf-1 (apoptotic protease activating factor) in the cytosol and promotes the assembly of the apoptosome. Caspase-9 is recruited to the apoptosome through interactions of CARD (caspase activating and recruiting domain) and is activated to form the active caspase 9. Once the initiator caspases are activated, they cleave

Pathways of apoptosis mediated by the mitochondria



and activate executioner caspases namely caspase 3,6 and 7 (Igney and Krammer, 2002). The active executioner caspases then cleave each other and in this way, an amplifying proteolytic cascade of caspase activation is started. Eventually the active executioner caspases cleave cellular substrates- the death substrates, which leads to characteristic biochemical and morphological changes (Rathmell and Thompson, 1999). Cleavage of nuclear lamins is involved in chromatin condensation and nuclear shrinkage. Cleavage of the cytoskeletal proteins such as actin, plectin, Rho kinase I (Rock I), and gelsolin leads to cell fragmentation, blebbing, and the formation of apoptotic bodies. After exposure of "eat me" signals, the dying cells are engulfed by phagocytes (Savill and Fadok, 2000). Various proteins that regulate the apoptotic process at different levels tightly control the apoptotic machinery. FLIPs interfere with the initiation of apoptosis directly at the level of death receptors (Krueger *et al.,* 2001). Members of the Bcl-2 family are major regulators of apoptosis. The IAPs (inhibitor of apoptosis proteins) constitute other class of regulatory proteins. IAPs bind to and inhibit caspases; they also function as ubiquitin ligases promoting the degradation of the caspases they bind to.

1.4 Targeted Cancer Therapy.

The term "targeted therapy refers to a new generation of cancer drugs designed to 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 approach contrasts with the conventional, more empirical approach used to develop cytotoxic chemotherapeutics-the mainstay of cancer drug development in past decades (Sawyers, 2004).

Over the last 20 years, there has been a fundamental shift in the way target identification in cancer is approached. Advances in molecular biology now allow us to identify genes that go awry in cancer, and offer the opportunity to dissect the molecular mechanisms underlying the disease. At first, there were just a handful of cancer genes, and the challenge was to find out how the gene products functioned. Now, many genes are known to affect tumorigenesis and tumor growth, and the key is to decide which ones to exploit in the areas of signal transduction, cell-cycle regulation, apoptosis, telomere biology and angiogenesis (Pitti, 1998).

1.4.1 Targeting apoptotic pathways in cancer cells.

Accruing evidence obtained in the last few years is beginning to establish that many (perhaps all) agents of cancer chemotherapy effect tumor cell killing in-vitro and in-vivo through launching the mechanisms of apoptosis. These lines of investigation have provided significant insight into the mechanisms involved in cell death in general and tumor cell death more specifically. Such insights may allow the identification of novel targets and the development of more specific chemotherapeutic agents that are designed to launch specifically the apoptotic machinery of the cells (Hannun, 1997). Some of the insight into mechanisms regulating apoptosis has come from the examination of chemotherapy-induced death. This is illustrated with the case of etoposide were the proteolytic cleavage of poly (ADP-ribose) polymerase or PARP occurs due to the activation of specific protease and that preceded endonuclease activation and DNA fragmentation (Lazebnik and Kaufmann, 1994). It now appears that many inducers of cell death, including cytokines and other chemotherapeutic agents, ultimately converge on the activation of this and related proteases, which then appear to launch the terminal and execution stages of apoptosis. Many chemotherapeutic agents, such as cytosine arabinoside, vincristine, and daunorubicin have been shown to cause accumulation of ceramide (Strum *et al.,* 1994; Zhang *et al.,* 1996; Bose *et al.,* 1995; Jaffrezou *et al.,* 1996).

When taken together the hypothesis for chemotherapeutics induced apoptosis goes this way- each chemotherapeutic agent interacts with a specific target causing dysfunction and injury, which is then interpreted by susceptible cancer cells as an instruction to undergo apoptosis. Thus it may be considered that chemotherapy-induced cell death proceeds through there distinct general phases.

1. Phase I-an insult generating mechanism. Each class of chemotherapeutic agents interacts with a specific target such as DNA, RNA and microtubules and the action of these agents on their respective targets causes injury or dysfunction.
2. Phase II- signal transduction. The cell is able to decipher and assess the specific injury to the chemotherapy target. For example, DNA damaging agents may use the c-Abl tyrosine kinase to induce cell cycle arrest in a p53 dependent mechanism (Yuan *et al.,* 1996).

3. Phase III- induction of apoptosis. A decision point may exist such that susceptible cells react to the signals generated in response to chemotherapy-induced injury as a go ahead for the execution phase of apoptosis.

1.4.2 Transcription factors as targets for Cancer Therapy.

Given that small-molecule inhibitors of an over active process are thought to be the most useful mechanism of tumor inhibition (Gibbs, 2000), what are the most logical targets? The number of oncogenes that could go awry and that underlie deregulated transcription makes it likely that no one protein, or even a few proteins, would always be overactive in a given human cancer. However, a specific group of percentage of cancers has much to recommend them as the most appropriate targets. Three main groups of transcription factors are known to be important in human cancer. The first to be recognized were the steroid receptors- for example, estrogen receptors in breast cancer and androgen receptors in prostrate cancers. The second group that was recognized is resident nuclear proteins that are activated by serine kinase cascade. The third group of transcription factors that have oncogenic potential are latent cytoplasmic factors, activation of which is normally triggered by receptor ligand interaction at the cell surface (Brivanlou and Darnell, 2002). An over supply or over activity of one or more transcription factors from these three classes might well be required for the survival, unrestricted growth and metastatic behavior of all human cancers (Vogt, 2001; Brivanlou and Darnell. 2002; Denhardt, 1996). Inhibition of excess transcription factors activity therefore seems to offer a direct and promising target to develop effective anticancer therapy (Darnell, 2002).

1.4.3 Targeting NF-κB.

The nuclear factor κB family of proteins is crucial in the inflammatory response incident to cellular injury, and they are also important in the immune response (Perkins, 2000; Israel, 1997). They are cytoplasmic until cells encounter bacterial toxins, common virus infection or the extra cellular signaling proteins IL-1 or TNF-α. The duration of the active NF- κB cycle might be determined by the synthesis of IκB, which is itself transcriptionally activated by NF- κB (Tam *et al.,* 2001). In addition to the importance of IκB proteins in nuclear-cytoplasmic partitioning of NF- κB, a protein called A20 that blocks any further NF- κB

activation by binding to the IL-1 or TNF-α receptor is induced by NF- κB (Song *et al.,* 1996). The importance of NF- κB in cell transformation is emphasized by induction of apoptosis in multiple myeloma and Hodgkins disease cells when dominant negative NF- κB subunits were introduced into such cells. In considering the development of antitumor agents, the possibility of inhibiting cytoplasmic proteases that activate NF- κB is appealing. Protease inhibitors might block the destruction of IκB that releases p65 or the cleavage of p100 that produces p50. Second, if a limited number of serine kinases activate the degradation of IκB, or if the interaction site of several kinases with IκB is similar enough, this limited number of kinases might be a reasonable target. However, these upstream targets would not necessarily be as effective as drugs that were targeted directly to nuclear NF- κB protein interactions. NF- κB almost always drives transcription in association with other transcription co-factors like p300. NF- κB can interact with IRFs, AP-1, steroid receptors and cofactor proteins. These sites offer great potential for interruption that should be highly specific.

1.4.4 Targeting death receptors: FAS/CD95 receptor.

Death receptors are members of the TNF receptor gene super family, which consists of more than 20 proteins with a broad range of biological functions including the regulation of cell death, survival, differentiation or immune regulation (Ashkanazi and Dixit 1998; Krammer, 2000; Walczak and Krammer, 2000). Death receptors are defined by a cytoplasmic domain of about 80 amino acids called the death domain, which plays a crucial role in transmitting the death signal from the cells surface to intracellular signaling pathways.

The CD95 receptor/CD95 ligand system is a key signal pathway involved in the regulation of apoptosis in several different cell types (Walczak and Krammer, 2000). CD95, a 48kDa type I transmembrane receptor is expressed in activated lymphocytes, in a variety of tissues of lymphoid or non-lymphoid origin, as well as in tumor cells. CD95L, a 40kDa type II transmembrane molecule, occurs in a membrane bound and in a soluble form generated through cleavage by metalloproteases. CD95L is produced by activated T cells, which play critical role in the regulation of the immune system by triggering autocrine suicide or paracrine death in neighboring lymphocytes or other target cells. Also, CD95L is constitutively activated in several tissues and has been implied in immune privilege of certain organs such as the testis or the eye (Green and Ferguson, 2001). 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 and Krammer, 2001). Several studies demonstrated that apoptosis induced by anticancer therapy involves the CD95 system. Treatment with anticancer drugs led to an increase in CD95 expression, which triggered the receptor pathway in a autocrine or paracrine manner by binding to its receptor CD95 (Friesen *et al.,* 1996,1997,1997, Fulda *et al.,* 1997 a,b, 1998a-c,2000 a-c; Leverkus *et al.,* 1997; Her *et al.,* 1997; Houghton *et al.,* 1997; Kasibatla *et al.,* 1998). Up regulation of CD95L was observed in many different tumor cell lines, for example, leukemia, neuroblastoma, malignant brain tumor, hepatoma, colon or breast *in vitro* and also ex-vivo in primary, patients derived tumor cells. The CD95 receptor/ligand system has also been implicated in thymine-less death in colon carcinoma cells following treatment with 5-flurouracil (5-FU) (Houghton *et al.,* 1997). Activation of transcription factors AP-1 and NF- κB was shown to mediate the increase in CD95L transcription and mRNA levels in response to chemotherapy (Herr *et al.,* 1997; Kasibatla *et al.,* 1998; Eichhorst *et al.,* 2000). CD95 expression on the cell's surface increased upon drug treatment, in particular, in cells harboring wild type p53 (Fulda *et al.,* 1998 a-c; Muller *et al.,* 1997, 1998). In addition to the up regulation of CD95L and CD95, anticancer agents have been reported to activate the CD95 pathway by modulating expression and recruitment of pro-or antiapoptotic components of the CD95 disc to activated receptors (Fulda *et al.,* 2000). Up regulation of FADD and procaspase 8 was found upon treatment with doxorubicin, cisplatin or mitomycin C in colon carcinoma cells (Micheau *et al.,* 1989 a,b). Also increased recruitment of FADD and caspase 8 to the CD95 receptor to form CD95 DISC was observed in certain tumor cells upon drug treatment in a CD95L dependent or independent manner (Beltinger *et al.,* 1999). In T-cell receptor-positive leukamia cells, treatment with doxorubicin, similar to CD3 triggering led to induction of functionally active CD95L, which caused cell death via CD95/CD95L interaction in a paracrine manner (Frieson *et al*., 1996; Fulda *et al.,* 2000). Death receptors can instruct tumor cells to commit apoptotic suicide independently of p53. So, targeting of death receptors in cancer might be a useful therapeutic strategy. In tumors that retain some responsiveness to conventional therapy, death receptor engagement in combination with

chemotherapy might lead to synergistic apoptosis activation and reduce resistant cells from proliferating.

1.5 Molecular mechanisms of Drug resistance in cancer.

Drug resistance is a major problem that limits the effectiveness of chemotherapy used to treat cancer. Tumors may be intrinsically resistant to chemotherapy prior to treatment. However, drug resistance can also be acquired during treatment by tumors that are initially sensitive to chemotherapy. There are two general classes of resistance to anti cancer drugs to tumor cells: those that impair delivery of anticancer drugs to tumor cells and those that arise in the cancer cell itself due to genetic and epigenetic alterations that affect drug sensitivity. Impaired drug delivery can result from poor absorption of orally administered drugs, increased drug metabolism or increased excretion, resulting in lower levels of drug in the blood into the tumor mass (Pluen, 2001; Jain, 2001). Recent studies have emphasized the importance of the tumor vasculature and an appropriate pressure gradient for adequate drug delivery to the tumor. In addition, some cancer cells those are sensitive to chemotherapy as monolayer cells in culture become resistant when transplanted into animal models (Green *et al.,* 1999). This indicates that environmental factors, such as the extra cellular matrix or tumor geometry, might be involved in drug resistance. Cancer cells grown in culture as three-dimensional spheroids, mimicking their in-vitro geometry, have been shown to become resistant to cancer drugs (Pluen, 2001; Green *et al.,* 1999; Durand and Olive, 2001). Much remains to be learned about this type of drug resistance and its role in clinical oncology.

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.

1.5.1 Deregulation of the apoptotic machinery.

The ultimate goal of cytotoxic chemotherapies is to induce cell death in tumor cells. The onset of apoptosis is regulated by multiple intra and extra cellular signals; amplification of these signals is by second messengers and activation of the effectors of apoptosis, the caspases. The failure to induce apoptotic machinery in cancer cells could be envisaged into two broad cellular behaviors of these cells.

1.5.1.1 Expression of anti-apoptotic proteins.

Tumor cells can acquire resistance to apoptosis by various mechanisms that interfere at different levels of apoptosis signaling. A common feature of follicular B-cell lymphoma is the chromosomal translocation (14:18), which couples the Bcl-2 gene to the immunoglobulin heavy chain locus, leading to enhanced Bcl-2 expression (Fluda, 1997; Tsujimoto *et al.,* 1985; McDonnell *et al.,* 1989; Reed *et al.,* 1988). Bcl-2 co-operates with the oncoprotein c-Myc or, in acute promyelocytic leukemia, the promyelocytic fusion protein, thereby contributing to tumorigenesis (Strasser *et al.,* 1990; Vaux *et al.,* 1988; Kogan *et al.,* 2001). It has been shown both in in-vitro and in-vivo models that Bcl-2 expression confers resistance to many kinds of chemotherapeutic drugs (Weller *et al.,* 1995; Miyashita and Reed. 1992; Schmitt *et al.,* 2000; Findley *et al.,* 1997). Bcl-X_L can confer resistance to multiple apoptosis-inducing pathways in cell lines and seem to be up regulated by a constitutively active mutant epidermal growth factor receptor (EGFR) in-vitro (Findley *et al.,* 1997; Boise *et al.,* 1993; Dole *et al.,* 1995; Nagane *et al.,* 1998; Minn *et al.,* 1995). Human melanomas and a murine B-cell lymphoma cell line were shown to express high levels of FLIP which interferes with apoptosis induction at the level of CD95/CD95L induced activation of caspase 8 (Krueger *et al.,* 2001; Irmler *et al.,* 1997; Mueller and Scott, 2000). Tumors with high level of FLIP were shown to escape from T-cell mediated immunity in-vivo, despite the presence of the perforin/granzyme pathway (Medema *et al.,* 1999), so tumor cells with elevated FLIP levels seems to have a selective advantage. The expression of soluble receptors that acts as decoys for death ligands interferes with death-receptor mediated apoptosis. Two distinct soluble receptors: soluble CD95 (sCD95) and decoy receptor 3(DcR 3) have been shown to competitively inhibit CD95 signaling. Soluble CD95 is expressed in various malignancies

and elevated levels can be found in the sera of cancer patients (Cheng *et al.,* 1994; Midis *et al.,* 1996; Ugurel *et al.,* 2001; Gerharz *et al.,* 1999). Ectopic expression of DcR3 in a rat glioma model resulted in decreased immune-cell infiltration, which indicates that DcR 3 is involved in immune evasion of malignant glioma (Roth *et al.,* 2001). Expression of the IAP-family protein surviving is found in most human tumors but not in normal adult tissues making it highly tumor specific (Reed, 2001; Ambrosini *et al.,* 1997). Survivin acts as an apoptosis inhibitor by binding to caspase 9 or interacting with SMAC/DIABLO. Survivin might also be necessary for completion of the cell cycle (Reed, 2001). Expression of the serine protease inhibitor PI-9/SPI-6 that inhibits granzyme B, results in the resistance of tumor cells to cytotoxic lymphocytes, leading to immune escape (Medema *et al.,* 2001).

1.5.1.2 Inactivation of pro-apoptotic genes.

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 functions are common (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). Metastatic melanomas have been found to escape mitochondria dependent apoptosis by under expressing Apaf 1, as shown by high rate of allelic loss at the Apaf 1 locus (Soengas *et al.,* 2001). The remaining allel is transcriptionally inactivated by gene methylation thereby not responding to chemotherapy. In neuroblastoma N-myc oncogene was amplified (Teitz *et al.,* 2000). Also in these tumors, the gene for the initiator caspase 8 is frequently inactivated by gene deletion or methylation. Caspase-8 deficient neuroblastoma cells are resistant to death-receptors and doxorubicin-mediated apoptosis. The death receptor CD95 is reduced in some tumor cells like in hepatocellular carcinomas, neoplastic colon epithelium and melanomas (Strand *et al.,* 1996; Moller *et al.,* 1994; Leithauser *et al.,* 1993; Volkmann *et al.,* 2001). Loss of CD95, probably by down regulation of transcription, down regulation by oncogenic Ras or loss of expression due to p53 aberrations contribute to chemo resistance. In primary samples of myeloma and T-cell leukemia several CD95 gene mutations have been reported. The mutated forms of CD95 interfere in a dominant negative way with apoptosis induction by CD95 (Strand *et al.,* 1996;

Moller *et al.,* 1994; Leithauser *et al.,* 1993; Volkmann *et al.,* 2001). Deletions and mutations of the death receptors TRAIL-R1 and TRAIL-R2 have been observed in tumors. Mutations in the ecto domains or death domain of TRAIL-R1 or R2 result in truncated forms of these receptors or other anti-apoptotic forms (Shin *et al.,* 2001; Fisher *et al.,* 2001; Pai *et al.,* 1998; Lee *et al.,* 1999). Reduced expression of pro-apoptotic protein XAF1 (XIAP-associated factor 1) has been observed in various cancer cell lines (Liston *et al.,* 2001). XAF1 binds to XIAP and antagonizes its anti-apoptotic function at the level of the caspases.

1.5.2 Drug influx and efflux.

The mechanism by which many chemotherapeutic drugs are taken up by cells is unknown. It has been established that folate pathway inhibitors (anti-folate drugs) such as the dehydrofolate reductase (DHFR) inhibitor methotrexate (MTX) and the thymidyate synthase (TS) inhibitor tomudex (TDX) enter the cell predominantly via the reduced folate carrier (RFC) (Bertino, 1993; Jackman *et al.,* 1991). Decreased expression of RFC and inactivating mutations are documented mechanisms of MTX resistance (Gorlick and Bertino, 1999; Guo *et al.,* 1999). Impaired uptake of TS-targeted anti-folates by the RFC has also demonstrated to be a resistance mechanism to this agent in cell line models (Wang *et al.,* 2003), suggesting that impaired uptake by the RFC may constitute an important general resistance mechanism to anti-folates. 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 and Coley, 2003; Krishna and Mayer, 2000). Furthermore, expression of these proteins has been reported to correlate with resistance to chemotherapy in-vitro. Multi-drug resistance has most often been linked to over expression of P-gp in many drug resistant cell lines and in a number of leukemias and solid tumors (Thomas and Coley, 2003; Goldman, 2003).

1.5.3 Drug inactivation

Mechanisms that inactivate drugs can diminish the amount of free drug available to bind to its intracellular target. More than 80% of the anti-metabolite 5-flurouracil (5-FU) is normally

catabolized by dehydropyrimidine dehydrogenase (DPD), primarly in the liver (Diasio and Harris, 1989). In-vitro studies have demonstrated that DPD over expression in cancer cell lines confers resistance to 5-FU (Takebe *et al.,* 2001). The formation of conjugates between the thiol glutathione (GSH) and platinum drugs such as cisplatin, carboplatin and oxaliplatin is a key step in the inactivation of these drugs (Meijer *et al.,* 1992). Cytochrome P450 enzymes can inactivate CPT-11 and its active metabolite SN-38 is a target for glucouronidation by uridine diphosphoronyl transferase. 1A1 (UGI1A1) (30 L). Decreased drug inactivation can also play an important role in drug resistance. Drugs such as 5-FU and CPT-11 must be converted to their active metabolites to exert their anti-cancer effects. CPT-11 is converted to SN-38 by carboxylesterase (CE) (Xu and Villalona-Calero, 2002) and studies have indicated that the level of CE activity in cancer cells is an important determinant of CPT-11 sensitivity (Boyer *et al.,* 2004; Kojima *et al.,* 1998). The activation of 5-FU involves enzymes like thymidine phosphorylase (TP), uridine phosphorylase (UP), and orotate phosporibosyl transferase (OPRT) and these have been shown to be associated with 5-FU sensitivity (Schwartz *et al.,* 1985; Houghton and Houghton, 1983; Evrard *et al.,* 1999).

1.5.4. Drug targets.

Alterations in expression levels or mutation of a chemotherapeutic drug target can have a major impact on drug resistance. The 5-FU metabolite flurodeoxyuridine monophosphate (FdUMP) is a potent inhibitor of TS, and it is the inhibition of TS that is believed to be the primary anticancer activity of 5-FU (Peters and Kohne, 1999). Genotyping studies have found that patients homozygous for a particular polymorphism in the TS promoter that increases TS expression are less likely to respond to 5-FU based chemotherapy than patients who are heterozygous or homozygous for the alternative polymorphism (Marsh and McLeod, 2001). The CPT-11 active metabolite SN-38 is a potent inhibitor of DNA topoisomerase-I (topo I) (Xu and Villalona-Calero, 2002). It was found that topo-I mRNA was highly downregulated in a CPT-11 resistant colon cancer cell line (Boyer *et al.,* 2004). Similarly DNA topo-I activity correlated with CPT-11/SN-38 sensitivity in a panel of human colon cancer cell lines (Jansen *et al.,* 1997). Topoisomerase-II (topo-II) is a target for anthracyclines such as doxorubicin and epipodophyllotoxins such as etoposide. Alterations in topo-II protein or mutation of topo II have been correlated to resistance in cell lines (Deffie *et*

al., 1992; Friche *et al.,* 1991). Taxanes such as paclitaxel and docetaxal and vinca alkaloids such as vinblastine and vincristine suppresses microtubule polymerization dynamics, which results in the slowing or blocking of mitosis at the metaphase-anaphase boundary further leading to apoptosis (Jordan *et al.,* 1993, 1996). Changes in microtubule dynamics and levels of tubulin isotypes have been correlated with resistance to placitaxel and vinca alkaloids (Dumontet and Sikic, 1999).

1.5.5. Alterations of the p53 pathway.

As p53 has a central function in apoptosis induction alterations of the p53 pathway influence the sensitivity of tumors to apoptosis (Ryan *et al.,* 2001; Lowe *et al.,* 1994). 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). In about 70% of breast cancers, wild type p53 is expressed but fails to suppress tumor growth due to a lack of the ASPP (apoptosis stimulating protein of p53) family of proteins, which interacts with p53 and specifically enhance the DNA binding and transactivation function of p53 on the promoters of pro-apoptotic genes in-vivo (Igney and Krammer, 2002).

1.5.6 DNA damage repair.

The capacity for a cancer cell to repair DNA can determine resistances to chemotherapeutic drugs that induce DNA damage either directly (e.g. Platinum drugs) or indirectly (e.g. Topoisomerase inhibitors). The response to DNA damage is either repair or cell death and therefore has a profound effect on tumor chemo sensitivity and chemo resistance. Signaling pathways have evolved to arrest the cell cycle following DNA damage to allow time for repair. Only when repair is incomplete, during extensive DNA damage, cells undergo apoptosis. However the relationship between cell cycle arrest and apoptosis following chemotherapy is complex and dependent on both the chemotherapy used and the molecular phenotype of the tumor cell. Nucleotide excision repair (NER) is the major pathway for platinum DNA adducts removal and repair of platinum drug induced DNA damage. Both oxaliplatin and cisplatin induced DNA adducts are removed with similar efficiency by NER

(Reardon *et al.,* 1999). The importance of NER is highlighted by the finding these defects in this pathway result in hypersensitivity to cisplatin, and that restoration of NER activity reduces sensitivity to more normal levels (Chanet and Sancar, 1996; Furuta *et al.,* 2002). Inherited defects in the DNA mismatch repair (MMR) genes are common in certain familial forms of cancer such as heredity non-polyposis colon cancer (HNPCC) and are also observed in a variety of sporadic tumors, including colorectal, breast and ovarian (Issa, 2000; King *et al.,* 1995; Paulson *et al.,* 1996; Herfarth *et al.,* 1999). The main function of the MMR system is to scan newly synthesized DNA and remove single nucleotide mismatches that arise during replication. Data implicate MMR deficiency in the development of resistance to a wide range of DNA damaging agents, including platinum drugs (Fink *et al.,* 1998).

1.5.7 Alterations in the survival signaling.

Most tumors are independent of the survival signals that protect normal cells from death by neglect. The alterations in the survival pathways give a strong proliferative advantage to tumors often conferring chemo resistance by evasion of apoptosis. Over expression and oncogenic mutations of many protein tyrosine kinases (PTKs) have been described in human cancers (Blume-Jensen *et al.,* 2001). Best characterized PTK are the members of epidermal growth factor receptor (EGFR) family. EGFR has been strongly implicated in tumor progression, where receptor over expression is associated with advanced disease (Olayioye *et al.,* 2000). In-vitro over expression of EGFR has been shown to increase resistance to chemotherapeutic drugs (Pegram *et al.,* 1997; Chen *et al.,* 2000; Nagane 1998). One of the downstream pathways activated by PTK is mediated by P13K. Activation of P13K generates PIP3 a lipid second messenger for translocation of Akt to plasma membrane where it is phosphorylated and activated by PDK-1. Akt phosphorylates Bad thereby achieving negative regulation of anti-apoptotic Bcl-X_L by the later. Bad is inactivated by Ras-Raf-MEK-ERK cascade, which is activated by PTKs (Hayakawa *et al.,* 2000). Akt is frequently constitutively activated in human cancers due to gene amplification or mutations in signaling pathways that regulate Akt activity, such as over expression of EGFR or mutation of PTEN, which is one of the most frequently mutated tumor suppressor genes (Blume-Jensen, 2001). In-vitro studies have demonstrated that inhibiting the P13K/Akt pathway enhances the cytotoxic effects of a variety of chemotherapeutic agents (Ng *et al.,* 2001; Nguyen *et al.,* 2004).

The transcription factor NF-κB is a key regulator of oncogenesis through its promotion of proliferation and inhibition of apoptosis. NF-κB exerts its anti-apoptotic effects by up-regulating a member of anti-apoptotic proteins, including IAPs, TNF receptor associated factors (TRAFs), c-FLIP, Bcl-2, BfL-I(A1) and Bcl-X_L (Lin and Karin, 2003). NF-κB is also activated by Akt and has been connected with multiple pathways involved in oncogenesis, including cell cycle regulation and apoptosis (Lin and Karin. 2003; Karin *et al.,* 2002; Baldwin 2001). Whether constitutive or induce, NF-κB appears to be a critical determinant of drug resistance, with NF-κB activation blunting the ability of chemotherapy to induce cell death (Baldwin, 2001). Furthermore, increased NF-κB activity in patients with esophageal cancer has been correlated with reduced response to non-adjuvant chemotherapy (Abdel-Latif *et al.,* 2004). Thus inhibiting NF-κB signaling may prove to be an effective strategy to enhance drug-induced apoptosis in a range of cancers.

1.6 Strategy to overcome chemoresistance.

The problem of drug resistant tumors is alarming. Various strategies have been devised to overcome this problem like the combination of radiation therapy and chemotherapy. The most effective especially for non-solid tumors seems to be of therapy involving a combination of different chemotherapeutic drugs. A thorough knowledge of the molecular pathway(s) of apoptosis mediated by the chemotherapeutic agent is of utmost importance in such combination therapies. Hence research efforts over the past decades have been focused in understanding the various pathway(s) of apoptosis triggered by the numerous chemotherapeutic agents available and also putative ones.

1.6.1 Combination therapy.

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 help stimulate 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 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. Therefore, the combination therapy outlined by the new study is a promising general strategy for treating many cancers that are refractory to current therapies. 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.

1.7 Cardiac glycosides.

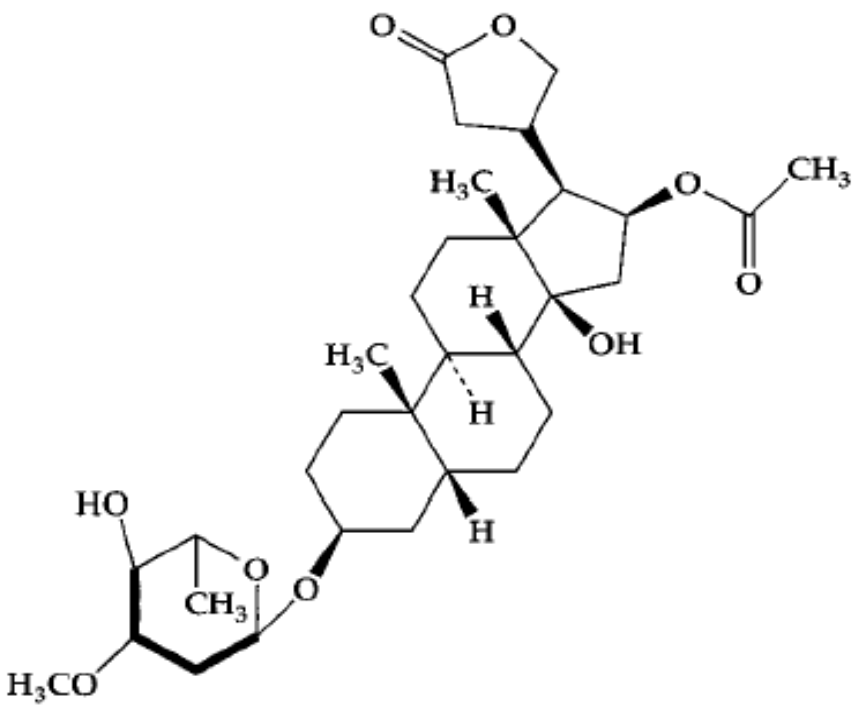
Cardiotonic steroids (CS) encompasses a group of compounds that share the capacity to bind to the extracellular surface of the major ion transport protein, the membrane inserted sodium,

potassium-ATPase thereby conferring cardiac contractile force. It's a family of compounds that includes plant-derived cardiac glycosides and putative endogenous vertebrate counterparts. Also members of this group of compounds include plant-derived pharmaceuticals such as the digitalis steroid glycoside drugs as well as the more polar plant monoglycoside, ouabain and vertebrate derived aglycone CS such as bufalin and marinobufagenin. There has been accumulating evidence of adrenocortical release, biosynthesis, and regulation of CS. Also there is growing evidence of the ability of plant derived glycosides to mediate effects on second messenger signaling with effects on pathways influencing cytoskeletal reorganization, cell growth and cell division, as well as further evidence of involvement of endogenous CS in regulation of blood pressure and renal function (Dmitrieva and Doris, 2002). *In vitro* inhibition of malignant cells growth by cardiac glycosides was reported long back in 1960's and since then several anticancer effects of digitalis have been observed (Shiratori *et al*., 1967; Inada *et al*., 1993). A long term follow-up (22.3 years) of 175 breast cancer patients of which 32 were on digitalis treatment, have again indicated a lower death rate (6%) compared with patients not on digitalis (34%) (Stenkvist, 1999).

1.7.1 Oleandrin.

Nerium oleander (family Apocynaceae) is a tropical flowering plant with white, red and pink flowers. The ayurvedic name of this plant is Karavira and it has been used in the treatment of hemorrhoids ulcers, leprosy and snakebite and in the induction of abortion. The leaves of *Nerium oleander* have been used to extract cardiac glycosides, oleandrin, adynerin, neriatin, oloroside A and other compounds (Abe and Yamaguchi, 1978, 1979). Cardiac glycosides are a class of natural products that are used to increase cardiac contractile force in patients with congestive heart failure and cardiac arrhythmias, the most familiar are digoxin/digitoxin and ouabain. Oleandrin has been used for treating cardiac abnormalities in Russia and China for years. Interestingly, anecdotal evidence has emerged from this experience suggesting that they may produce beneficial side effects in patients with leiomyosarcoma, erwings sarcoma, prostrate cancer and breast cancer. Indeed there are also scattered reports of breast tumour regression in Scandinavian patients taking other cardiac glycosides. Pre-clinical studies have demonstrated that oleandrin has excellent activity against a variety of human solid tumour

Chemical structure of oleandrin



{(3b,5b,16b)-16-(Acetyloxy)-3-[(2,6-dideoxy-3-O-methyl-a-L-arab ino-hexopyranosyl)oxy]-14-hydroxycard-20(22)-enolide};

MW 576.73

cell lines. However, a thorough understanding of the antineoplastic activities of oleandrin has not been addressed. Hence in this scenario the objectives of the study were to: -

1. decipher the molecular signal transduction pathway(s) mediated by oleandrin in relation to its anti-tumor activities.

2. validate oleandrin's effectiveness in countering the life lines of a tumor cell.

Chapter Two

Materials and Methods

2.1 Materials :

2.1.1 Chemicals used :

Oleandrin, C2 ceramide (*N*-acetyl-D-sphingosine), glycine, lipopolysacharide (LPS), bovine serum albumin (BSA), arabinoside cytosine (AraC), MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], calyculin A, zinc chloride, doxorubicin, vincristine, taxol, cis-platin, etoposide, adriamycin, lactacystin, z-VAD-fmk, thiobarbituric acid, 4-methyl umbelliferyl phosphate, antimycin, epidermal growth factor (EGF), nerve growth factor (NGF), cephalin, cholesterol, lecithin, sphingosine, amphotericin B, FMLP, nitroblue tetrazolium (NBT), diphenyl hexatriene (DPH), melanocyte growth stimulatory activity (MGSA, also known as GROα), histopaque, dextran, ortho-phenylenediamine (OPD), *p*-nitrophenyl phosphate, *p*-nitrophenyl b-D-glucuronide, monodansyl cadaverine (MDC), leupeptin, PMSF, bestatin, TPCK, TLCK, polymixin B-sulphate, acrylamide, bis-acrylamide, bromophenol blue, iodogen, RII peptide, sodium molybdate and giesma stain used were obtained from **Sigma,** St. Louis, MO, USA.

Recombinant TNFα, recombinant human IL-8, IL-1 were obtained from **PeproTech Inc**., Rocky Hill, NJ, USA.

Fluoroscent substrates (paranitroaniline conjugated) for Caspase-3, 8 and 9 were obtained from **Calbiochem**, San Diego, CA, USA.

Alexa-Fluor conjugated IgG, mounting medium with DAPI and live and dead cell assay kit were obtained from **Molecular Probes**, Eugene, OR, USA.

RPMI-1640 medium, DMEM medium, phosphate free DMEM, Opti-MEM medium, trypsin-EDTA, antibiotic-antimycotic, freezing medium, fungizone, L-glutamine, fetal bovine serum (FBS) and TRIzol were obtained from **Gibco BRL.**

M-MuLV reverse transcriptase, DNA-polymerase, RNase inhibitor, dNTPs and $MgCl_2$ were obtained from **Invitrogen Corporation**, Carlsbad, CA, USA.

2.1 Antibodies used :

Anti-IκBα, p65, p50, c-Rel, Rel B, IKK, cyclin D1, PP1, PP2A, PP2B-A, CRM1, Bcl-2, Fas L, Fas, retinoblastoma (Rb), p-Rb, p-Akt, casein kinase IIα, PKAα, IL-8, IL-8R1, IL-8R2, JNK, ERK1/2, tubulin and actin used were obtained from **Santa Cruz Biotechnology, Inc**., Santa Cruz, CA, USA.

Anti-Poly(ADP-ribose) polymerase (PARP), caspase-3, caspase-8, caspase-9, Akt and NFAT used were obtained from **PharMingen**, Palo Alto, CA, USA.

Anti-FKHR and -Akt were obtained from **Cell Signaling Technologies**, Beverly, MA, USA.

2.2 Cell Lines :

2.2.1 Human Origin :

HeLa (cervical epithelial cells), Jurkat (T cells), Daudi (B-cells), MCF-7 (breast epithelial cells), HuT-78 (T cells), U937 (histiocytic lymphoma), THP-1 (monocytic macrophages) and HL-60 (monocytic cells) were obtained from American Type Culture Collection.

Mn-SOD-MCF-7 cells were a gift from Professor Bharat B Agarwal, MD Anderson Cancer Centre, Houston, Texas, USA.

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.

2.2 Buffers and Media :

2.2.1 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 ——— To be added just before use
0.5 µg/ml benzamidine

2.2.2 SDS-PAGE :

2.2.2.1 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.

2.2.2.2 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.

2.2.2.3 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

2.2.2.4 10X SDS-PAGE Running Buffer :

0.25 M Trizma Base
1.92 M Glycine
1% SDS

2.2.3 Western Blot :

2.2.3.1 10X Blotting Buffer :

0.25 M Trizma Base
1.92 M Glycine
1% SDS

2.2.3.2 To make 2 litres of 1X Blotting Buffer :

400 ml of methanol
200 ml of 10X blotting buffer
1400 ml of Milli-Q water.

2.2.3.3 PBS :

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
2 mM KH_2PO_4

2.2.3.4 HEPES Buffer Saline :

20 mM HEPES (pH 7.5)
150 mM NaCl

2.2.3.5 Blocking Buffer :

5% Fat free milk or 3% BSA
0.05% Tween
Make up volume with PBS.

2.2.3.6 Stripping Buffer :

100 mM β-mercaptoethanol
2% (w/v) SDS
62.5 mM Tris-HCl (pH6.7)

2.2.4 Electrophoretic Mobility Shift Assay (EMSA) :

2.2.4.1 Cytoplasmic Extraction Buffer :

1 M HEPES (pH 7.9)
2 M KCl

0.1 M EGTA (pH 7)
0.5 M EDTA (pH 8)

2.2.4.2 Nuclear Extraction Buffer :

1 M HEPES (pH 7.9)
5 M NaCl
0.1 M EGTA (pH 7)
0.5 M EDTA (pH 8)

2.2.4.3 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

2.2.4.4 10X EMSA Binding Buffer :

200 mM HEPES (pH 7.9)
4 mM EDTA (pH 8.0)
40 mM DTT
50% glycerol

2.2.4.5 5x EMSA buffer :

0.25 M Tris
2.0 M Glycine
0.01 M EDTA (pH 8.5)
pH made upto 8.5

2.2.4.6 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

2.2.4.7 NFAT-EMSA Binding Buffer :

10 mM Tris (pH 7.5)
30 mM NaCl
0.5 mM EDTA
1 mM DTT
5% glycerol

2.2.5 Kinase Assay :

2.2.5.1 Assay Buffer (10X) :

20 mM HEPES (pH 7.4)
10 mM $MgCl_2$
1 mM DTT

2.2.5.2 Wash Buffer :

20 mM HEPES (pH 7.4)
25 mM NaCl
1 mM DTT

2.2.6 Genomic DNA isolation by Silica Method :

2.2.6.1 Lysis Buffer :

4 M guanidine thiocyanate
1% N-lauryl sarcosyl
10 mM DTT

2.2.6.2 Wash Buffer :

70% ethanol
10 mM EDTA
10 mM Tris-Cl (pH 8.0)

2.2.6.3 Elution Buffer :

10 mM Tris-Cl (pH 8.0)
0.1 mM EDTA (pH 8.8)

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 Neutrophils and macrophages differentiation and isolation:

THP1 cells were stimulated with 10 ng/ml PMA for 16 hours and then adherent cells were used as macrophages (Sarkar *et al*., 2003). HL-60 cell line was maintained in complete medium of RPMI-1640. These cells were cultured in presence of 1.3% DMSO for 2 days. These cells were differentiated into neutrophils (Kanayasu-Toyoda *et al*., 1999, 2003). These cells were well characterized as neutrophils by detecting the level of IL-8R expression, morphology, IL-8 induced chemotaxis, and PMA-induced oxidative burst response and enzyme release compared to normal HL-60 cells. Hence these cells were considered as neutrophils for further studies.

2.3.3 Radiolabeling of IL-8 and other ligands :

Interleukin-8 was iodinated by Chloramine T following the method of Grob *et al.,* with slight modification. The $Na^{125}I$ was mixed with 50 µl of 100 mM phosphate buffer (pH 7.4) in a 1.5ml polypropylene tube. After gentle mixing the tubes were kept at 23°C water bath. 10 µg IL-8 was then added with this mixture. The idonated reaction was started by addition of 30 µl

of freshly prepared Chloromine T (1 mM) solution. After proper mixing, the reaction was continued for 60 seconds and then the reaction was terminated by addition of 30 µl of 57.7 mM sodium metabisulfide. Next, 190 µl D-PBS containing 5 mg/ml BSA was added to the reaction mixture. The entire mixture was transferred to a Sephadex G-10 column (20cm x 1cm) equilibrated with D-PBS containing 5 mg/ml BSA for separation of free iodine. The iodinated protein was eluted by addition of 800µl-equilibrated buffer in each fraction. About 16-column fraction was collected and the count was taken in each fraction in Gamma counter. The first peak, obtained from the counts was due to labeled protein and the second peak for free iodine. The labeled protein (IL-8) containing fraction was aliquoted and kept at -20°C. The specific activity of labeled IL-8 was $1x10^7$ to $4x10^7$ cpm/µg of the protein. The anti-rabit IgG was $3x10^7$ to $5x10^7$ cpm/µg protein.

2.3.4 Receptor binding Assay :

Freshly prepared human neutrophils were suspended in RPMI-1640 (10^7 cells/ml) containing 20 mM HEPES buffer pH 7.2, 5 mg/ml BSA. Cell suspension (200 µl) was taken in each tube for subsequent experiments. After incubation under different condition the cells were cooled on ice. Radiolabelled IL-8 binding was carried out at 4°C for 2 hours using ^{125}I IL-8 (4ng/tube). Then the cells were centrifuged at 4°C for 20 seconds at 10,000xg. The supernatant discarded and the cell pellet was resuspended in ice cold medium and layered onto ice cold 800 µl of 10% sucrose in D-PBS and centrifuged at 4°C for 2 minutes at 10,000xg. Only neutrophils passed through the sucrose solution and unbound ^{125}I IL-8 remained above sucrose solution. The supernatant was removed carefully and the pellet containing part of the tube was taken. The radiolabelled ligand bound to the cell was measured in Gamma counter (Grob *et al.,*).

2.3.5 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 different enzyme assay.

2.3.5.1. Myeloperoxidase : Myeloperoxidase activity was measured taking the 25µl supernatant and 100µl substrate solution of ortho phenylene diamine (OPD)(0.75mg/ml) and H_2O_2 (0.01%) in citrate phosphate buffer (0.1 M, pH5.2). After 30 minutes at room temperature, the reaction was stopped with addition of 50 µl of 4 N H_2SO_4 and absorbance was measured at 492 nm.

2.3.5.2. Alkaline phosphatase : 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 .

2.3.5.3. β-D-glucuronidase : 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.

2.3.5.4. Lysozyme : For lysozyme assay, 100 µl supernatant was added in substrate solution containing Micrococcus lysodykticus cell wall (1.5 mg/ml) in 0.05 M potassium phosphate buffer pH 6.2 and the absorbance was taken immediately at 450 nm. The gradual decrease in absorbance was taken in every minute of incubation upto 10 minutes.

2.3.6 Chemical Crosslinking :

The level of receptor on the surface of neutrophils was detected through chemical crosslinking. The neutrophils ($1x10^7$ cells/tube) suspended in PBS were incubated with or without modulator(s) at 37°C. After incubation, the cells were washed, cooled and incubated with ^{125}I IL-8 (20 ng) at 37°C. After binding, unbound ^{125}I IL-8 was removed by washing with D-PBS and finally the cells were suspended in 100 µl D-PBS. A bifunctional crosslinker DSS (1 mg/ml) was added to the cell suspension and incubated at 4°C for 1 hour. The cells were washed and mixed with 80 µl CHAPS (9 mM) in D-PBS with PMSF (200 µM), leupeptin (1 µM), pepstatin (1 µM) and EDTA (100 µM). After mixing thoroughly, centrifugation was carried out at 4°C at 10,000xg for 15 minutes. The supernatant was removed carefully,

analyzed in 10% SDS-PAGE under reducing condition following the method of Laemmli. The gel was dried and autoradiograph was carried out using a Fuji Bas1000 phosphorimager.

2.3.7 Membrane Preparation :

Freshly prepared neutrophils ($1x10^8$/ml) were suspended in hypotonic buffer (10 nM HEPES containing 1 mM PMSF, 100 μM leupeptin, 100 μM pepstatin and 0.5 mM EDTA) were kept in ice for 30 minutes. Then the cells were homogenised in a Dounce homogeniser, centrifuged for 10 minutes at 160 x g to remove unbroken cells and cell debris. The supernatant was layered on a discontinuous gradient of 5ml of 15%(w/v) sucrose resting on 10ml of 40%(w/v) sucrose and centrifuged at 1,00,000 x g for 60 minutes at 4°C. The pellet containing membrane was used for the experiment.

2.3.8 Measurement of Membrane fluorescence :

1,6 Diphenyl 1,3,5-hexatriene (DPH), a widely used membrane interacting fluorescent probe was used for the alteration of membrane fluidity. DPH is insoluble in aqueous solvent but is soluble in tetrahydrofuran (THF). For the preparation of aqueous solution, 10 μl of DPH solution (2 mM in tetrahydrofuran) was taken in 10ml of rapidly stirring PBS. No trace odour of THF was detected after stirring for 4 hours in dark place. For interaction with neutrophils, cell suspension was mixed with equal volume of DPH solution (final conc. 1 μ M) and incubated at 37°C for 2 hours. After incubation, the cells were washed thrice and suspended in PBS. The emission spectra of DPH bound to cells were recorded at 430nm when the fluorescent probe was excited at 365nm in a Perkin Elmner spectrofluorometer.

2.3.9 Measurement of Fluidity and microviscosity:

The lipid fluidity of neutrophil membrane was determined by fluorescence depolarization measurement using diphenyl hexatriene (DPH) as a fluorescent probe. The measurement of fluorosence anisotropy was carried out in a fluorescence polarization spectrometer at 37°C using excitation wavelength 365 nm and an emission wavelength of 430 nm for DPH. The fluorescence anisotropy(r) was calculated using the equation :

$r = (I_{II} - I_I)/(I_{II} + 2I_I)$

Where, I_{II} and I_I are the fluorescence intensities oriented respectively parallel and perpendicular to the direction of the polarization of the excited light. The microviscosity parameters $[(r_o/r\ )-1]^{-1}$ were calculated in each case knowing the maximal limiting fluorescence anisotropy r_o which for DPH is 0.362.

2.3.10 MTT assay :

The drug-induced cytotoxicity was measured by the MTT assay. Briefly 5000 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 multiscanner auto reader (Coda, Bio Rad) with the extraction buffer as blank.

2.3.11 Total RNA isolation from cultured mammalian cells :

To the cell pellet (approximately 2 million cells) 800 µl of TRIzol was added and the cells suspended in it by repeated pipetting. Later 200 µg of glycogen was added followed by vigorous vortexing or power homogenization. This was vortexed at high speed for 10 seconds and the contents passed twice through a 26-guage needle connected to a 1ml syringe. The contents were transferred to a 1.5 ml microcentrifuge tube and 160 µl of choloroform was added followed by vortexing for about 30 seconds. The tube was centrifuged at maximun 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 pellet 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.

2.3.12 Detection of p65 by semiquantitative 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). The PCR war performed using primers for *p65* (5'-GGTCCACGGCGGACCGGT- 3' and 5'-GACCCCGAGAACGTGGTGCGC-3'). 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* gene.

2.3.13 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).

2.3.14 Preparation of Silica Slurry for DNA isolation :

50% (w/v) silica (powder silica SIGMA) was taken in sterile milli Q water and centrifuged to pellet. The supernatant was discarded and again loaded with milliQ water and the process was repeated 3 times. Finally fresh milliQ water was added to make 50% silica slurry. The slurry was aliquoted and used for DNA isolation.

2.3.15 Radioactive End Labelling of oligos :

The Oligonucleotides, γ^{32}-ATP, T_4Polynucleotide 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
γ^{32}-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 37ºC 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 200 µl milli-Q water on the top of the column. After collecting 5 to 6 fractions the tubes where 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.

2.3.16 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 –70ºC. 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. Then centrifuged for 5 min at 10000xg. The supernatant (nuclear extract) was stored at –70ºC.

2.3.17 Protein estimation by Bradford method :

In the first well of a 96-well plate 50 µl of milli-Q water was added in duplicate. To the subsequent wells 50 µl BSA standards (i.e. 50 µg/ml, 100 µg/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 570 nm. The unknown protein concentration was calculated as follows

$$\frac{\frac{\text{BSA Std 1(Conc)}}{\text{OD}} + \frac{\text{BSA Std 2 (Conc)}}{\text{OD}}}{2}$$

Concentration of unknown sample (mg/ml) = **Required quantity of protein / Conc of sample mg/ml**

2.3.18 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 NF-kB 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 37ºC 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 every thing 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 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 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 80ºC for 1 hr. under suction. After drying the gel, gel was removed from the gel-dryer, the corners were taped and was exposed on a Molecular-Dynamics Phosphorimager Screen to read the protein of interest.

2.3.19 NF-κB-dependent Reporter Gene Transcription :

Briefly, 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). 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). The relative promoter activity was normalized with β-galactosidase activity as the transfection efficiency.

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 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 ceramide at 37°C for 2 h, 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).

2.3.22 H-Thymidine incorporation assay :

The viable and proliferating cell number was detected by ^{3}H-Thymidine incorporation assay. HeLa cells (10^4 cells/well of 96-well plate) were incubated with test sample in a final volume of 0.2 ml for 72 h at 37°C. Cell proliferation was measured by thymidine incorporation by adding 50 µl of ^{3}[H]thymidine (0.5 µCi/well diluted in Hank's buffered salt slution) last 18 h. Cells were harvested and washed, and thymidine incorporation was measured in a beta counter (Packard).

2.3.23 Immunoprecipitation of p65 from orthophosphate-labeled cells :

To determine the phosphorylation of the p65 subunit of NF-κB (Manna et al., 2000a-Oncogene Manuscript), Jurkat, HuT-78, and SA-LPS stimulated Jurkat cells ($5x10^6$) were labeled with [^{32}P]orthophosphate (Amersham) in phosphate-free medium for 4 hours 37°C, and then the cells were treated with TNF (1 nm), araC (50 µm), and ceramide (10 µm) for 4 hours at 37°C.Then the cellextracted proteins were immunoprecipitated with anti-p65 polyclonal Ab (Santa Cruz Biotechnology) followed by protein A/G sepharose beads.Washed beads were then boiled with SDS sample buffer for 5 minutes, and subjected to SDS–PAGE (9%).The gel was dried, exposed to PhosphorImager screen, and analysed by a Phosphorimager (Fuji, Japan).To confirm equal loading, 50 µg protein was resolved on 10%

SDS PAGE, electrotransferred to nitrocellulose filters, and probed with the anti-p65 Ab, and the bands were detected by chemiluminescence (ECL, Amersham).

2.3.24 In-vitro phosphatase activity assay :

Jurkat cells ($5x10^6$), labeled with [^{32}P]orthophosphate (Amersham) in phosphate-free medium for 4 hours at 37°C, were stimulated with TNF (1 nm) for 1 hour. Cell extract proteins were incubated with anti-p65 antibody followed by immunoprecipitation with protein A/G sepharose beads. To assay activities of different phosphatases these beads were used. Jurkat cells were stimulated with araC for different times and cell extract was prepared. Cells extracts were incubated with different anti-phosphatase antibodies followed by immunoprecipitation by protein A/G sepharose. Then these beads were incubated with ^{32}P-labeled p65 in 20mm HEPES, pH 7.4, 10mm MgCl2, and 1 mm DTT for 2 hours at 37°C. Reactions were stopped with the addition of 15 µl of 2X SDS sample buffer, boiled for 5 min, and subjected to SDS–PAGE (9%). The gel was dried and ^{32}P-p65 was analysed by a Phosphorimager (Fuji, Japan).

2.3.25 Immunocytochemistry :

The level of p65 was examined by the immunocytochemical method as described with slight modifications. Briefly, 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 anti-p65 Ab 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.

2.3.26 Live and dead assay :

The cytotoxic effects were also determined by the Live/Dead assay (Molecular Probes, Eugene, OR). Briefly, after different treatment 1 x 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).

2.3.27 Ultra Competent Cells Preparation :

All the salts (10 mM PIPES, 15 mM $CaCl_2.2H_2O$, 250 mM KCl, 55 mM $MnCl_2 . 2H_2O$) except $MnCl_2$ were dissolved in milliQ water and pH was adjusted to 6.7 with 1N KOH. $MnCl_2$ was dissolved separately in mill Q water. $MnCl_2$ was added drop wise while stirring ($MnCl_2$ if added directly will give a brown color to the solution and precipitate out, hence it needs to be dissolved separately). Solution was then filter sterilized and stored. To prepare competent cells pre-inoculum was prepared. A single bacterial colony was picked from LB agar plate that has been incubated for 16-20 hours at 37°C and inoculated into 3 ml LB medium and incubated overnight at 37°C temperature with 200 rpm shaking. 1% of this pre-inoculum was subcultured in 100 ml LB-broth and incubated at 18°C until OD_{600} reached 0.5 - 0.6 (approx.). Culture was kept on ice for 10 min. with constant shaking. Cells were pelleted by centrifugation at 2000xg/4°C/8 min. Pellet was resuspended in 40 ml of ice-cold TB buffer. Bacterial suspension was kept on ice for 30 min, re-spun at 200xg/4°C /8 min. Pellet was resuspended in 8 ml of TB buffer in which final concentration of DMSO was 7% and left on ice for 10 min.100 µl aliquots were made and snap freezed in liquid nitrogen and stored at -80°C.

2.3.28 Preparation of Plasmid DNA by alkaline lysis :

Grown culture was pelleted by centrifugation at 10000xg/4°C/3 min supernatant was discarded. Pellet was resuspended in 250 µl of ice-cold alkaline lysis solution - 1. 300 µl of alkaline solution - 2 was then added and the tuber was inverted very gently 3-4 times and incubated at room temperature for 5 min. 350 µl solution - 3 was added and mixed by inverting the tube 3 or 5 times, gently. Suspension was incubated on ice for 10 min. Bacterial lysate was spun at 10000xg/12 min/4°C. Supernatant was transferred to a fresh tube. 0.4 volume of phenol:chloroform was added to the supernatant. Inverting the tubes several times mixed contents. It was then spun at 10000xg/12 min/4°C. Aqueous phase was taken out in a fresh tube and 0.6 volume of isopropanol was added, mixed properly and incubated at room temperature for half an hour and spun at 10000xg/RT/20 min. Supernatant was discarded. Pellet was washed with 70% ethanol. The tube was stored at room temperature until the

ethanol has dried. The pellet was resuspended in 20 µl of milliQ water and 20 µg/ml RNase added. The tube was incubated at 50°C for 45 min. the tube was vortexed for few seconds. Concentrations of the vectors were then estimated by running 1% agarose gel.

2.3.29 Nuclear fragmentation assay using propidium iodide(PI) :

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.

2.3.30 Preparation of GST-cJun.

2.3.30.1 PCR cloning:

PCR amplification of the N-terminal domain encoding the phosphorylation region of c-Jun from peripheral blood

Primer	**Sequence**
1) c-JunBam+	5'-ATC GG**G GAT CC**G ATG ACT GCA AAG ATG GAA-3'
2) c-JunXho-	5'-CTA GGG **CTC GAG** TCA GGG GCA CAG GAA CTG GGT-3'

Reaction Mixture (25 µl):-

Template	1.0 µl
Primer 1	0.5 µl
Primer 2	0.5 µl
dNTPs(2.5mM)	1.0 µl
10X buffer	2.5 µl
Enzyme Deepvent	0.5 µl
Milli Q	19.0 µl
	25µl

PCR parameters:

1) Pre-denaturation	94°C 2min
2) Denaturation	94°C 30secs
3) Primer annealing	54°C 1min
4) Elongation	72°C 45secs
5) Final elongation	72°C 7min

The PCR amplified product was purified through agarose gel. (Shown in fig.). c-Jun was ligated to pET41b (linearised prior by Bam and Xho) vector, using T4 DNA ligase at 16°C for 12 hours. The constructs so obtained were then transformed into DH5α competent cells and the positive colonies were selected.

2.3.30.2 Protein extraction and purification using Glutathione Sepharose 4B :

A primary inoculum of 3 ml of LB broth containing 30 μg/ml kanamycin was prepared by inoculating it with one colony of E. coli BL21 (DE3) harboring pET41b construct and grown O/N at 37°C. 100 ml of LB broth with kanamycin was inoculated with overnight grown pre-inoculum and incubated at 37°C with vigorous shaking until OD reached 0.5. At this stage it was induded with 1mM IPTG and grown further for 4 hours at 37°C.The culture was spun at 1000 rpm for 15min at 4°C. Supernatant was discarded and the pellet resuspended in lysis buffer (pH 8) (50 mM NaH_2PO_4 and 300 mM NaCl) and 1 mM PMSF added and was sonicated for 10 min at 20% power output and 30 sec on -off cycle. After sonication, lysate was spun at 500xg/30 min/4°C.

The overexpressed protein was purified using Glutathione Sepharose 4B matrix column. The column was packed with Glutathione Sepharose 4B along with 500 μl of 10%Triton x 100 and the supernatant collected after the lysis, sonication and spinning of 100 ml culture (induced with IPTG.incubated at RT with gentle agitation for 40 min. Spun at 700 rpm for 5min. Pellet was washed with 10 bed volumes of PBS. After repeated wash at100xg to the sedimented matrix, 1.0 ml of elution buffer per ml bed volume of GSH Sepharose 4B was added. Resuspended gently, incubated at room temperature for 10 minutes, and the elutes were collected and saved for SDS-PAGE analysis.

2.3.31 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, 2 μg/ml leupeptin , 2 μg/ml aprotinin, 1 mM PMSF, 0.5 μg/ml benzamidine and 1 mM DTT . Cell extracts (250 μg/sample) were immunoprecipitated with 0.3μg of anti-JNK antibody or anti ERK 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-c-Jun or matrix protein (p17) as substrates in 20 mM HEPES,(pH7.4) , 10 mM $MgCl_2$, 1 mM DTT, and 10 μCi of [γ^{32} 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-Jun or matrix protein (p17) was visualized by staining with coomassie blue and the dried gel was analysed by a Phosphor Imager.

2.3.32 Annexin V-PE staining :

The cells after various treatments were washed with cold PBS and then resuspended in 1X binding buffer (Annexin V-PE apoptosis detection kit I, BD Biosciences) at a concentration of 1 x 10^6 cells per ml. Then 100 μl of the cell suspension was transferred to a 5 ml culture tube and to it 5 μl of annexin V-PE was added. The cells were gently vortexed and incubated for 15 minutes at room temperature in the dark. Then 400 μl of 1X binding buffer was added to the tube and analyzed by flow cytometry with one hour.

Chapter Three

Oleandrin mediates inhibition of ceramide induced NF-κB activation but potentiats apoptosis

3.1 Introduction.

Research over the last few years has shown that different plant products exhibit chemopreventive effect (Wattenberg, 1990). As many as 70% of the therapeutic drugs used today are derived from plants. Oleandrin is polyphenolic cardiac glycoside derived from the leaves of *Nerium oleander*. It has been used to treat congestive heart failure and is known to be toxic to a wide variety of tumor cells (Gupta *et al*, 1985; Gupta *et al*, 1986; Gupta *et al*, 1987). Two of the active components of *Nerium* are the cardiac glycosides oleandrin and oleandrigenin. Previous *in vitro* studies using two human prostate cancer cell lines, DU145 and PC3, have demonstrated that cardiac glycosides may inhibit fibroblast growth factor-2 (FGF-2) export through membrane interaction with the Na^+, K^+-ATPase pump (Smith *et al.,* 1999). The mechanism of the cytotoxic effects of oleandrin, however, is not understood. Because NF-κB and AP-1 are known to play major roles in cell proliferation, tumor promotion, and drug resistance (Wang *et al.*, 1999; Waddick and Uckun, 1999; Karin *et al.*, 1997; Hannun, 1996), the possibility that the cytotoxic effects of oleandrin are mediated through suppression of NF-κB and AP-1 was looked into. Therefore, in this report the effect of oleandrin on NF-κB and AP-1 activation induced by ceramide was investigated.

Given the fact that the carcinogenic, inflammatory, and growth-modulatory effects of many chemicals are mediated by NF-κB, it is hypothesized that the suppression of NF-κB activation pathway accounts for oleandrin's activities. Numerous lines of evidence suggest this possibility. For example, various agents that promote tumorigenesis are known to activate NF-κB (Baeuerie and Baichwal, 1997), including phorbol ester, okadaic acid, and TNF where ceramide is produced as an intermediate molecule. Most agents that activate NF-κB also activate AP-1 (Karin *et al.*, 1997). That AP-1 activation mediates tumorigenesis and invasiveness has also been described (Smith *et al*., 1999). The activation of NF-κB and AP-1 is regulated by several protein kinases that belong to the mitogen-activated protein kinase (MAPK) family (Karin *et al.*, 1998). The activation of NF-κB and AP-1 and its associated kinases is in most cases dependent on the production of reactive oxygen species (Manna *et al.*, 1998; Manna *et al.*, 1999; Li and Karin., 1999; Kumar *et al.*, 1999).

In this study, the hypothesis that the anti-inflammatory and anticarcinogenic effects of oleandrin are mediated through its modulation of NF-κB and AP-1 activation, and caspase-mediated apoptosis was tested. It was demonstrated that oleandrin is a potent inhibitor of NF-κB and AP-1 activation in a variety of cell lines.

3.2 Results

In this study, the effect of oleandrin on ceramide-induced signal transduction was examined. The chemical structure of oleandrin is shown elsewhere (Manna *et al.*, 2000 a). It is highly soluble in DMSO. For most of the studies, HeLa cells were used because these cells are well characterized in our laboratory. At the concentration of oleandrin and duration of exposure employed in these studies, there was no effect on cell viability (95.52 ± 2.88, 93.26 ± 5.82, and 92.24 ± 4.76 percentage of cell viability was observed at 0.2, 0.5, and 1.0 μg/ml oleandrin respectively for 6 h of incubation).

3.2.1 Inhibition of ceramide-induced NF-κB activation by oleandrin

HeLa cells were plated in 60 mm petridish and pretreated at 60% confluent state with different concentrations of oleandrin for 4 h and then stimulated with 10 μM ceramide for 1 h. Nuclear extracts were prepared and assayed for NF-κB by EMSA. As shown in Fig.3.1.1, ceramide induced 10-fold activation of NF-κB, and oleandrin inhibited this activation in a dose-dependent manner; full inhibition occurred at 0.2 μg/ml and at this concentration itself did not activate NF-κB.

To detect the composition and specificity of the retarded band visualized by EMSA, nuclear extracts from ceramide-activated 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 m.w. (Fig.3.1.2), thus suggesting that the ceramide-activated 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 cold NF-κB indicating the specificity of NF-κB.

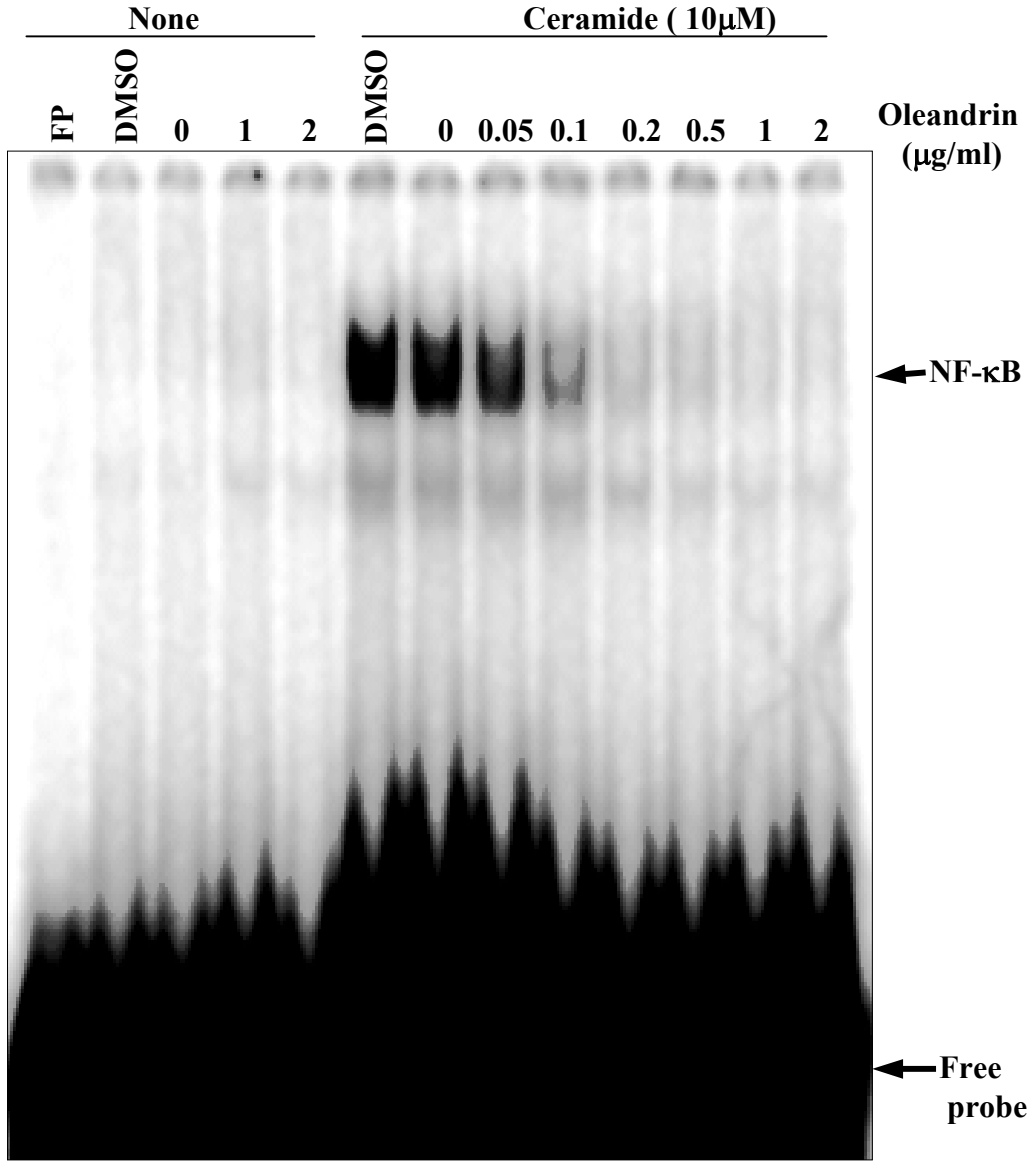


Fig 3.1 Effect of oleandrin for the inhibition of ceramide-dependent NF-κB activation. HeLa cells were cultured in 60 mm petridish at 37°C CO_2 incubator. At 60% confluency cells were preincubated for 4 h with different concentrations (0-2 μg/ml) of oleandrin, followed by 1 h incubation with 10 μM ceramide. After these treatments, nuclear extracts were prepared and then assayed for NF-κB, as described in Materials and Methods.

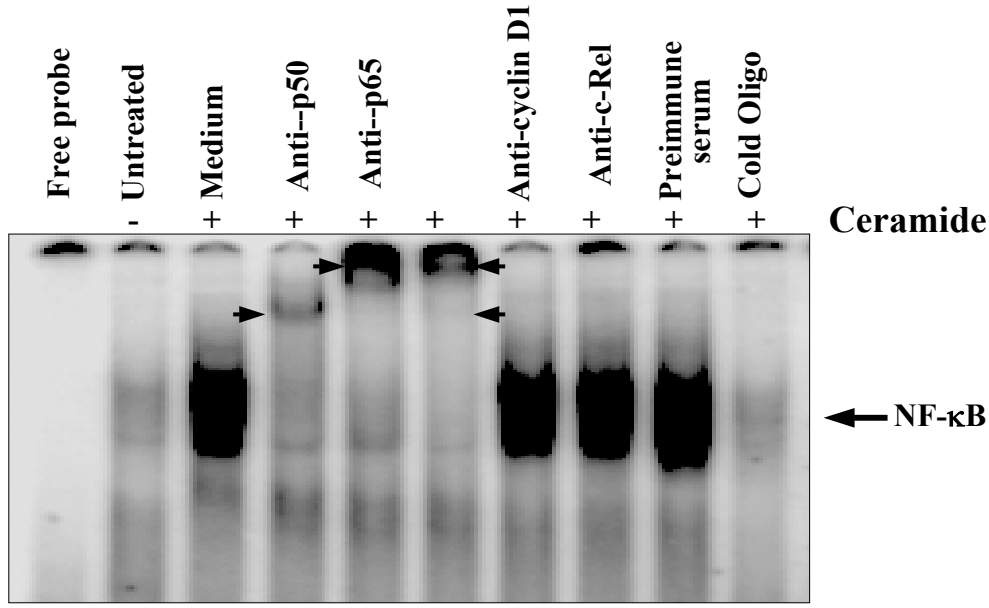


Fig 3.1.2 Supershift of NF-κB band. Nuclear extracts were prepared from untreated or ceramide-treated HeLa cells, incubated for 15 min with different Abs and cold NF-κB oligo, and then assayed for NF-κB, as described in Materials and Methods.

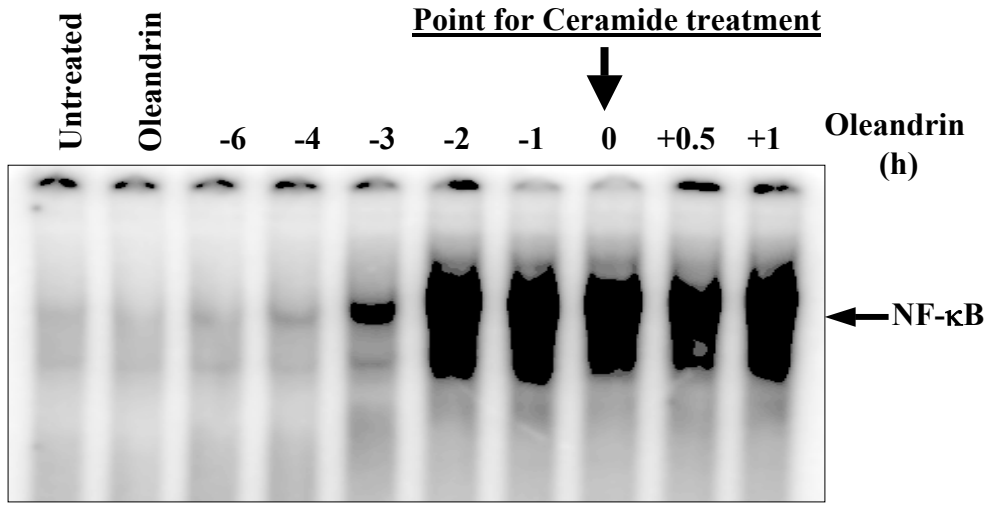


Fig 3.1.3 Oleandrin mediated NF-κB inhibition is time dependent. Cells were preincubated at 37°C with 0.2 μg/ml oleandrin for the indicated times and then stimulated with or without 10 μM ceramide at 37°C for 1 h. After these treatments, nuclear extracts were prepared and then assayed for NF-κB.

The effect of changes in the length of incubation with oleandrin on NF-κB activation by Ceramide was examined. Cells were incubated with 0.2 μg/mL oleandrin for different times and then stimulated with 10 μM ceramide for 1 h and assayed for NF-κB. The results in Fig.3.1.3 show that oleandrin inhibited ceramide-induced NF-κB activation with increased time of incubation. At 4 h, complete inhibition was observed.

To determine the effect of oleandrin on NF-κB activation at even higher concentrations of ceramide, both untreated and oleandrin-pretreated cells were incubated with various concentrations of ceramide (0-25 μM) for 1 h and then assayed for NF-κB by EMSA. Although the activation of NF-κB by 25 μM ceramide was strong (Fig.3.1.4), oleandrin completely inhibited it as efficiently as it did at higher concentration of ceramide. These results show that oleandrin is a very potent inhibitor of NF-κB activation.

3.2.2 Blocking of ceramide-induced NF-κB-dependent reporter gene expression by oleandrin

It was shown earlier that oleandrin blocks ceramide-induced NF-κB activation. To detect the role of oleandrin on NF-κB dependent reporter gene expression, HeLa cells were transiently co-transfected with NF-κB-containing plasmid linked to the *SEAP* and *β-galactosidase* genes with or without dominant negative IκBα plasmids. Cells either pretreated or treated with different concentrations of oleandrin for 4 h and then stimulated with 10 μM ceramide for 12 h. The SEAP activity was assayed in culture supernatant and the pellet was taken for β-galactosidase activity assay. Results are expressed as fold activity over the nontransfected control. The results showed in Figure 3.2.1 & 3.2.2 that oleandrin decreased ceramide-induced SEAP activity in a dose dependent manner and IκBα-DN transfected cells showed no induction of SEAP activity by ceramide suggesting oleandrin's inhibitory action of active NF-κB-dependent gene transcription. The β-galactosidase activity from cell extracts showed almost similar reduction of absorbance (as per Promega protocol) at 420 nm (data not shown) suggesting the transfection control for each treatment.

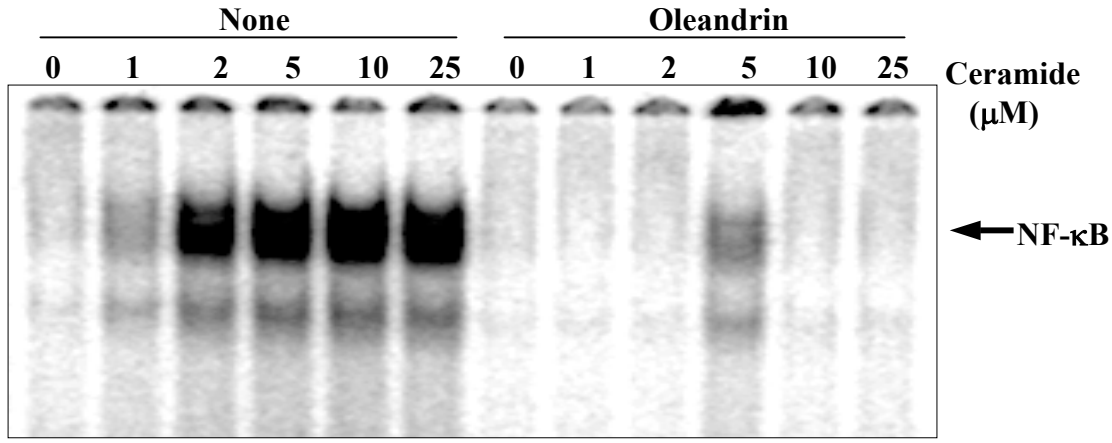


Fig 3.1.4 Inhibition of ceramide induced NF-κB activation by oleandrin. Cells were preincubated at 37°C with 0.2 μg/ml oleandrin for 4 h and then treated for 1 h with different concentrations of ceramide at 37°C and tested for NF-κB activation.

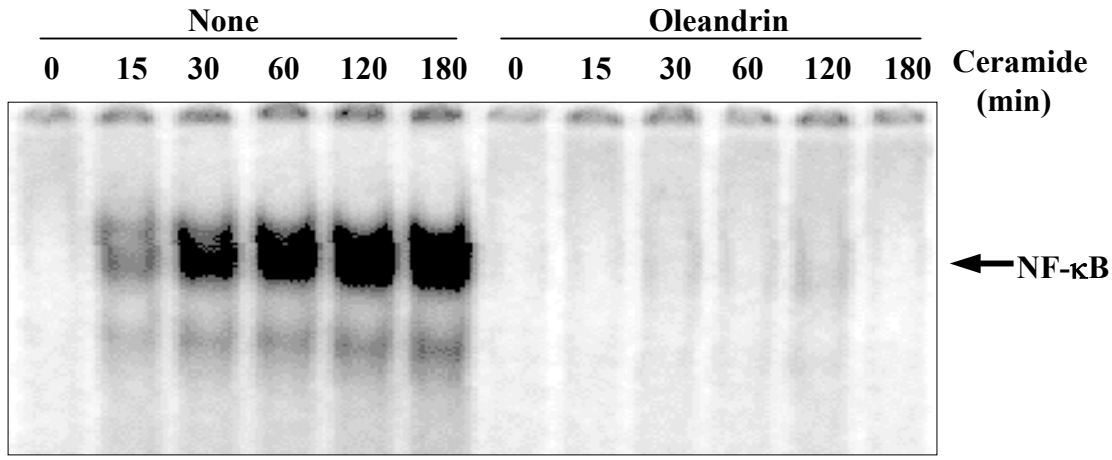


Fig 3.1.5 Effect of oleandrin for the inhibition of ceramide-dependent NF-κB activation. Cells were incubated at 37°C with 0.2 μg/ml oleandrin for 4 h and then stimulated with 10 μM ceramide at 37°C for different times, as indicated, and then tested for NF-κB activation by EMSA from nuclear extracts

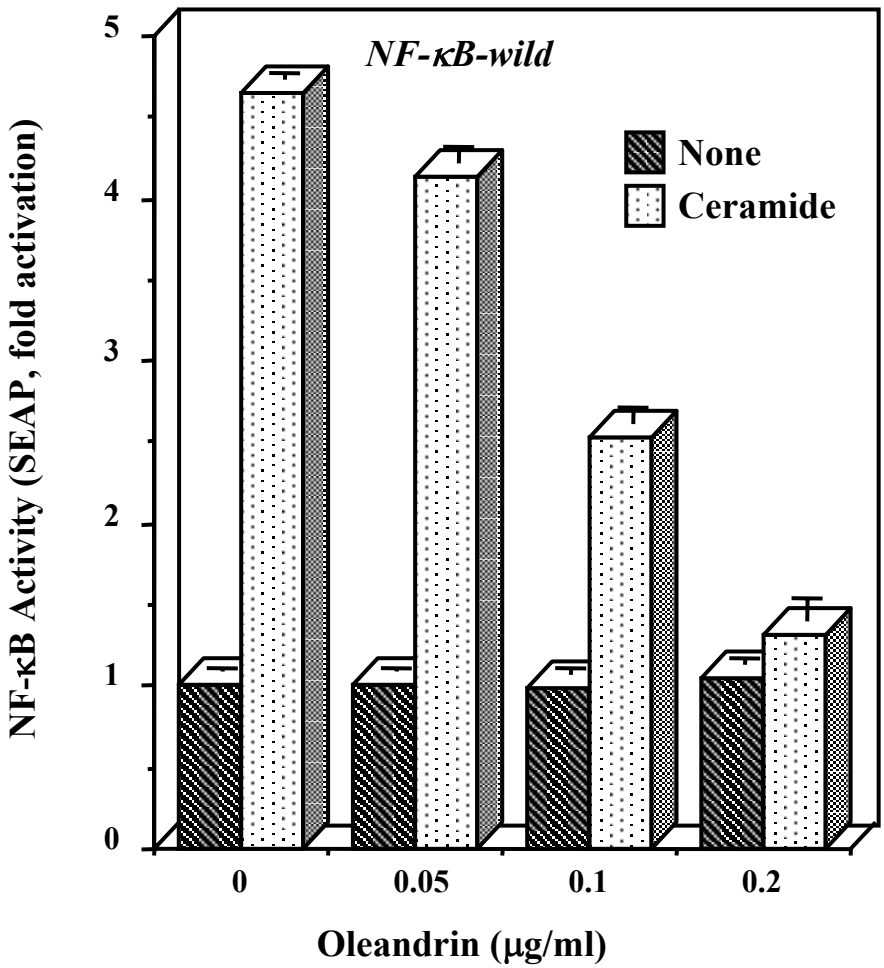


Fig 3.2.1 Oleandrin inhibits ceramide induced NF-κB activity. HeLa cells, either pretreated with different concentrations of oleandrin for 4 h were transiently transfected with NF-κB-containing plasmid linked to the SEAP gene. Cells were then stimulated with 10 μM ceramide for 12 h. Culture supernatants were assayed for secreted alkaline phosphatase activity as described in Materials and Methods. Results are expressed as fold activity over the vector transfected control.

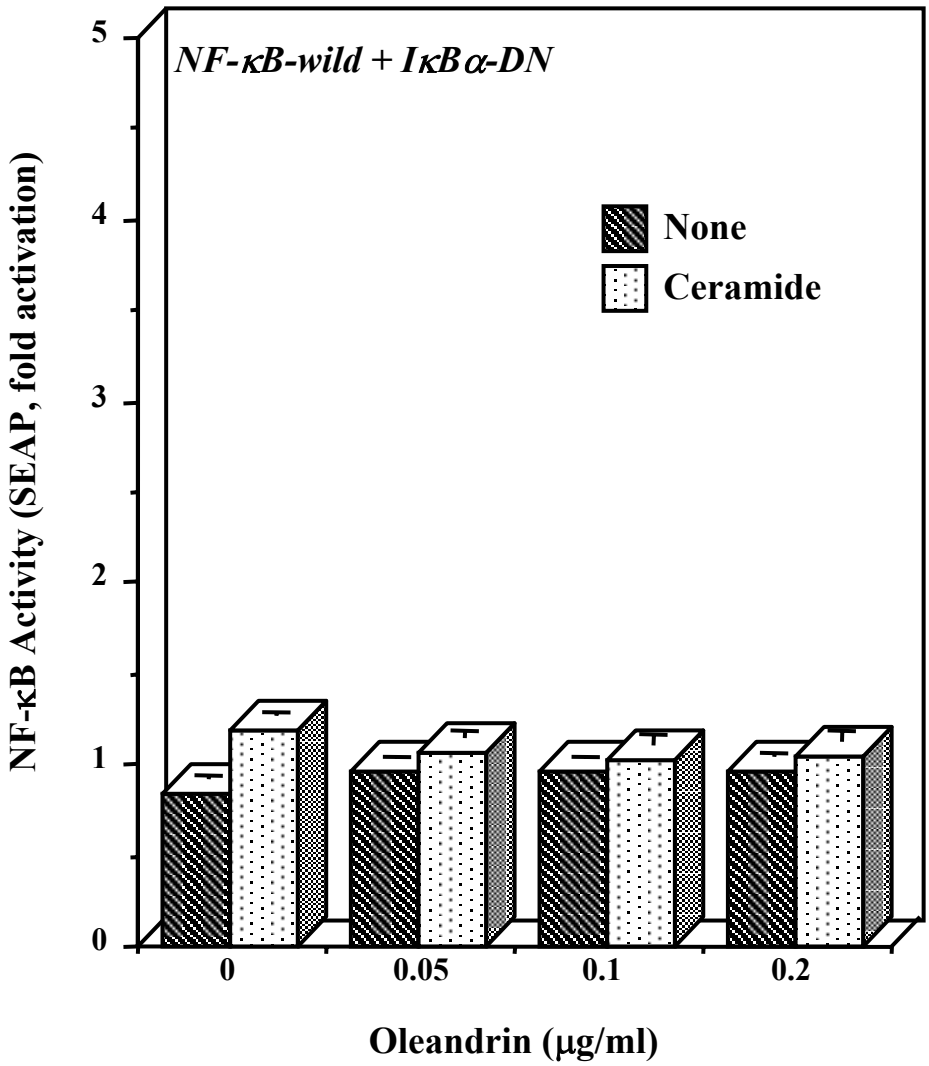


Fig 3.2.2 Oleandrin inhibits ceramide induced NF-κB activity. HeLa cells, either pretreated with different concentrations of oleandrin for 4 h were transiently transfected with NF-κB-containing plasmid linked to the SEAP gene with dominant negative IκBα plasmid. Cells were then stimulated with 10 μM ceramide for 12 h. Culture supernatants were assayed for secreted alkaline phosphatase activity as described in Materials and Methods. Results are expressed as fold activity over the vector transfected control.

3.2.3. Inhibition of ceramide-dependent IκBα degradation and nuclear translocation of p65 subunit of NF-κB by oleandrin

The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα. To determine whether the inhibitory action of oleandrin was due to its effect on IκBα degradation, the cytoplasmic levels of IκBα protein were examined by Western blot analysis and nuclear extracts were assayed for NF-κB. Both untreated and oleandrin-pretreated cells were incubated with ceramide (10 μM) for different times and then assayed for NF-κB. In untreated cells, ceramide activated NF-κB in a time-dependent manner (Fig.3.1.5). In oleandrin-pretreated cells, however, a little activation of NF-κB was detected after ceramide exposure of up to 60 min. Upon treatment with ceramide the level of IκBα decreased within 15 min and then reappeared slowly from 30 min, indicating degradation followed by re-synthesis of IκBα (Fig.3.3, first panel). Oleandrin pre-treated cells exhibited a sustained IκBα band at all time points indicating that oleandrin treatment leads to inhibition of ceramide-induced IκBα degradation. Whether oleandrin affects the ceramide-induced nuclear translocation of the p65 subunit of NF-κB was also examined by Western blot analysis. As shown in Fig. 3.3, second panel, upon ceramide treatment, p65 disappeared from the cytoplasm, and oleandrin prevented the disappearance. Oleandrin alone had no effect on p65 level. These results indicate that oleandrin block the nuclear translocation of NF-κB. Level of p50 remained the same in ceramide-induced cells at different times both in presence and in absence of oleandrin (Fig.3.3, third panel). All the lanes exhibited equivalent intensities of tubulin, assayed by Western blot as the loading control (Fig.3.3, lower panel).

3.2.4. Effect of oleandrin on DNA-binding ability of NF-κB proteins in vitro

It has been shown that N-tosyl-L-Phe-chloromethylketone, a serine protease inhibitor, and herbimycin A, a protein tyrosine kinase inhibitor, and caffeic acid phenylethyl ester down-regulate NF-κB activation by chemical modification of the NF-κB subunits, thus preventing its binding to DNA (Mahon *et al.*, 1995; Finco *et al.*, 1994; Natarajan *et al.*, 1996). To determine whether oleandrin also modifies the DNA binding of NF-κB, the cytoplasmic extracts was incubated with 0.8% deoxycholate (as DOC has shown to dissociate IκBα and release NF-κB) for 15 min at room temperature. DOC-treated cytoplasmic extracts were then

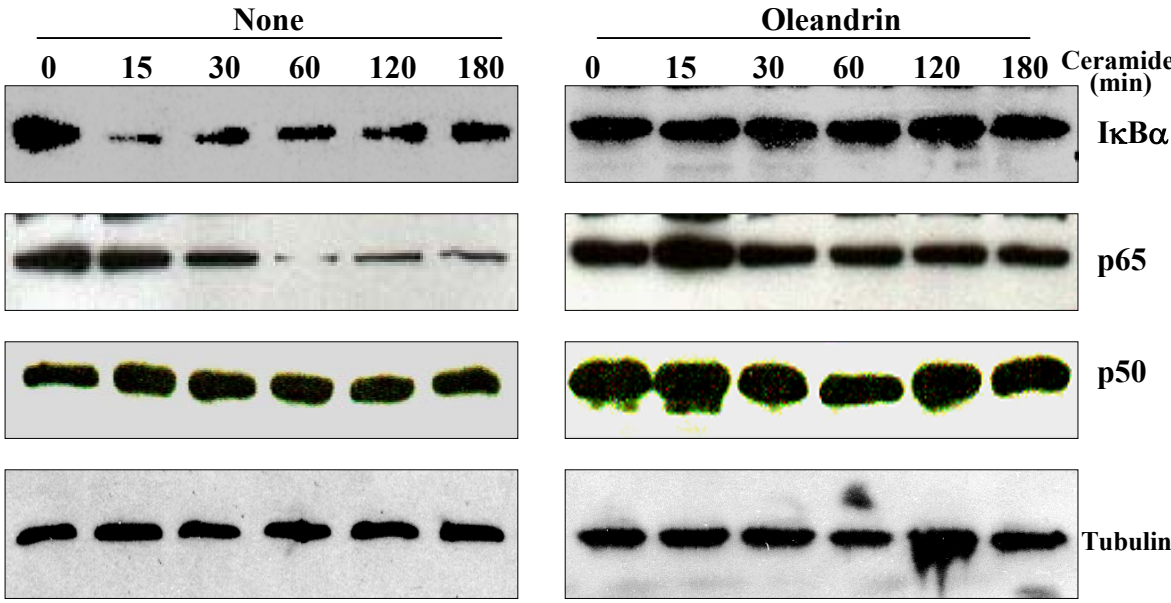


Fig 3.3 Effect of oleandrin for the inhibition of ceramide-dependent NF-κB Activation and IκBα degradation. Cells were incubated at 37°C with 0.2 μg/ml oleandrin for 4 h and then stimulated with 10 μM ceramide at 37°C for different times, as indicated, and then cytoplasmic extracts were assayed for IκBα, p65, p50, and tubulin by Western blot analysis.

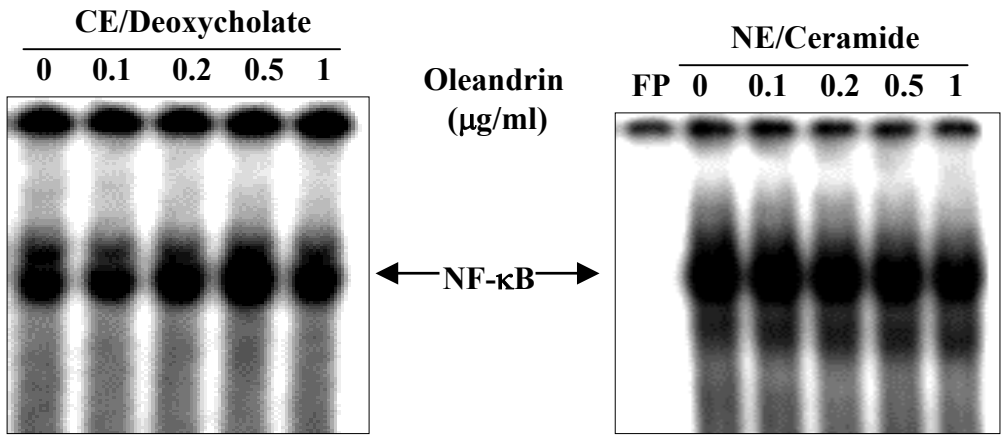


Fig 3.4 In vitro effect of oleandrin on DNA binding of NF-κB protein. Cytoplasmic extracts (**CE**) from untreated HeLa cells (10 μg protein/sample) were treated with 0.8% DOC for 15 min at room temperature, incubated with different concentrations of oleandrin for 4 h at room temperature, and then assayed for DNA binding by EMSA.**.** Nuclear extracts (**NE**) were prepared from 10 μM ceramide-treated HeLa cells; 6 μg/sample NE protein was treated with indicated concentrations of oleandrin for 4 h at room temperature and then assayed for NF-κB by EMSA.

exposed to various concentrations of oleandrin and assayed for DNA binding by EMSA. As shown in Fig.3.4, oleandrin had no effect on the binding of NF-κB to the DNA. Whether oleandrin modifies the nuclear fraction of NF-κB in ceramide-treated cells was also examined. The nuclear extracts from ceramide-induced cells were treated with various concentrations of oleandrin and then examined for DNA-binding activity by EMSA. The results in Fig.3.4 show that oleandrin did not modify the DNA-binding ability of NF-κB proteins prepared from TNF-treated cells either. Therefore, oleandrin inhibits NF-κB activation through a mechanism different from that of TPCK, herbimycin A, and CAPE.

3.2.5. Inhibition of NF-κB activation by oleandrin is not cell type specific

As NF-κB activation pathways differ in different cell types (Bonizzi *et al.*, 1997), it was therefore studied whether oleandrin affects other cell type as well. It has been demonstrated that distinct signal transduction pathways could mediate induction in epithelial and lymphoid cells. All the effects of oleandrin described above were conducted with HeLa cells, an epithelial cell line. It was found that oleandrin blocks ceramide-induced NF-κB activation in human T-cells, B cells, breast cancer cells, and monkey kidney cells (Fig.3.5). An almost complete inhibition of NF-κB in all the cell types suggests that this effect of oleandrin is not restricted to specific cells.

3.2.6. Inhibition of ceramide-induced AP-1 activation by oleandrin

The activation of JNK causes the activation of AP-1. Ceramide is also a potent activator of AP-1 (Manna *et al.*, 2000 b). Ceramide induced AP-1 expression was completely inhibited by oleandrin in a dose-dependent manner and complete suppression occurred at 0.5 μg/ml concentration in HeLa cells (Fig.3.6). Oleandrin also suppressed ceramide-induced AP-1 activation in Jurkat and Daudi cells (Fig.3.6). Disappearance of the AP-1 bands by competition with unlabeled nucleotides showed the specificity of the assay procedure employed in this experiment.

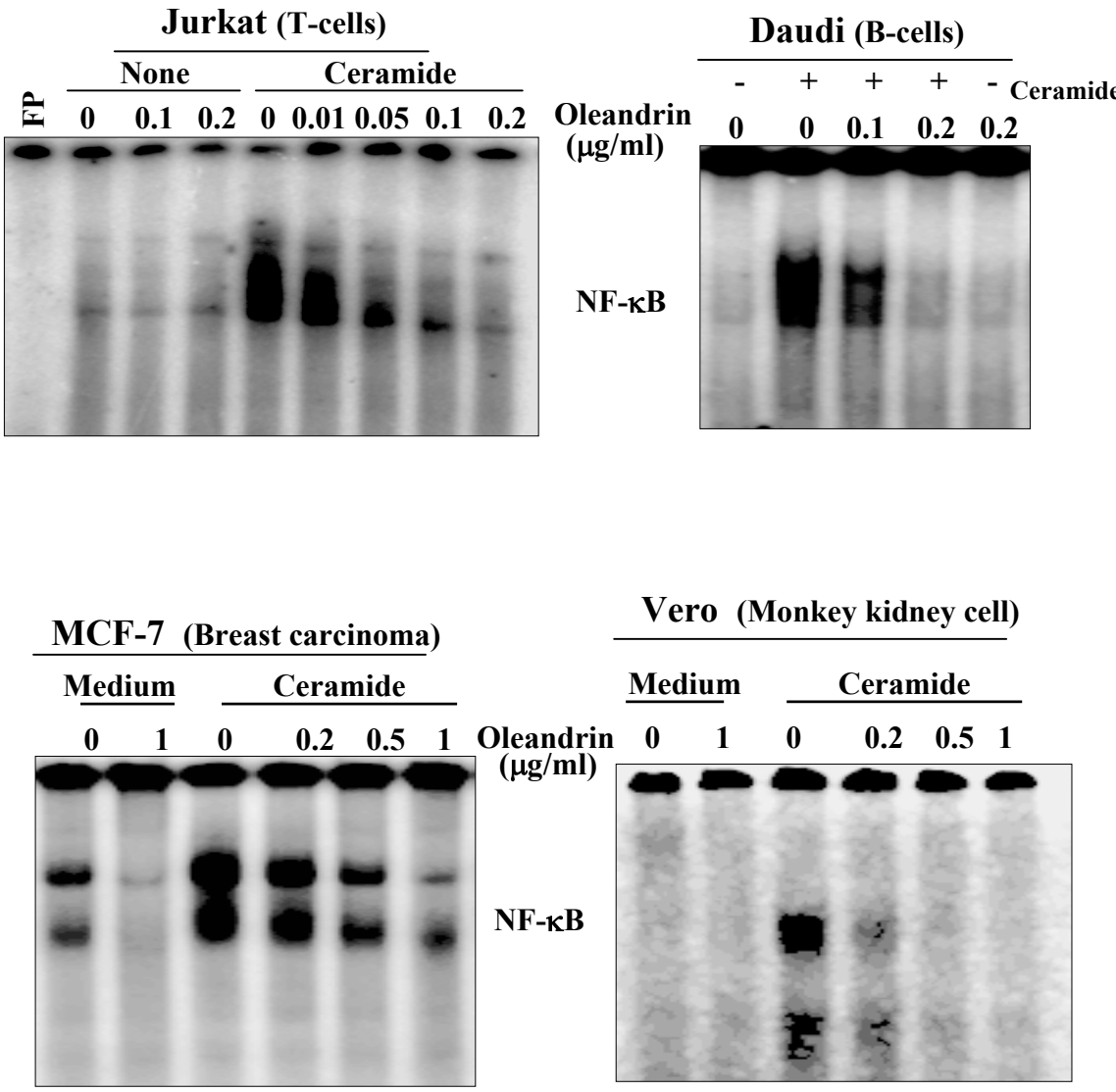


Fig 3.5 Effect of oleandrin on activation of NF-κB induced by ceramide in different cell lines. Human Jurkat, Daudi, and MCF-7 cells, murine macrophage, and monkey kidney cells were incubated at 37°C with 0.2 μg/ml oleandrin for 4 h and then treated at 37°C for 1 h with 10 μM ceramide. After these treatments, nuclear extracts were prepared and then assayed for NF-κB.

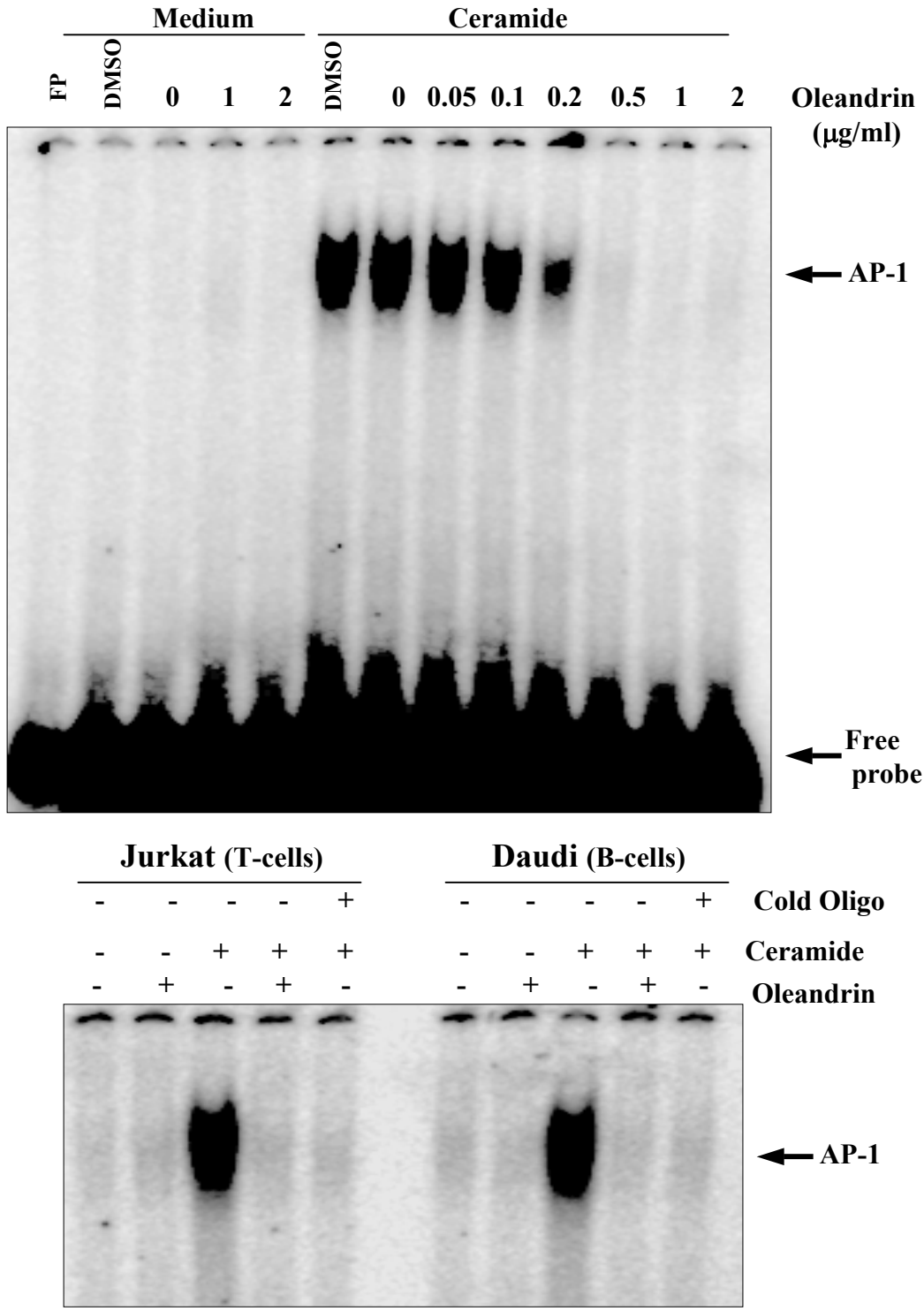


Fig 3.6 Oleandrin inhibits ceramide-dependent AP-1 activation. HeLa cells were pretreated with the indicated concentrations of oleandrin for 4 h at 37°C. Then cells were stimulated with 10 μM ceramide for 1 h and assayed for AP-1, as described in Materials and Methods. B. Jurkat and Daudi cells were preincubated at 37°C with 0.2 μg/ml oleandrin for 4 h, and then stimulated with 10 μM ceramide for 1 h and tested for AP-1 activation with excess 100 fold cold AP-1 oligo in ceramide stimulated nuclear extract.

3.2.7. Potentiation of ceramide-induced reactive intermediates generation, lipid peroxidation, and cytotoxicity by oleandrin

Whether oleandrin mediates its effects through suppression of reactive intermediates (reactive oxygen and nitrogen intermediates) production has been examined by flow cytometry. As shown in Fig.3.7, ceramide treatment led to induction of reactive intermediates generation in a concentration-dependent manner and oleandrin pre-treated cells showed potentiation of reactive intermediates generation. Oleandrin treatment alone at 0.2 μg/ml concentration generated 65% more reactive intermediates than that observed in resting cells. As oleandrin potentiated ceramide-induced reactive intermediates generation, the effect of oleandrin on ceramide-induced lipid peroxidation in term of TBA-reactive substances production was also examined. Results in figure 3.8 showed that ceramide induced lipid peroxidation in HeLa cells was in a concentration-dependent manner, and it was potentiated by oleandrin. Oleandrin alone induced 230% lipid peroxidation above unstimulated cells at 0.2 μg/ml concentration (Fig.3.8).

Among all the inducers, ceramide is one of the most potent inducers of apoptosis (Sen and Baltimore, 1986; Manna *et al.*, 2000 b). Whether oleandrin modulates ceramide-induced apoptosis was also investigated. The viable cell number was reflected on cell proliferation and oxidative burst response. The proliferating cell number was assayed by ^{3}H-Thymidine incorporation assay. As shown in Figure 3.10, ceramide-induced thymidine incorporation was decreased in a dose-dependent manner. Oleandrin potentiated ceramide-induced thymidine incorporation at any concentration of ceramide. The results indicate that ceramide-mediated cell killing is potentiated by oleandrin. HeLa cells were treated with variable concentrations of ceramide for 72 h either in the absence or presence of oleandrin (0.2 μg/ml) and then examined for cytotoxicity by the MTT method. Results in figure 3.9 show that the cytotoxic effects of ceramide in HeLa cells were dose dependent, with almost 45% killing occurred at 2 μM concentration of the ceramide. This cytotoxicity was potentiated by treatment of cells with 0.2 μg/ml oleandrin and this concentration of oleandrin alone induced 60% cell killing. To show that the cell death mediated by oleandrin was not due to necrosis, the cytosolic marker enzyme lactate dehydrogenase (LDH) was assayed from the culture supernatant of oleandrin-treated cells (Manna *et al.*, 1997). Culture supernatant from 0.5 μg/ml of oleandrin-treated for 0, 24, 36, and 48 h when incubated with substrate solution (0.23 M sodium

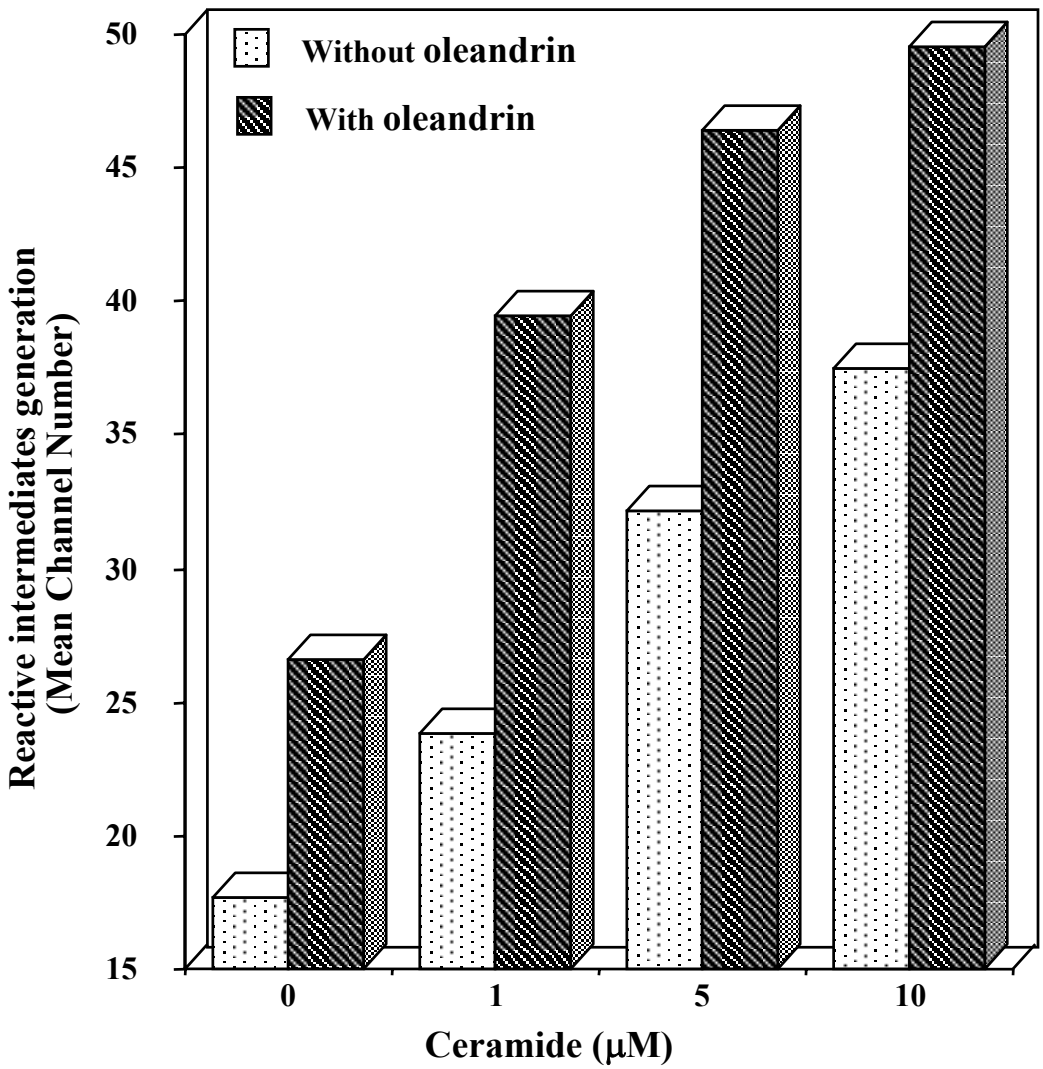


Fig 3.7 Effect of oleandrin on ceramide-induced reactive intermediates generation. HeLa cells were treated with 0.2 μg/ml oleandrin for 4 h and then exposed to different concentration of ceramide for 2 h in a CO_2 incubator. Reactive intermediates production was then determined by the flow cytometry method, as described in Materials and Methods. The results shown are representative of two independent experiments.

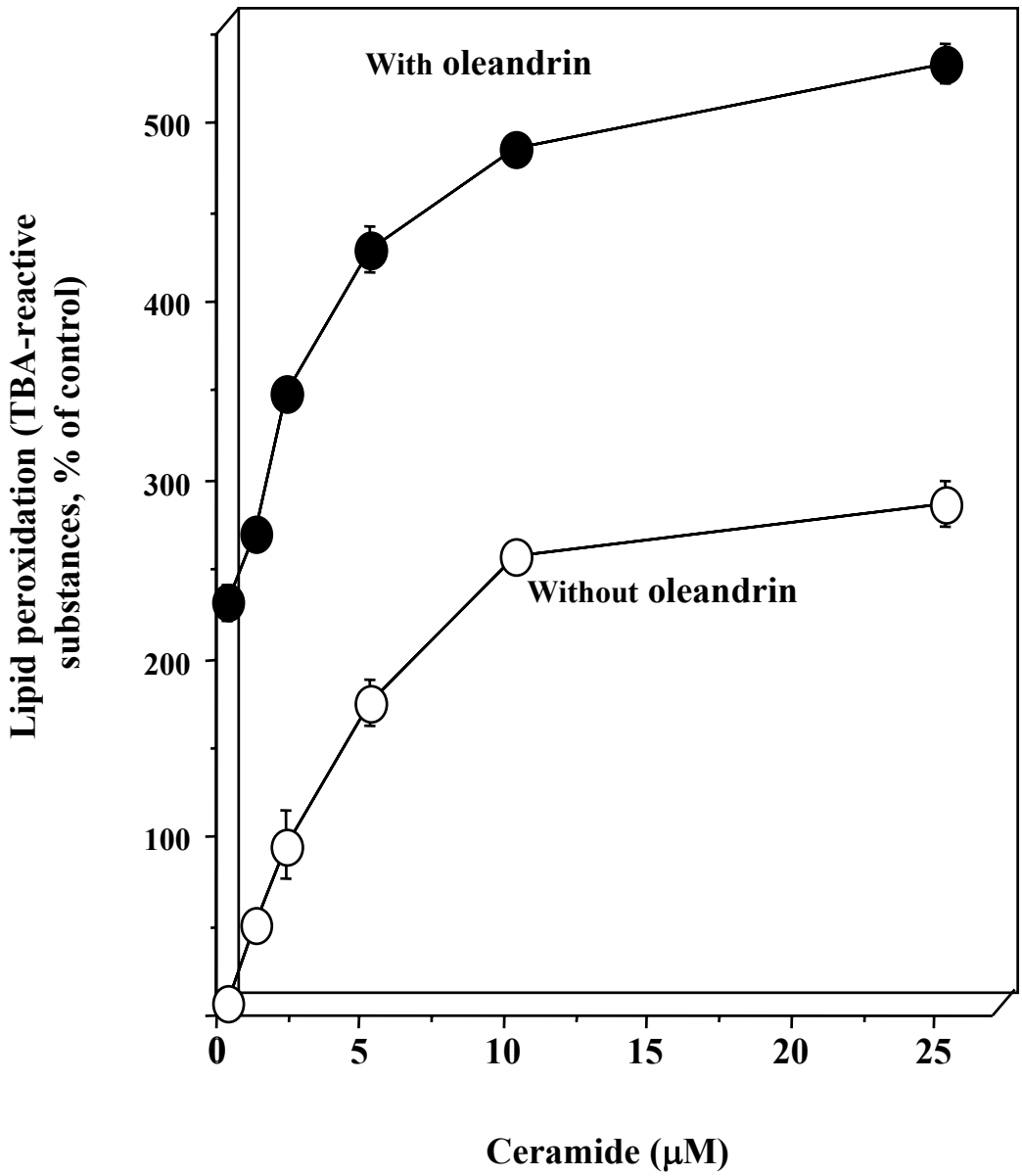


Fig 3.8 Effect of oleandrin on ceramide-induced lipid peroxidation. HeLa cells were treated with 0.2 μg/ml oleandrin for 4 h and then exposed to different concentration of ceramide for 6 h in a CO_2 incubator. Cell pellets were extracted by 3 times freeze-thaw method with addition of 200 μl of water and 180 μl 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.

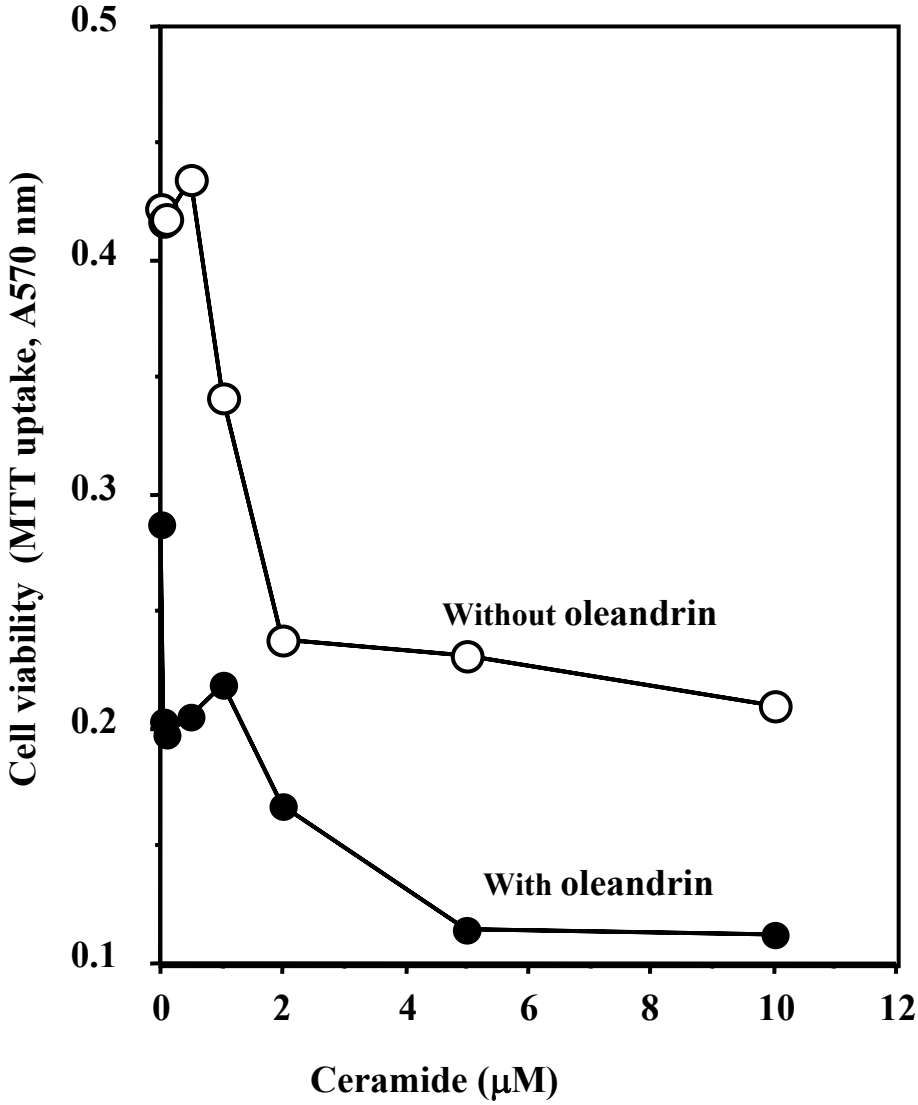


Fig 3.8 Effect of oleandrin on ceramide-induced cytotoxicity. HeLa cells, untreated or pretreated with 0.2 μg/mL oleandrin for 4 h at 37°C were incubated with the indicated concentrations of ceramide for 72 h at 37°C, in a CO_2 incubator. Then MTT assayed and absorbance was taken at 570 nm. The result indicated was mean OD of triplicate assays.

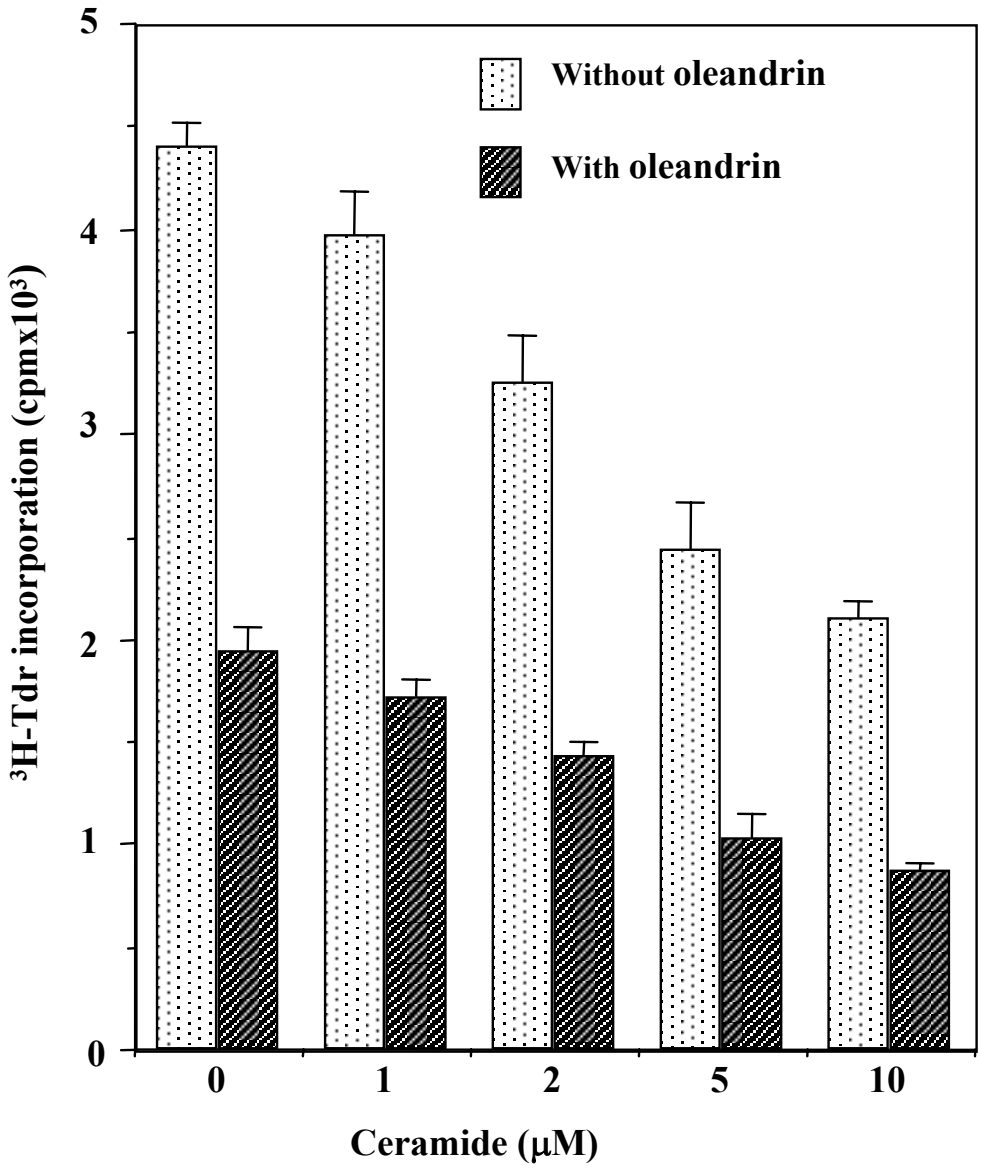


Fig 3.10 Effect of oleandrin and ceramide in ^{3}H-Thimidine incorporation. HeLa cells treated with 0.2 μg/ml oleandrin for 4 h and then stimulated with different concentrations of ceramide for 72 h at 37°C, in a CO_2 incubator. Last 18 h cells were incubated with 0.5 μCi (^{3}H-Thimidine) and then assayed for ^{3}H-Thimidine incorporation assay. Results were mean cpm ± SD of triplicate assays.

pyruvate and 5 mM NADH in 0.1 M phosphate buffer, pH 7.5) did not decrease absorbance at 420 nm significantly (data not shown) indicating cell death was not due to leakage of cytoplasm i.e necrosis.

As oleandrin induces ROI generation and also apoptosis, the role of ROI was detected in manganese superoxide dismutase (Mn-SOD) overexpressed MCF-7 cells. Both MCF-7 *neo* (control vector) and *Mn-SOD* cells were treated with different concentrations of oleandrin for 4 h followed by stimulation with ceramide (10 μM) for 72 h, subsequently cell viability was assayed by MTT uptake. Oleandrin potentiated ceramide-induced cell viability in MCF-7 (*neo)* cells (Fig.3.11.1). In MCF-7 (*Mn-SOD)* cells, Oleandrin induced cell death by 10-25% at 0.2-1 μg/ml concentrations (Fig.3.11.2). Ceramide partially induced cell death in MCF-7 (*Mn-SOD*) cells and about 0-10% potentiation of cell death was observed at varying concentrations of oleandrin pre-treated MCF-7 (*Mn-SOD*) cells. The data indicates that detoxification of ROI partially block oleandrin-mediated cell death.

3.2.8. Potentiation of ceramide-induced PARP cleavage and DNA fragmentation by oleandrin

Because the cytotoxic effects of ceramide and oleandrin or in combination, the effect of oleandrin on ceramide-induced caspase activation in the form of PARP protein cleavage was also examined. As shown in figure 3.12, ceramide induced partial cleavage of PARP at 10 μM, and oleandrin pre-treated cells showed potentiation of PARP cleavage even at 1 μM concentration of ceramide were, complete cleavage of PARP was observed. Oleandrin and ceramide each induced DNA fragmentation in HeLa cells, and in combination they potentiated DNA fragmentation (Fig.3.13).

DNA fragmentation was also detected in oleandrin and ceramide-induced cell's nuclei as detected by PI staining, and oleandrin enhanced ceramide induced DNA fragmentation (Fig.3.14).

3.2.9. Potentiation of cell death by oleandrin in NF-κB overexpressed cells

In order to detect the role of NF-κB on oleandrin-mediated cell death, HeLa cells were co-transfected either vector or p65 plasmids with β-galactosidase and SEAP reporter DNA. As shown in Figure 3.15, p65 transfected HeLa cells showed NF-κB activation and oleandrin

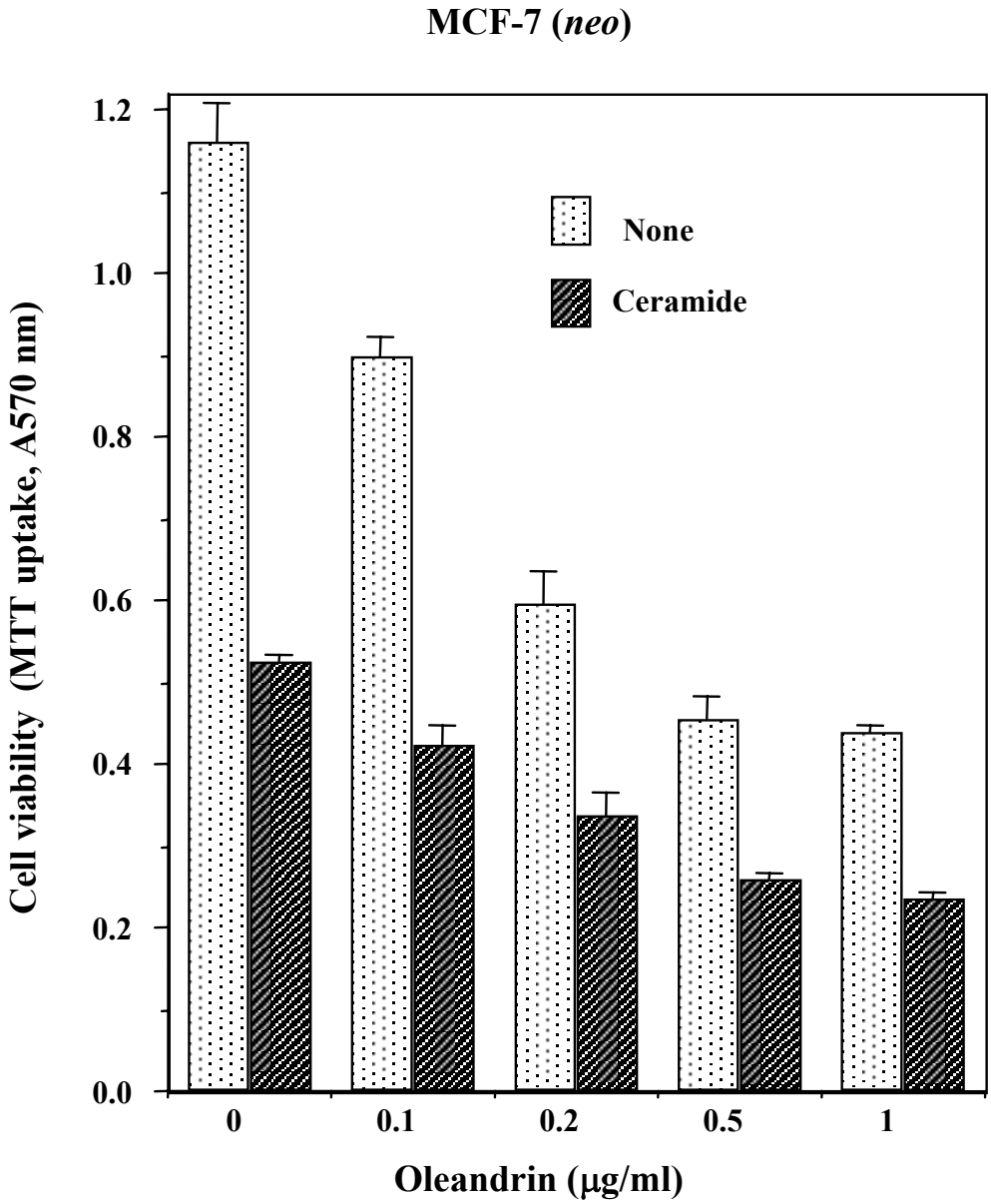


Fig 3.11.1 Effect of oleandrin on ceramide induced cytotoxicity. MCF-7 (neo) cells were treated with different concentrations of oleandrin for 4 h and then stimulated with ceramide (10 μM) for 72 h. Cell viability was assayed by MTT uptake. Results were mean absorbance of triplicate assays.

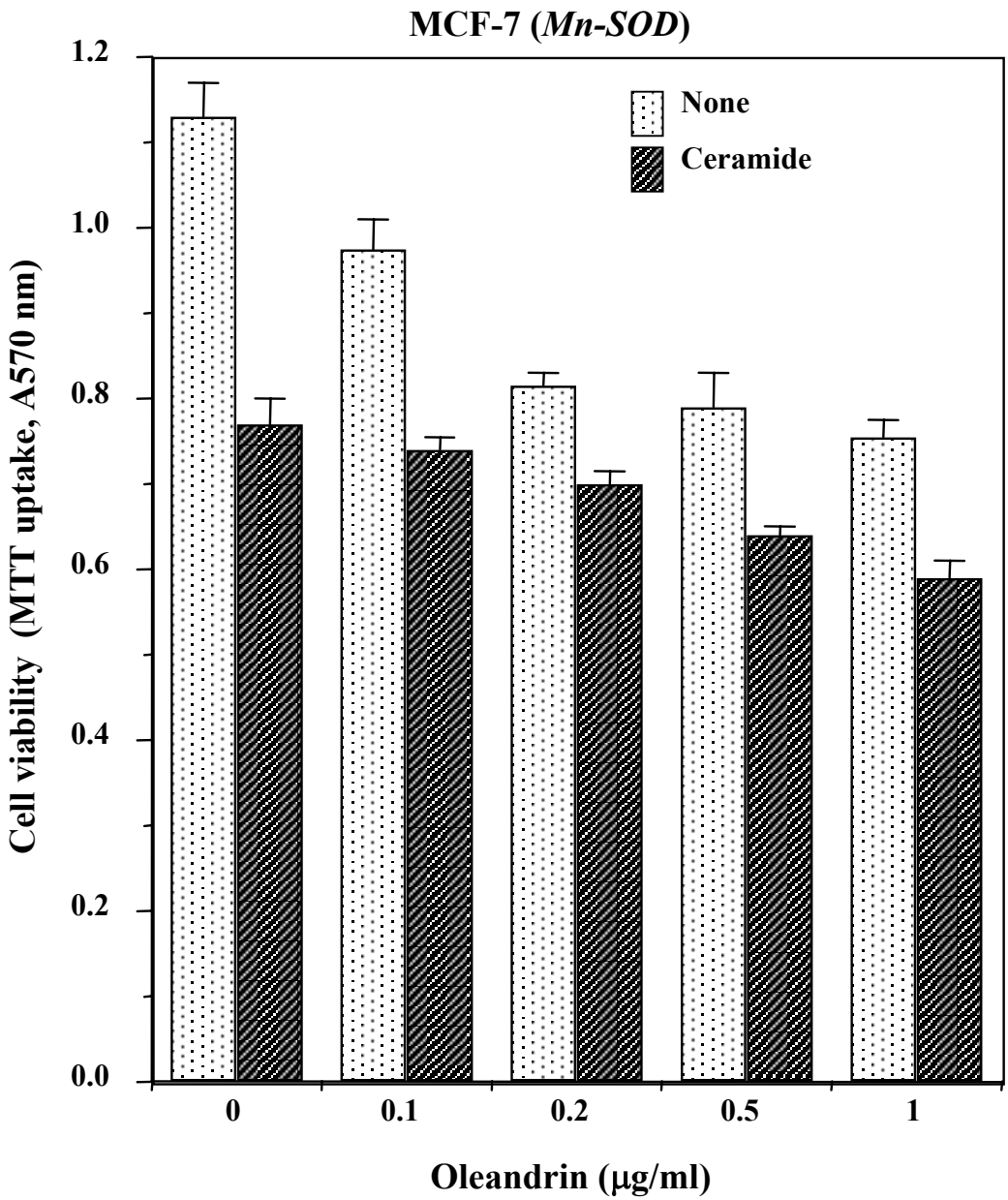


Fig 3.11.2 Effect of oleandrin on ceramide induced cytotoxicity. MCF-7 (Mn-SOD) cells were treated with different concentrations of oleandrin for 4 h and then stimulated with ceramide (10 μM) for 72 h. Cell viability was assayed by MTT uptake. Results were mean absorbance of triplicate assays.

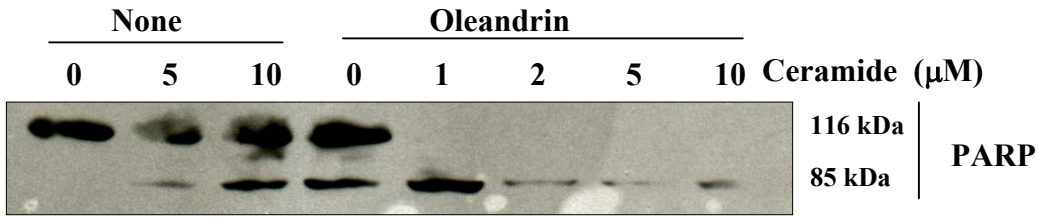


Fig 3.12 Effect of oleandrin on ceramide-induced PARP cleavage. HeLa cells, untreated or pretreated with 0.2 μg/ml oleandrin for 4 h at 37°C, were incubated with the indicated concentrations of ceramide for 24 h at 37°C, in a CO_2 incubator. Then cell extracts were prepared and 50 μg protein was analyzed by Western blot using anti-PARP mAb. The bands were located at 116 and 85 kDa.

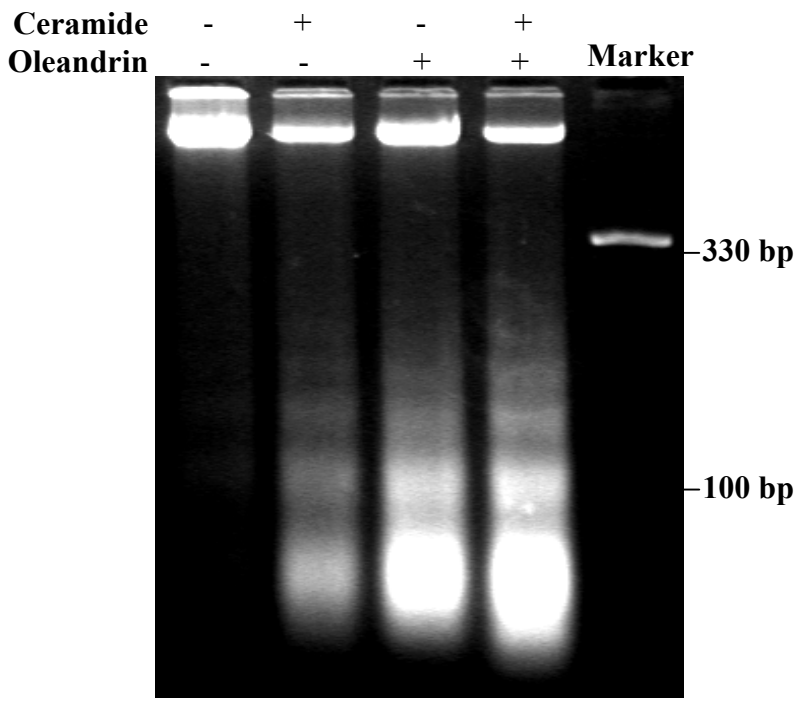


Fig 3.13 Effect of oleandrin on ceramide-induced DNA fragmentation. HeLa cells, untreated or pre-treated with 0.2 μg/mL oleandrin for 4 h at 37°C, were incubated with 10 μM ceramide for 24 h at 37°C, in a CO_2 incubator. Then cells were scrapped, washed, DNA was prepared, and 2.0 μg DNA was analyzed by 2% agarose gel.

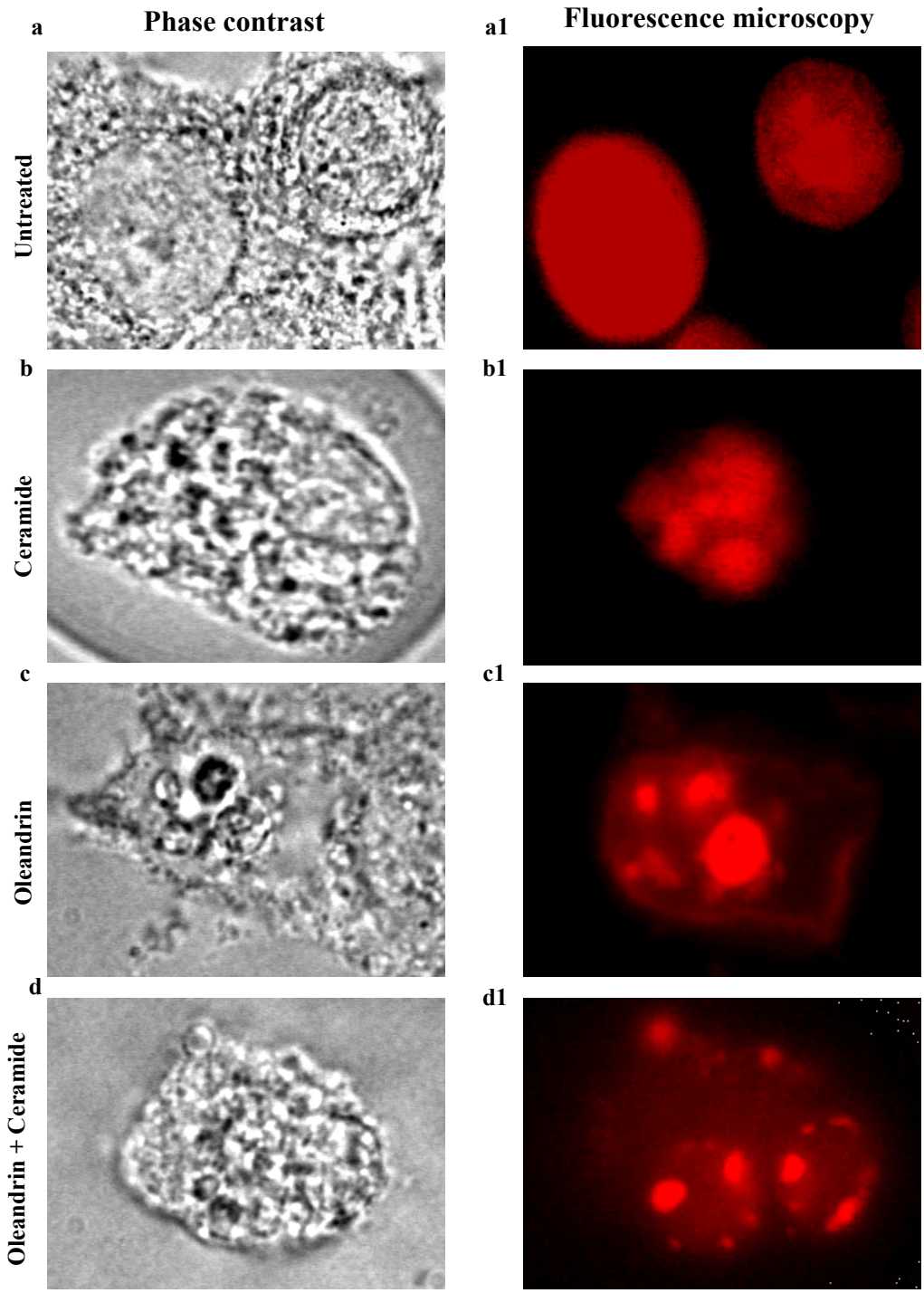


Fig 3.14 Effect of oleandrin on ceramide-induced nuclear fragmentation. HeLa cells, untreated or pretreated with 0.2 µg/ml oleandrin for 4 h at 37°C, were incubated with 10 µM ceramide for 24 h at 37°C, in a CO_2 incubator. Then cells were scrapped, washed, fixed with methanol and stained with propidium iodide (PI). Cells were then taken in slide and visualized in fluorescence microscope. The picture showed the phase contrast view of untreated (a), ceramide-treated (b), oleandrin-treated (c), and oleandrin pretreated followed by ceramide-treated (d) cells. Same cells were shown as untreated (a1), ceramide-treated (b1), oleandrin-treated (c1), and oleandrin pretreated followed by ceramide-treated (d1) under fluorescence microscope view.

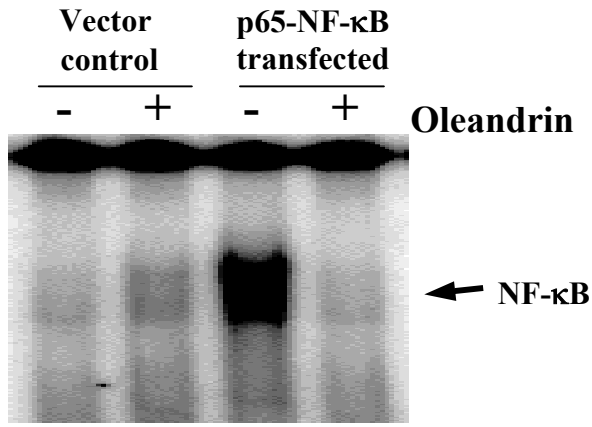


Fig 3.15 Effect of oleandrin on NF-κB activation and cell viability in NF-κB. overexpressed cells. HeLa cells were transfected with control plasmid pCMVFLAG1 or *p65-NF-κB* DNA for 12 h and then treated with oleandrin (0.2 μg/ml) for 4 h. Then NF-κB was assayed from nuclear extracts.

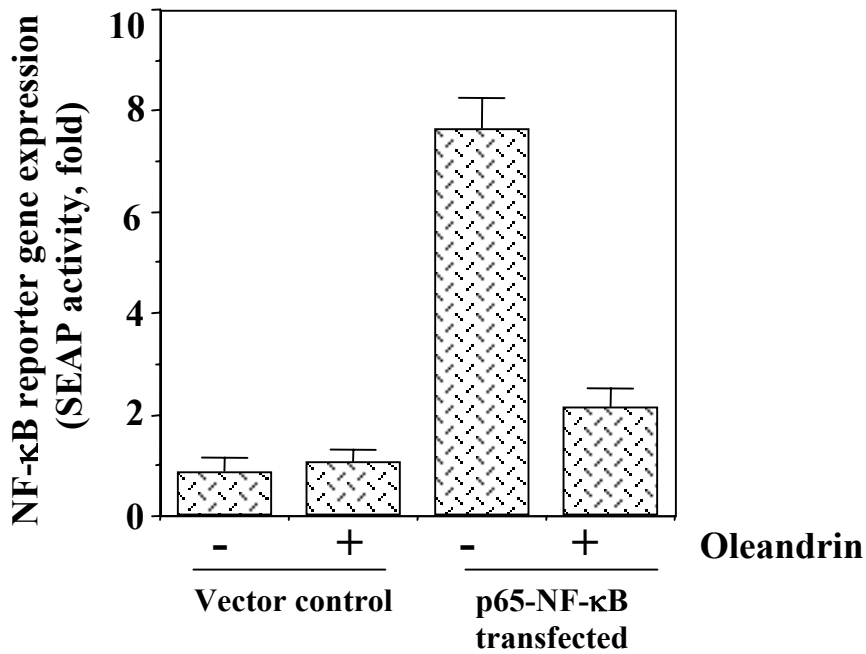


Fig 3.16 Effect of oleandrin on NF-κB activation and cell viability in NF-κB overexpressed cells. HeLa cells were transfected with control plasmid pCMVFLAG1 or *p65-NF-κB* DNA with β-galactosidase and SEAP reporter genes for 12 h and then treated with oleandrin (0.2 μg/ml) for 4 h. Then SEAP was assayed from culture supernatant

downregulated NF-κB activation. The SEAP activity was observed in p65 transfected cells about 7-fold compared to vector control. Oleandrin considerably downregulated the SEAP activity (Fig.3.16), similar to NF-κB activation. The activity of β-galactosidase, in all sets was equal (data not shown). Vector control and p65 transfected cells were incubated with oleandrin (0.2 μg/ml) for 4 h and then stimulated with ceramide (10 μM) for 36 h and the cytotoxicity was assayed by MTT uptake. The cell viability was decreased by oleandrin, ceramide, or in combination in vector transfected cells (Fig.3.17), which was similar to non-transfected HeLa cells as shown previously. In NF-κB overexpressed cells, ceramide was unable to induce cell death but oleandrin alone or in combination of oleandrin and ceramide cell death was observed (Fig.3.17). The data indicates that oleandrin-mediated cytotoxicity is due to downregulation of NF-κB.

3.2.10. Effect of oleandrin on ceramide-induced NF-κB activation, cytotoxicity and nuclear fragmentation in isolated PBMC and neutrophils

To detect the effect of oleandrin in primary cells, PBMC and neutrophils were isolated from whole human blood. The cells were treated with different concentrations of oleandrin for 4h followed by incubation of ceramide (10 μM) for 24 h. Then cells were examined for cytotoxicity by MTT method. The U937 cells showed the cell killing by oleandrin in a dose-dependent manner and ceramide potentiated the cell killing (Fig.3.17.1). In PBMC and neutrophils oleandrin unable to induce cell killing at any concentrations and surprisingly, ceramide or in combination with oleandrin were unable to kill these primary cells as shown by MTT assay (Fig.3.17.1 & 3.17.2).

To detect the effect of oleandrin in ceramide-induced NF-κB activation in primary cells, PBMC and neutrophils were treated with different concentrations of oleandrin for 4 h and then stimulated with 10 μM ceramide for 2 h. Nuclear extracts were prepared and analyzed for NF-κB by EMSA. The results showed in Figure 3.18.1 & 3.18.2 that ceramide potentially induced NF-κB in these cells but oleandrin was unable to block NF-κB activation at any concentration.

To detect the oleandrin mediated nuclear fragmentation neutrophils, pre-treated with oleandrin (0.2 μg/ml) for 4 h were stimulated with ceramide (10 μM) for 24 h. Nuclear

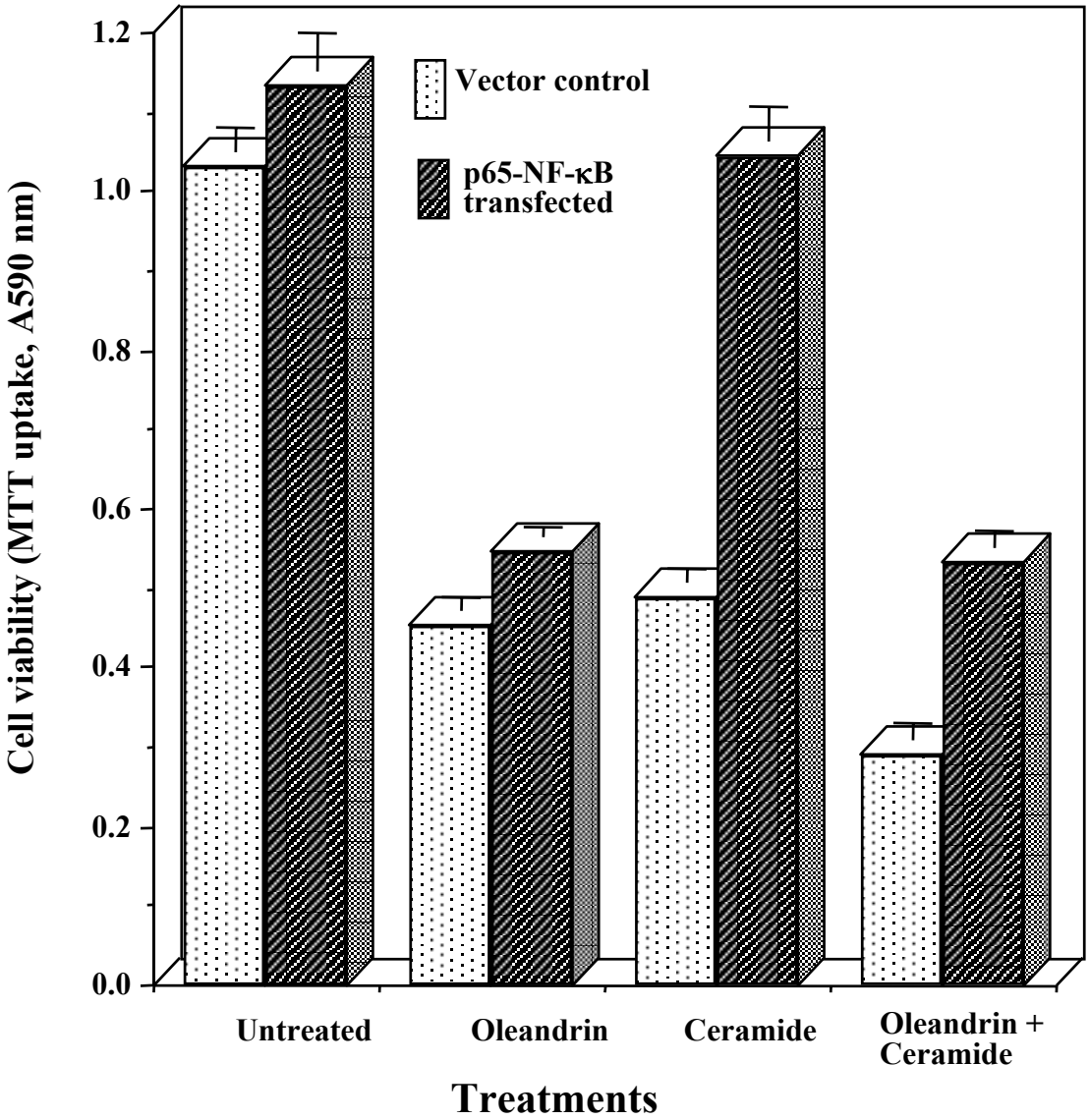


Fig 3.17 Effect of oleandrin on NF-κB activation and cell viability in NF-κB overexpressed cells. HeLa cells were transfected with control plasmid pCMVFLAG1 or *p65-NF-κB* DNA with β-galactosidase and SEAP reporter genes. Vector control and p65 transfected cells were treated with oleandrin (0.2 μg/ml) for 4 h and then stimulated with ceramide (10 μM) for 36 h. Cell viability was assayed by MTT dye uptake. Results are mean absorbance of triplicate assays.

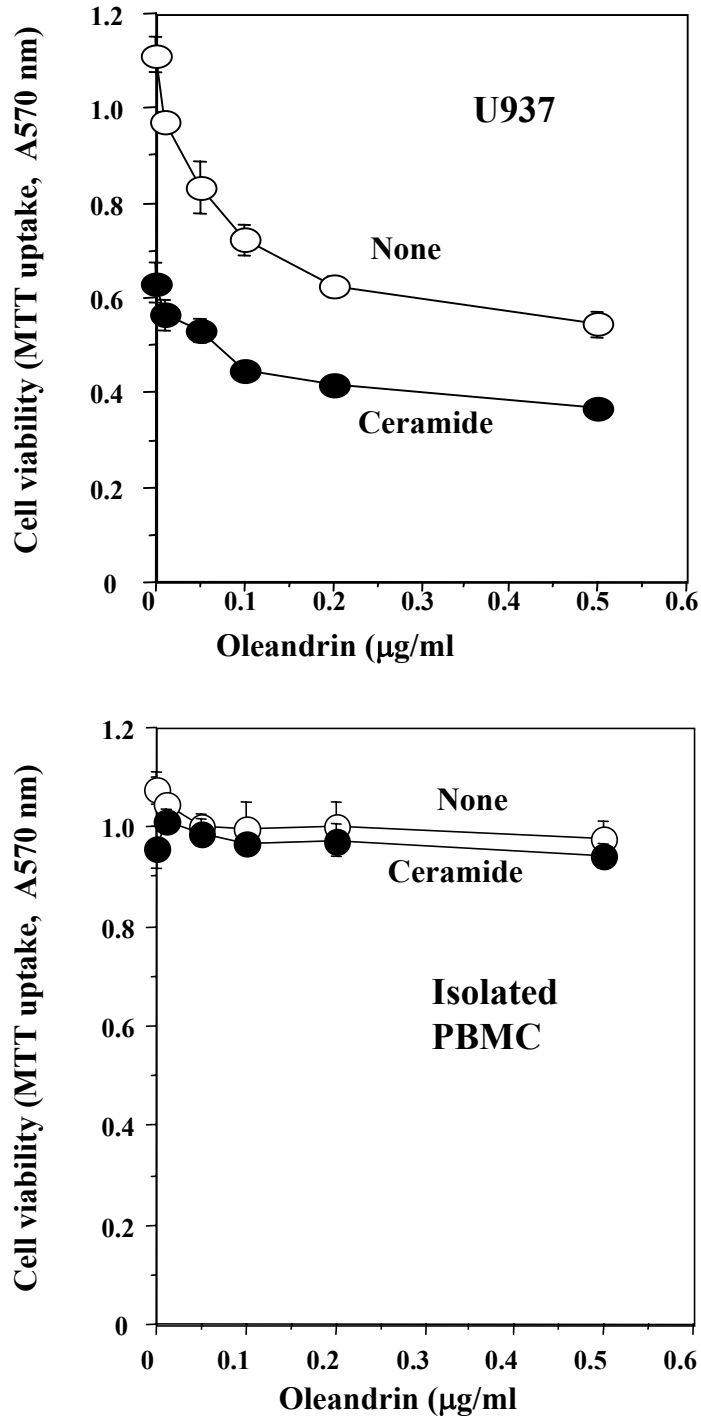


Fig 3.17.1 Effect of oleandrin on ceramide-induced cytotoxicity in U937 cells and PBMC. U937 and isolated PBMC were incubated with different concentration of oleandrin for 4 h were stimulated with 10 μM of ceramide for 24 h. Then MTT was assayed.

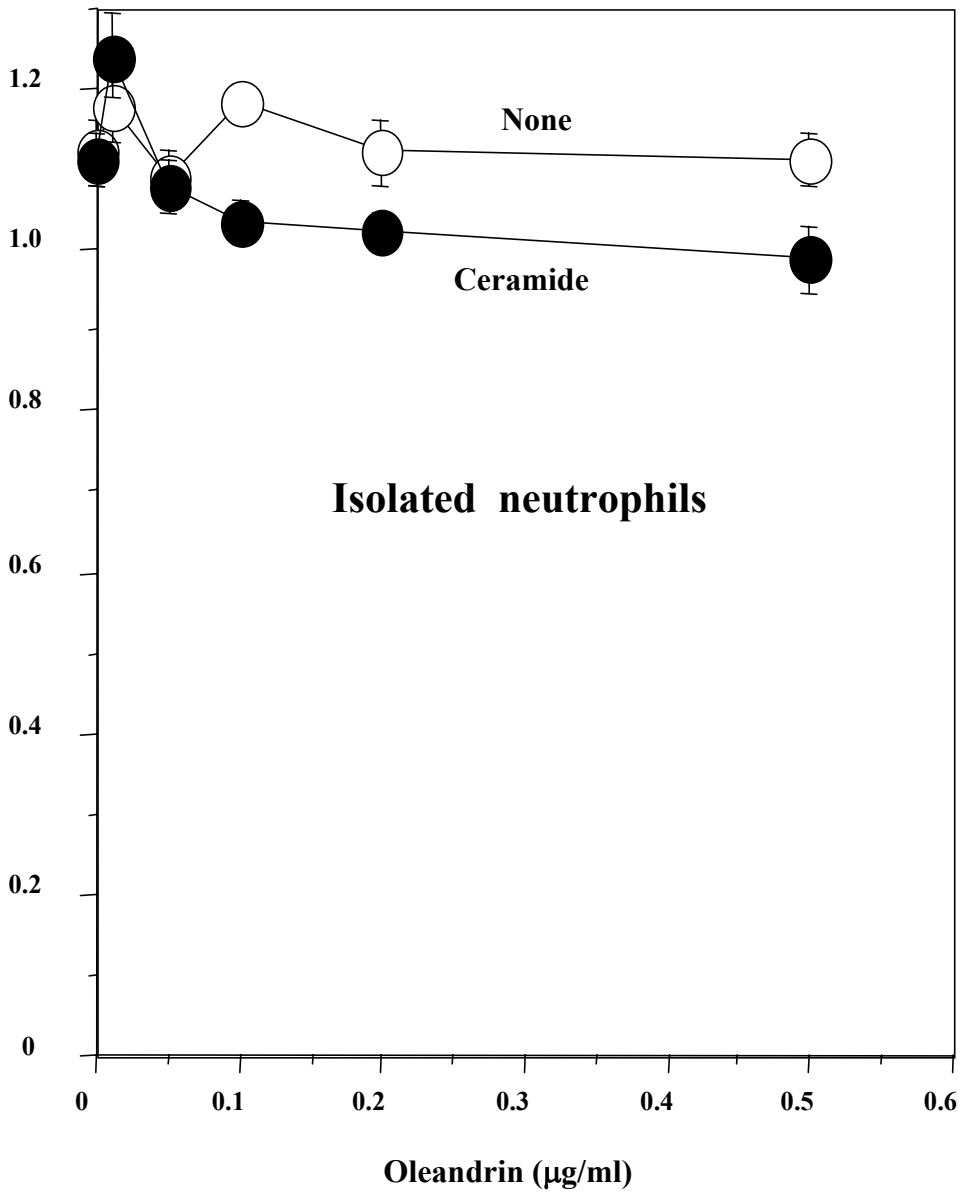


Fig 3.17.2 Effect of oleandrin on ceramide-induced cytotoxicity in neutrophils. Isolated neutrophils, incubated with different concentration of oleandrin for 4 h were stimulated with 10 μM of ceramide for 24 h. Then MTT was assayed.

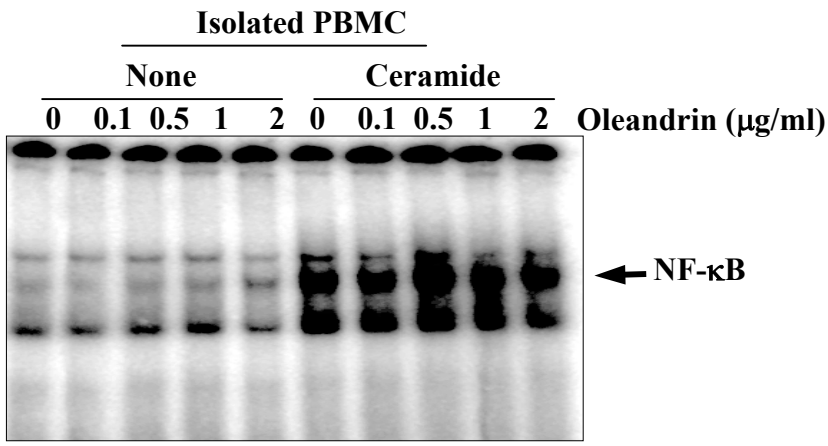


Fig 3.18.1 Effect of oleandrin on ceramide-induced NF-κB activation in Isolated PBMC. Cells, incubated with different concentrations of oleandrin were stimulated with ceramide (10 μM) for 2 h. The nuclear extracts were prepared and assayed for NF-κB.

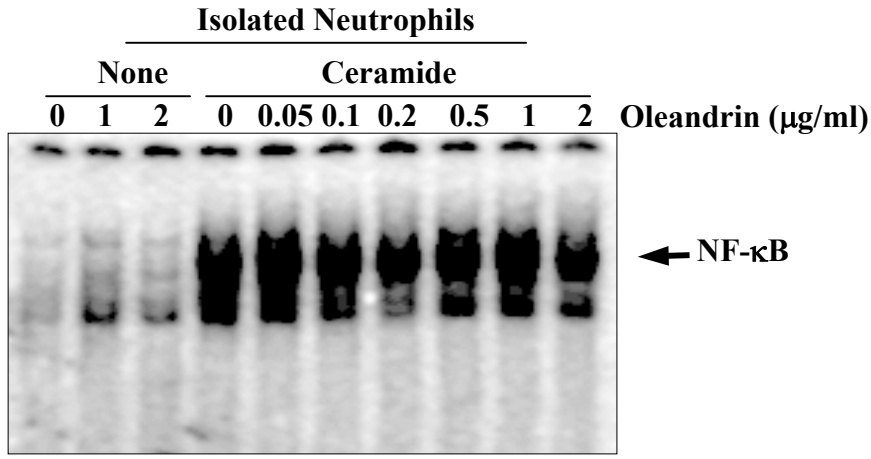


Fig 3.18.2 Effect of oleandrin on ceramide-induced NF-κB activation in isolated neutrophils. Cells, incubated with different concentrations of oleandrin were stimulated with ceramide (10 μM) for 2 h. The nuclear extracts were prepared and assayed for NF-κB.

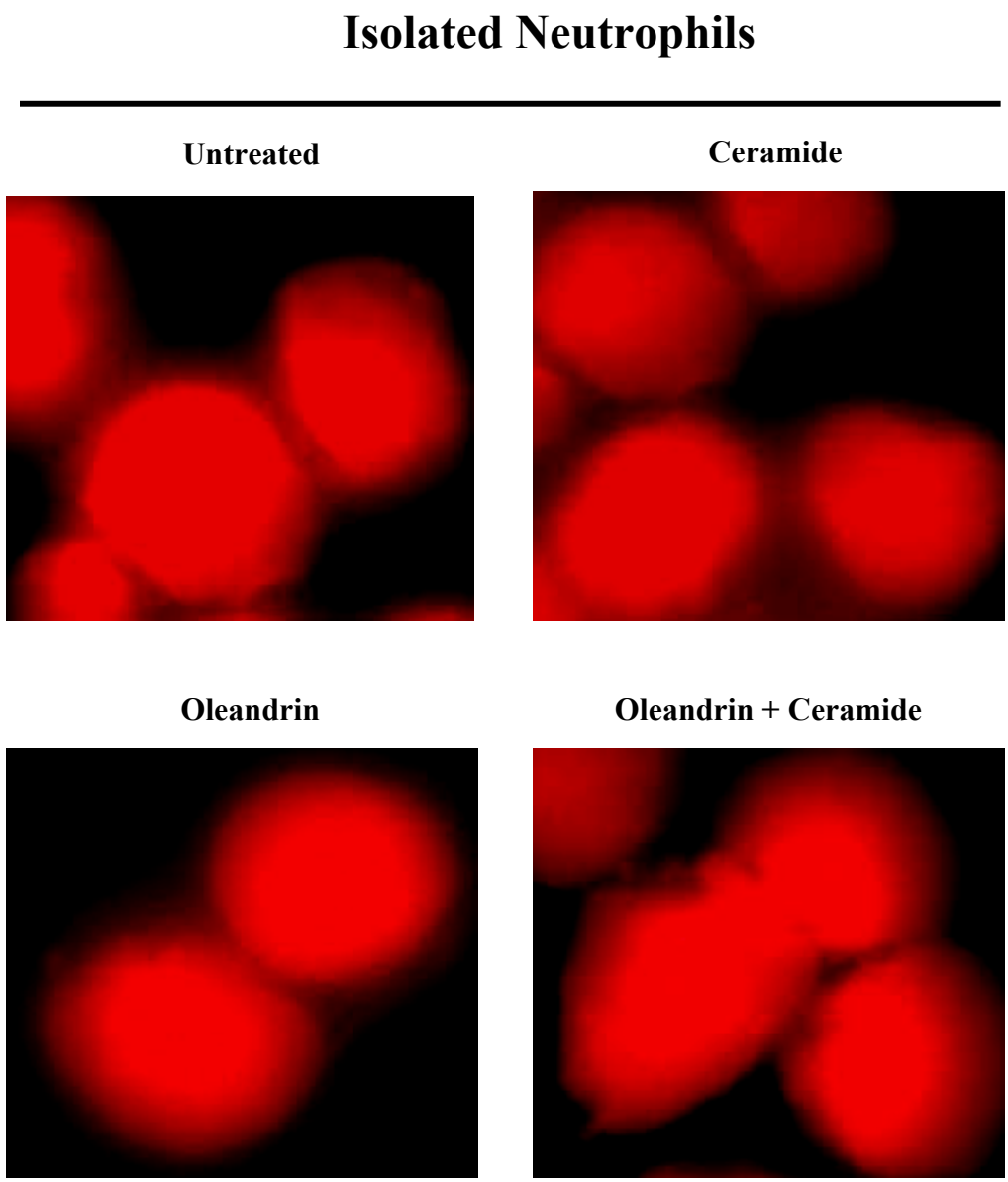


Fig 3.19 Effect of oleandrin on ceramide-induced nuclear fragmentation in neutrophils. Isolated neutrophils incubated with oleandrin (0.2 μg/ml) for 4 h and then stimulated with ceramide (10 μM) for 24 h. The cells were then fixed in 70% methanol, stained with PI and analyzed under fluorescence microscope to detect fragmented nuclei.

fragmentation was assayed by PI staining method. As shown in figure 7C, the nuclear fragmentation was not observed upon treatment with oleandrin, ceramide, or in combination in neutrophils indicating oleandrin or ceramide alone or in combination is unable to induce cell death.

3.3 Discussion

Because several *in vitro* and *in vivo* activities assigned to oleandrin require suppression of NF-κB activation, we tested the hypothesis that oleandrin directly blocks NF-κB activation and potentiates apoptosis. In the present report, it was demonstrated that oleandrin could block NF-κB activation, as determined by consensus DNA binding and dependent reporter gene transcription. It was found that oleandrin is indeed a potent inhibitor of ceramide-induced activation of NF-κB, and this inhibition is not cell line specific. Beside NF-κB, oleandrin blocked AP-1 activation. Oleandrin's ability is not only to increase but also potentiates ceramide-induced ROI generation, lipid peroxidation, and DNA fragmentation. In NF-κB overexpressed cells oleandrin was able to inhibit NF-κB activation and induce apoptosis. Surprisingly, in primary cells oleandrin was unable to block NF-κB activation as well as apoptosis.

Recent evidence indicates that different inflammatory agents may activate NF-κB through mechanisms that consist of some overlapping and some non-overlapping steps (Bonizzi *et al.*, 1997; Li and Karin, 1998; Imbert *et al.*, 1996). Ceramide is produced by the induction of those inflammatory agents, activates NF-κB and AP-1 and potentiates inflammation. Ceramide induced NF-κB and AP-1 activation was inhibited by oleandrin suggesting its anti-inflammatory property. Manna *et al*. previously reported that oleandrin, when pretreated for 1 h was unable to protect ceramide-induced NF-κB activation (Manna *et al.*, 2000 a). When cells were pretreated with oleandrin for 4 h then ceramide-induced NF-κB activation was completely blocked (Fig. 3.1.3). How oleandrin blocks ceramide-induced NF-κB activation is at present unclear. Most inhibitors of NF-κB activation mediate their effects through suppression of phosphorylation and degradation of IκBα (e.g. curcumin and silymarin) (Manna *et al.*, 1999; Singh and Aggarwal, 1995; Jobin *et al.*, 1999). Caffeic acid,

phenethyl ester and resveratrol block NF-κB activation without any effect on IκBα phosphorylation or degradation (Natarajan *et al.*, 1996; Egan *et al.*, 1999) but by inhibiting p65 phosphorylation followed by translocation to nucleus [Manna *et al*, 2000 c]. It was shown that oleandrin does not interfere with the NF-κB consensus DNA binding site, but that it did block the ceramide-induced translocation of NF-κB to nucleus and reporter gene transcription. It has been reported that oleandrin blocks TNF-induced NF-κB activation through inhibition of IκBα phosphorylation, degradation and nuclear translocation of NF-κB [Manna *et al*, 2000 a]. Similarly, oleandrin blocked ceramide induced IκBα degradation. Phosphorylation of IκBα is regulated by different kinases (IκB kinase -α, -β, or -γ, NF-κB-inducing kinase, and mitogen activated protein kinase kinase-1) (DiDonato *et al.*, 1997; Zandi *et al.*, 1997; Hirano *et al.*, 1996; Lin *et al.*, 1999; Sizemore 1999; Mercurio *et al.*, 1999). Therefore, it is possible that some of these kinases may involve in oleandrin-mediated inhibition of IκBα phosphorylation. Oleandrin also blocked ceramide-induced AP-1 activation. Most agents that activate NF-κB also activate AP-1 (Manna *et al.*, 1998; Manna *et al.*, 1999 a).

Ceramide-induced cytoxicity and caspase activation was also potentiated by oleandrin. Because NF-κB activation has been shown to play an antiapoptotic role (Lee and Burckart, 1998), the suppression of NF-κB by oleandrin may seem to be the cause of potentiation of apoptosis suggesting NF-κB's role in apoptosis. Previously, it was reported that oleandrin alone did not induce reactive intermediates generation or lipid peroxidation in 1 h incubation time [Manna *et al*, 2000 a] but 4 h treatment with oleandrin alone showed generation of reactive intermediates and lipid peroxidation (Fig.3.7 & 3.8). Though ROI generation causes NF-κB and AP-1 activation but we did not find any NF-κB activation by oleandrin alone still it induced reactive intermediates generation. Long time exposure with these toxic agents induced by oleandrin even by ceramide might have deleterious effect to induce cell death as shown by different apoptosis assay. MCF-7 cells, overexpressed with Mn-SOD protected almost 50% oleandrin-mediated cytotoxicity (Fig.3.11.1 & 3.11.2) indicating that oleandrin-induced ROI generation might cause apoptosis partially. Overexpression of other ROI detoxifying enzymes may be helpful to address the actual role of oleandrin-mediated apoptosis. The discovery that oleandrin potentiates ceramide-induced reactive intermediates

generation and lipid peroxidation explains the mechanism by which oleandrin exerts its effects.

Several reports indicate that constitutively or induced NF-κB induces resistance to apoptosis stimulated by a wide variety of agents (Wang *et al.*, 1999; Waddick and Uckun, 1999; Manna *et al.*, 1998; Dong *et al.*, 1999). Because oleandrin is known to be cytotoxic to various tumor cells (Pathak *et al.*, 2000; McConkey *et al.*, 2000), it is possible that this toxicity is mediated through the suppression of NF-κB. HeLa cells, when overexpressed with p65-NF-κB showed downregulation of NF-κB by oleandrin and induction of cell death (Fig. 3.15, 3.16 & 3.17) indicating the involvement of NF-κB for oleandrin-mediated apoptosis. Ceramide was unable to induce cell death in NF-κB overexpressed cells indicating the different mechanism involved for ceramide- and oleandrin-mediated apoptosis. Surprisingly, oleandrin was unable to downregulate NF-κB and thereby to induce cell death in primary cells. How the primary cells are protected from oleandrin mediated cell signaling and cell death needs to be elucidated. Oleandrin potentiates ceramide-induced cytotoxicity as shown by MTT assay, PARP cleavage, DNA fragmentation, and propidium iodide stained cells possibly by inhibiting NF-κB and AP-1 activation. Oleandrin also potentiates ceramide-induced lipid peroxidation and reactive intermediates generation influencing cells towards apoptosis as indicated through DNA fragmentation.

Several genes such as cytokines, cyclooxygenase-2, metalloproteases, urinary plasminogen activator, and cell surface adhesion molecules are involved in tumor promotion those are regulated by NF-κB (Hwang *et al.*, 1997; VonKnethen *et al.*, 1997; Sato *et al.*, 1993; Collins *et al.*, 1995; Iademarco *et al.*, 1992). As oleandrin blocked NF-κB-dependent reporter gene expression, so it may play a critical role in carcinogenesis and inflammation exhibiting anticarcinogenic and anti-inflammatory effects. Inhibiting NF-κB by adenoviral IκBα or proteosome inhibitors are currently being tested to overcome chemotherapy-induced resistance (Wang *et al.*, 1999). Therefore, NF-κB suppressive ability of oleandrin could be exploited by combination with chemotherapy. Evidence suggests that ceramide, generated inside the cells by the action of sphingomyelinases in response to a variety of cytotoxic agents may be used by cells to propagate apoptotic signals (Modrak *et al.*, 2002). It is possible that the intracellular pool of sphingomyelin used for signaling is decreased leading the cells

towards anti-apoptotic. An additional justification for the use of oleandrin may come from its ability to suppress AP-1, which known to play a critical role in tumorigenesis.

Because replication of certain viruses such as human immunodeficiency virus-1 is also dependent on NF-κB (Baeuerie and Baichwal, 1997; Baichwal and Baeuerie, 1997; Surh *et al.*, 2001; Lee and Burckart 1998), oleandrin may also abolish viral replication. Due to its ability to suppress COX-2 through NF-κB, aspirin is beneficial for preventing colon cancer (Wunsch, 1998). This suggests that oleandrin may also prove to be beneficial for colon cancer. Oleandrin's ability to suppress ceramide-induced NF-κB, AP-1, and other cellular responses may provide the molecular basis for the anticarcinogenic properties of oleandrin. Cardiac glycosides oleandrin, ouabain, and digoxin induce apoptosis in androgen-independent human prostate cancer cell lines *in vitro* by sustaining intracellular Ca^{++} for long time (McConkey *et al.*, 2000). Recently, oleandrin was also found to inhibit the fibroblast growth factor-2 export through membrane interaction with the Na^{+}, K^{+}-ATPase pump in human prostate cancer cell lines and arresting cell growth (Smith *et al.*, 2001). In addition, adenovirus-enforced overexpression of mitochondrial superoxide dismutase gene therapy has been used to treat ischemia/reperfusion injury of the liver through the own-regulation of NF-κB and AP-1 activation (Epperly *et al.*, 1998). The results indicate that suppressive effects of oleandrin on NF-κB and AP-1 activation and on other cellular responses may also explain its protective effects on liver and against cardiovascular diseases. Oleandrin's inhibitory activity on NF-κB activation and stimulatory activities on apoptosis were not observed in primary cells indicating its less or almost no side effects if it is design for therapy. Overall the results suggest that oleandrin may also have applications for various other diseases including inflammation and arthritis, where NF-κB activation has been shown to mediate pathogenesis. These possibilities require further investigation in detail. The apoptotic activity of oleandrin in tumor, but not in primary cells indicating its potential anti-cancer property with less side effects.

Chapter Four

Oleandrin induces the degradation of p65 via activation of the proteasome

4.1 Introduction

It is seen that constitutive or SA-LPS-induced NF-κB show resistance to cell death and p65 subunit transactivates NF-κB-dependent genes involved in cell progression and differentiation, the role of oleandrin on the p65 level in NF-κB-expressing cells was to be investigated. The results suggest that oleandrin downregulates p65 levels from NF-κB-expressing (SA-LPS induced Jurkat, constitutively expressed HuT-78, or p65 over expressed) cells. In this study, it is demonstrated for the first time that oleandrin mediates apoptosis in NF-κB expressing cells. This downregulation of NF-κB is due to downregulation of p65, which is independent of IκBα and caspases, but is mediated through proteasome degradation and thereby possible downmodulation of dependent genes, resulting in the induction of apoptosis. Oleandrin shows additive effect in cell killing induced by doxorubicin and sensitizes apoptosis in doxorubicin-resistant cells. Thus, this study might have potential importance in tumor cells, which are resistant to chemotherapeutic agents due to constitutively higher NF-κB levels.

4.2 Results :

In the present report the role of oleandrin on p65 levels in NF-κB-expressing cells were investigated. Two human T cell lines, Jurkat and HuT-78 were used. HuT-78 cells constitutively express the NF-κB were used for the studies. In Jurkat cells, NF-κB expression was induced upon treatment with serum-activated lipopolysaccharide (100 ng of LPS was incubated with 20 μl of human serum for 1 h at 37^0C and this mixture was SA-LPS). In this study Jurkat cells, induced with 100 ng/ml SA-LPS for 2 h and found sustainable NF-κB expression and is indicated as SA-LPS/Jkt throughout the manuscript were used.

4.2.1 Oleandrin inhibits NF-κB activation in NF-κB-expressing cells.

To detect the role of oleandrin on NF-κB activity in NF-κB-expressing cells, HuT-78 and SA-LPS/Jkt cells were incubated with different concentrations of oleandrin for 12 h at 37^0C. NF-κB activity in nuclear extracts was determined by gel retardation assay (EMSA) as indicated in Materials and methods. As shown in Figure 4.1.1, oleandrin alone did not activate NF-κB at any concentrations. Oleandrin decreased the NF-κB level in a concentration-dependent manner

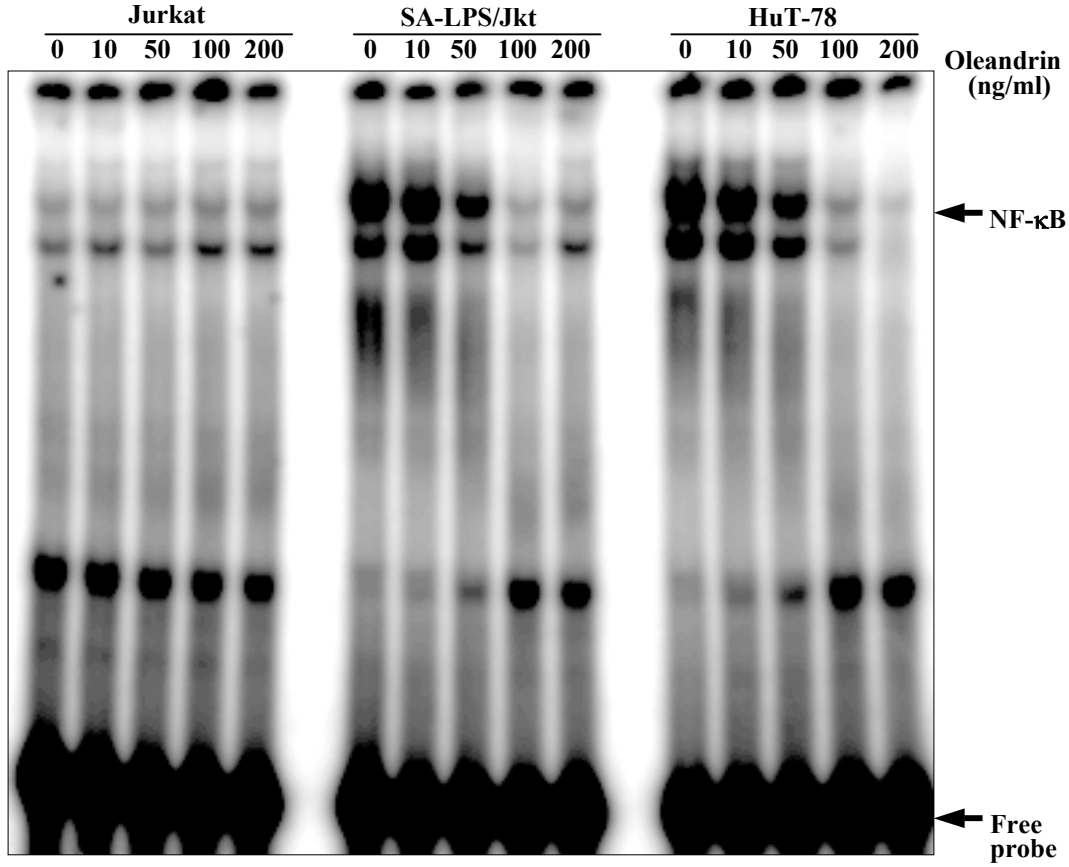


Fig 4.1.1 Effect of oleandrin on NF-κB activation in NF-κB-expressing cells. Jurkat cells, Stimulated with 100 ng/ml SA-LPS (the incubation mixture of 100 ng LPS with 20 µl of human serum for 1 h at 37^0C) for 2 h and HuT-78 cells were treated with different concentrations of oleandrin for 12 h at 37^0C. NF-κB was assayed from 8 µg nuclear extract (NE) proteins.

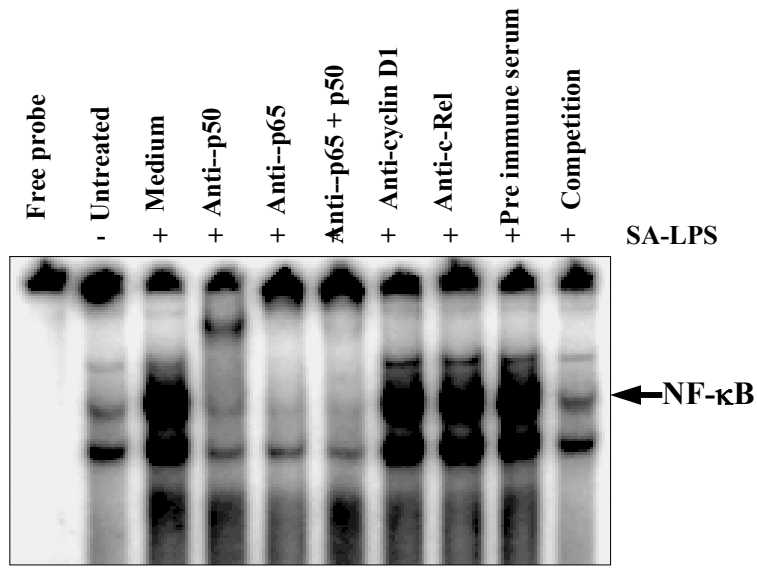


Fig 4.1.2 Supershift of NF-κB band. SA-LPS-induced NE was incubated for 15 min with different antibodies and cold NF-κB oligonucleotide and then assayed for NF-κB by gel shift assay.

in both SA-LPS/Jkt cells or HuT-78 cells, with total suppression at 100 ng/ml. The results suggest that the oleandrin mediates downregulation of NF-κB in NF-κB-expressing cells.

To detect the specificity and composition of the retarded band shown in EMSA, nuclear extracts from SA-LPS/Jkt cells were incubated with antibodies (Abs) to p50 and p65 alone or in combination, and then conducted EMSA. Abs to either subunit of NF-κB shifted the band to a higher M.W. (Fig.4.1.2), thus suggesting that the retarded complex consisted of both 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. This complex completely disappeared in the presence of 50-fold molar excess of cold NF-κB indicating its specificity.

4.2.2 Oleandrin inhibits NF-κB reporter gene activation in NF-κB-expressing cells.

As oleandrin inhibited NF-κB binding in the nuclear extracts from SA-LPS/Jkt or HuT-78 cells, the NF-κB dependent gene expression was also carried out. Jurkat or HuT-78 cells were transiently transfected with a NF-κB reporter plasmid containing SEAP gene, β-galactosidase, and/or dominant negative IκBα (IκBα-DN) plasmids for 6 h. Cells, cultured for 12 h (Jurkat cells were stimulated with 100 ng/ml SA-LPS for last 2 h) were treated with different concentrations of oleandrin for 12 h at 37^0C. Culture supernatant was collected and SEAP activity was assayed. The SEAP activity increased to almost 4-5 folds in SA-LPS/Jkt or HuT-78 cells alone (Fig.4.2). Oleandrin inhibited SEAP activity in SA-LPS/Jkt or HuT-78 cells in a dose-dependent manner. The IκBα-DN transfected cells showed the basal activity of SEAP in HuT-78 or SA-LPS/Jkt cells. The β-galactosidase activity from cell extracts showed almost similar absorbance from different treatments (as per Promega protocol) at 420 nm (data not shown) suggesting the transfection control for each treatment.

4.2.3 Oleandrin downregulates p65 level, but not IκBα or p50 in NF-κB-expressing cells.

As oleandrin downregulates NF-κB-dependent reporter gene activation, the levels of p50, p65, and IκBα were determined. As shown in Figure 4.3, the levels of IκBα and p50 were unchanged with different concentrations of oleandrin in SA-LPS/Jkt or HuT-78 cells. The level of p65 was decreased with increased concentrations of oleandrin suggesting the downregulation of p65. The same blot was re-probed with anti-tubulin antibody and the bands

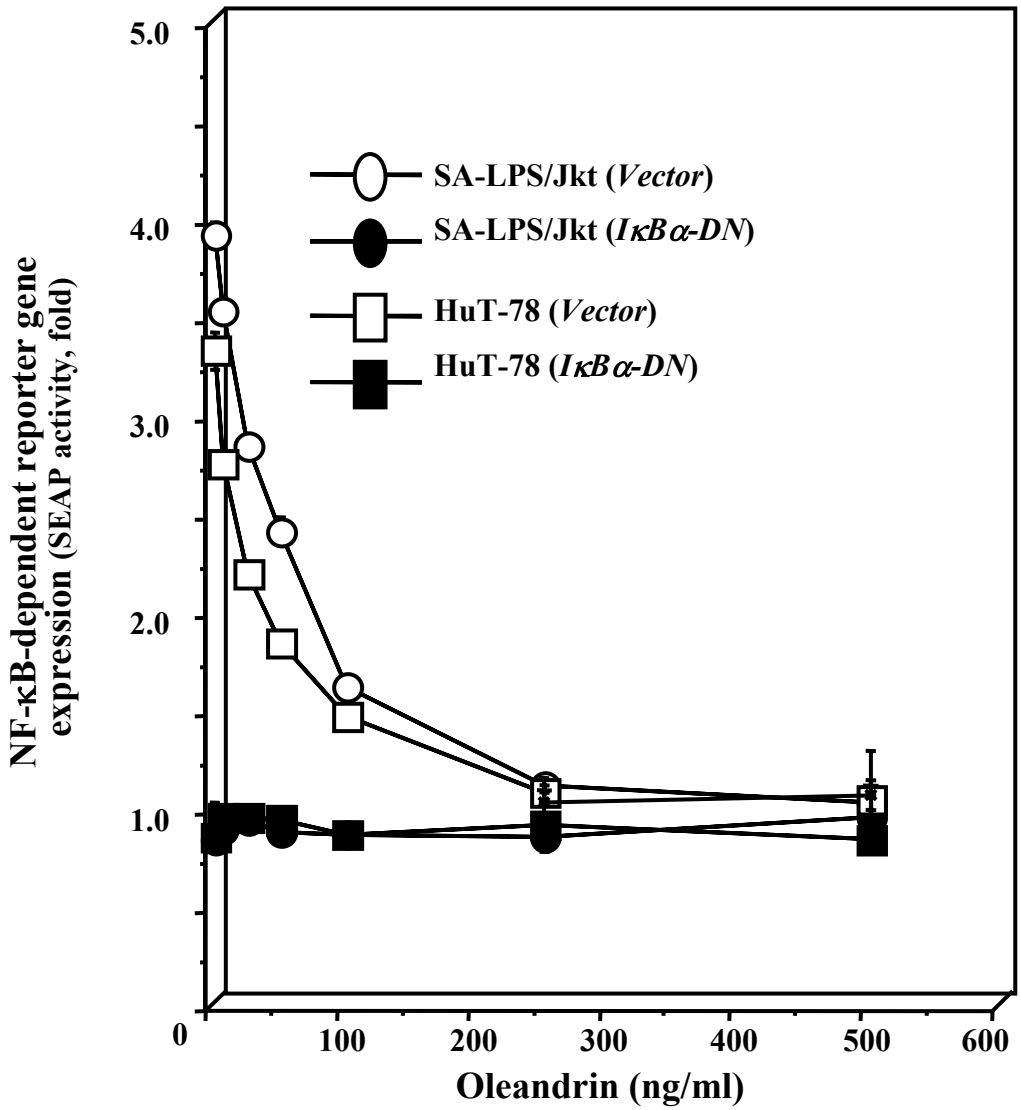


Fig 4.2 Effect of oleandrin on NF-κB-dependent reporter gene expression in NF-κB-expressing cells. Jurkat and HuT-78 cells were transiently co-transfected NF-κB-SEAP reporter gene, β-galactosidase with or without IκBα-DN constructs for 6 h. Then cells were washed and incubated for 12 h (Jurkat cells were stimulated with 100 ng/ml SA-LPS for last 2 h). Cells were washed and treated with different concentrations of oleandrin for 12 h. Culture supernatant was taken and assayed for SEAP and relative fluorescence was indicated as fold activation against vector transfected cells. The cell pellets were extracted and assayed for β-galactosidase activity. The data shown are representative of three independent experiments.

intensities were almost equal indicating equal loading of the proteins. These data suggest the level of p65 was decreased upon oleandrin treatment.

The oleandrin-mediated downregulation of p65 level was also detected by immunofluorescence using anti-p65 antibody followed by Alexa Flour-labeled anti-rabbit IgG. As shown in Figure 4.6, the p65 level was detected in the cytoplasm in un-stimulated cells whereas SA-LPS stimulated cells showed p65 mostly in the nucleus. Oleandrin-treated cells showed the p65 level neither in cytoplasm nor in nucleus indicating oleandrin-mediated downregulation of p65. The p65 level was observed in cytoplasm and nucleus of un-stimulated HuT-78 cells. The levels were gradually decreased with increased time of oleandrin (Fig.4.4). These data further suggest the oleandrin-mediated downregulation of p65.

4.2.4 Oleandrin downregulates p65 level in IκBα dominant negative (IκBα-DN)-transfected cells.

To detect the effect of oleandrin in either free or IκBα-masked p65, Jurkat cells were transfected with IκBα-DN construct and then treated with oleandrin for different times. The level of p65 decreased with increased time of oleandrin treatment (Fig.4.5) suggesting oleandrin-mediated down regulation of p65 level, irrespective of IκBα. The level of IκBα and tubulin remained same with different treatments as detected from the re-probed blot.

4.2.5 Oleandrin inhibits induced, but not expressed IKK activity.

To detect the role of oleandrin in expressed IKK activity in NF-κB-expressing cells, Jurkat cells, pre-incubated with oleandrin (100 ng/ml) for different times were stimulated with SA-LPS for 1 h. In another set, SA-LPS/Jkt cells were treated with oleandrin (100 ng/ml) for different times. The IKK activity and level of IκBα was detected from cell extracts. The results showed that upon oleandrin pre-treatment, SA-LPS induced IKK activity was decreased kinetically, consequently leading to IκBα degradation as detected by Western blot (Fig.4.7). In SA-LPS/Jkt cells, the high basal activity of IKK was observed and this activity remained unaltered with post-treatment of oleandrin (Fig.4.7). These results suggest that oleandrin blocks induced, but not expressed IKK activity.

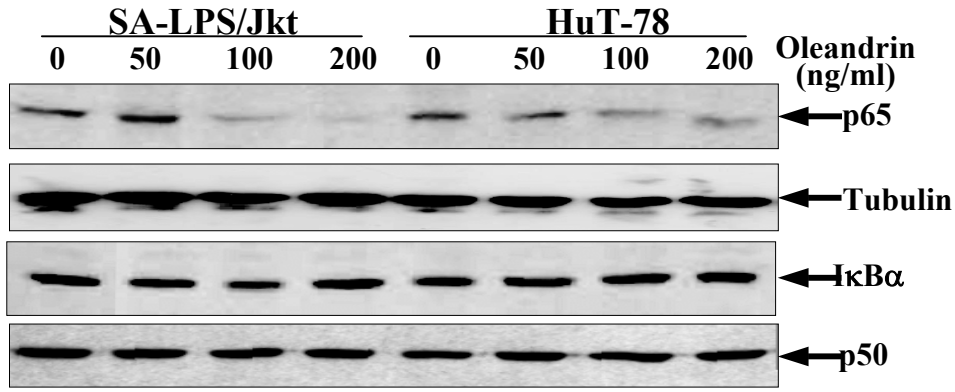


Fig 4.3 Effect of oleandrin on level of p50, IκBα, and p65 in NF-κB expressing cells. The SA-LPS/Jkt and HuT-78 cells were treated with different concentrations of oleandrin. The p65, p50 and IκBα were detected from whole cell extracts by Western blot. The p65 blot was re-probed with anti-tubulin antibody.

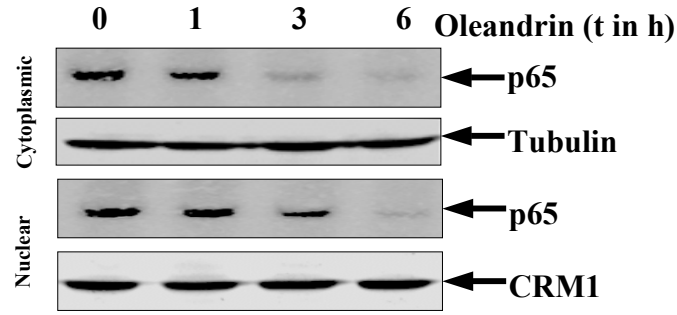


Fig 4.4 Effect of oleandrin on p65 level in cytoplasm and nucleus. HuT-78 cells were treated with 100 ng/ml oleandrin for different times and p65 was detected from cytoplasmic and nuclear extracts by Western blot.

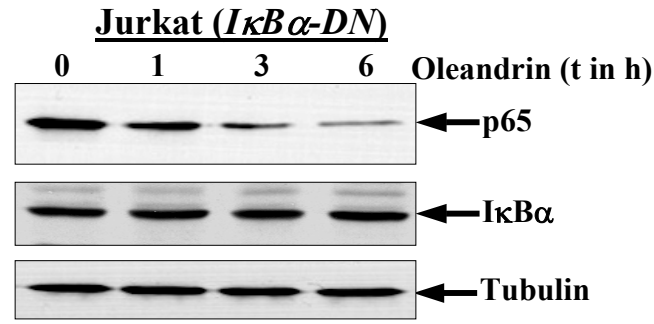


Fig 4.5 Effect of oleandrin on p65 level in IκBα-DN transfected cells. Jurkat cells, transfected with IκBα-DN construct were treated with oleandrin (100 ng/ml) for different times and the levels of p65 and IκBα detected by Western blot. The blot was re-probed with anti-tubulin antibody.

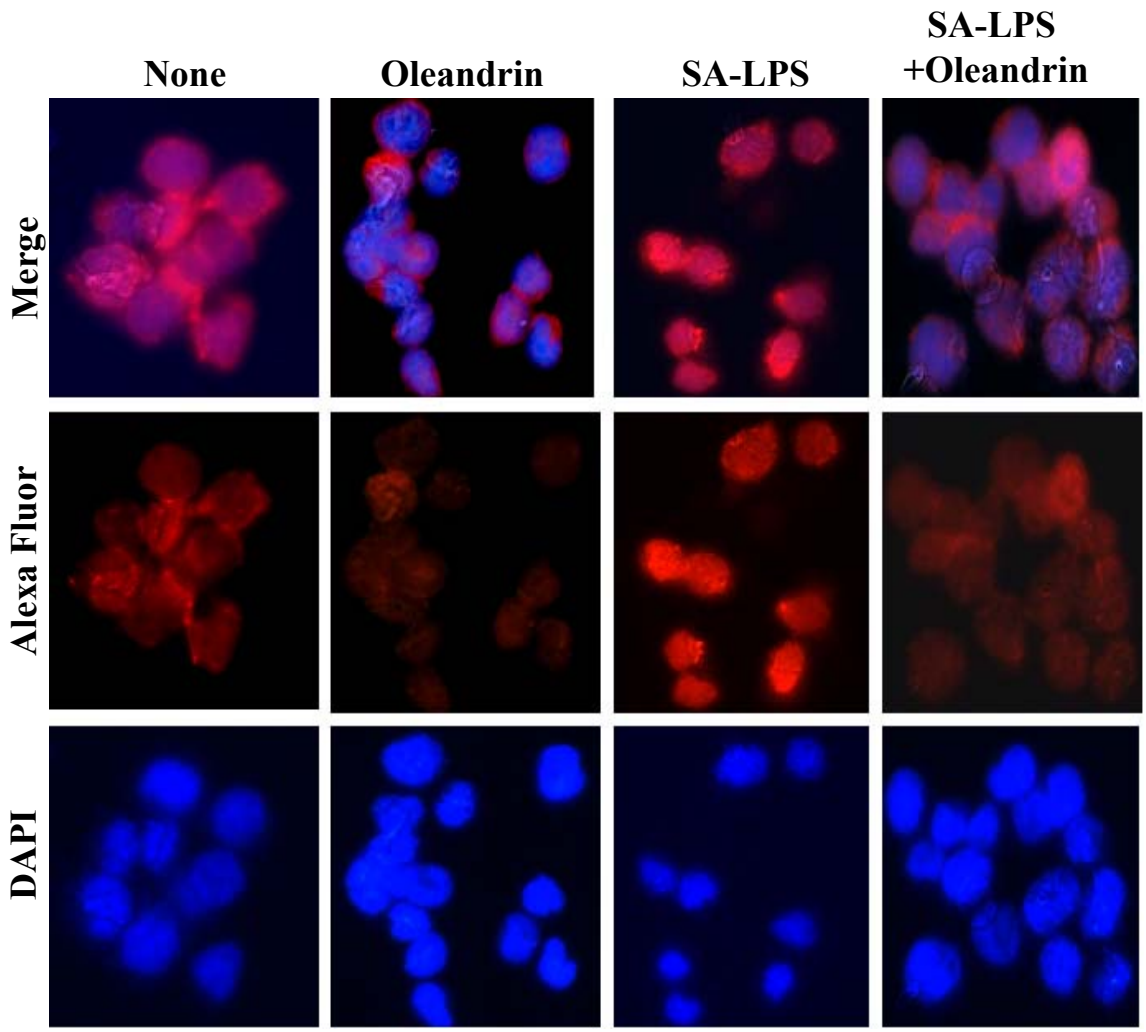


Fig 4.6 Effect of oleandrin on p65 level in SA-LPS/Jkt cells. Un-stimulated and SA-LPS/Jkt cells were treated with 100 ng/ml oleandrin for 12 h. The cells were fixed, incubated with anti-p65 antibody (1:100) followed by Alexa Flour-labeled anti-rabbit IgG and then mounted with mounting medium containing DAPI. The cells were visualized under fluorescent microscope

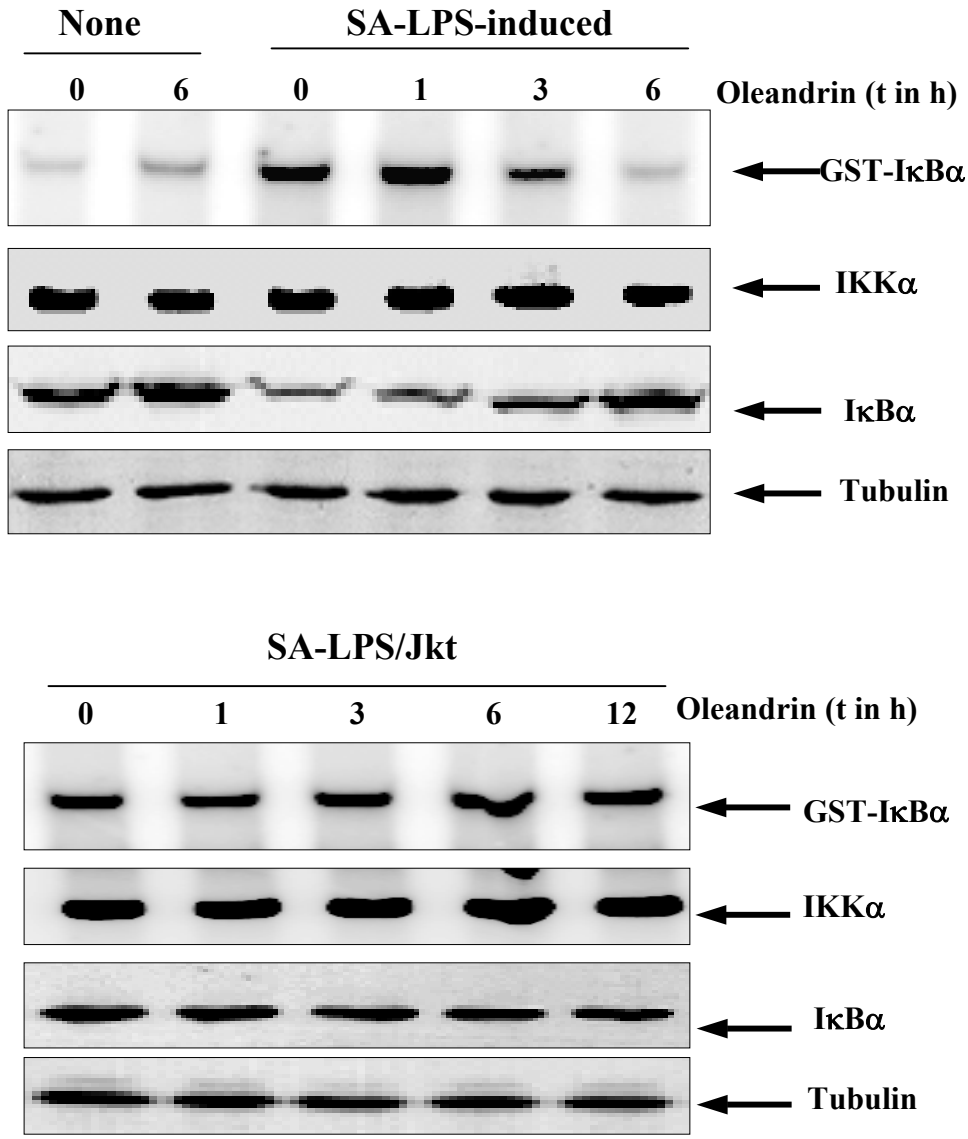


Fig 4.7 Effect of oleandrin on IKK activity in LPS-induced and NF-κB-expressing cells. Jurkat cells, pre-treated with 100 ng/ml oleandrin for different times were Stimulated with SA-LPS for 2 h. In other set, Jurkat cells, stimulated with SA-LPS for 2 h were treated with oleandrin (100 ng/ml) for different times. The cell extract were used to detect IKK activity using GST-IκBα as substrate. The levels of IKKα, IκBα, and Tubulin were detected from same extracts.

4.2.6 Oleandrin downregulates NF-κB, p65, but not Sp1 or p53 in HuT-78 cells.

To detect the level of other signaling molecules like Sp1 and p53 in the presence of oleandrin, HuT-78 cells were treated with 100 ng/ml oleandrin for different times. The nuclear extracts were then analyzed for NF-κB and Sp1 by gel shift assay. As shown in Figure 4.8, oleandrin downregulated NF-κB in 6 h of incubation completely without changing the binding of Sp1. Oleandrin downregulated p65 level in a dose-dependent manner without changing the levels of p53 or p50 in the total cell extracts of the same treated samples. Oleandrin downregulated NF-κB-dependent reporter gene as detected by SEAP gene expression in a time-dependent manner in HuT-78 cells, transfected with NF-κB-SEAP construct (Fig.4.8). These data suggest that 6 h incubation of oleandrin is sufficient to downregulate NF-κB or p65.

4.2.7 Oleandrin downregulates p65 and c-Rel, but not RelB or p50.

To detect the effect of oleandrin on other Rel family proteins, HuT-78 cells were treated with 0.1 μg/ml oleandrin for different times and then RelB, p65, c-Rel, and p50 were detected by Western blot. The levels of p65 and c-Rel were decreased with increased time of oleandrin treatment whereas the levels of RelB or p50 remained same with increased time of oleandrin treatment (Fig.4.9). The results suggest that oleandrin downregulates p65 and c-Rel, but not RelB or p50.

4.2.8 Oleandrin does not inhibit p65 mRNA.

As oleandrin downregulated p65 protein level and DNA binding activity, the levels of p65 mRNA were also determined by RT-PCR. The levels of specific p65 band as shown in agarose gel of RT-PCR products were not decreased with increased time of oleandrin treatment of HuT-78 cells (Fig.4.10) suggesting that its downregulating effect is not at the mRNA level.

4.2.9 Oleandrin decreases cyclin D1, cyclin E1, and phospho-Rb levels in NF-κB-expressing cells.

To detect the effect of oleandrin on other proteins related to cell cycle, HuT-78 cells were treated with oleandrin (100 ng/ml) for different times and cyclin D1, cyclin E1, or RB (phospho- and non-phospho-form) were detected by Western blot. The levels of cyclin D1, cyclin E1, and phospho-RB, but not RB were decreased with increased time of oleandrin treatment (Fig.4.11). All blots were re-probed with tubulin to show equal loading of the

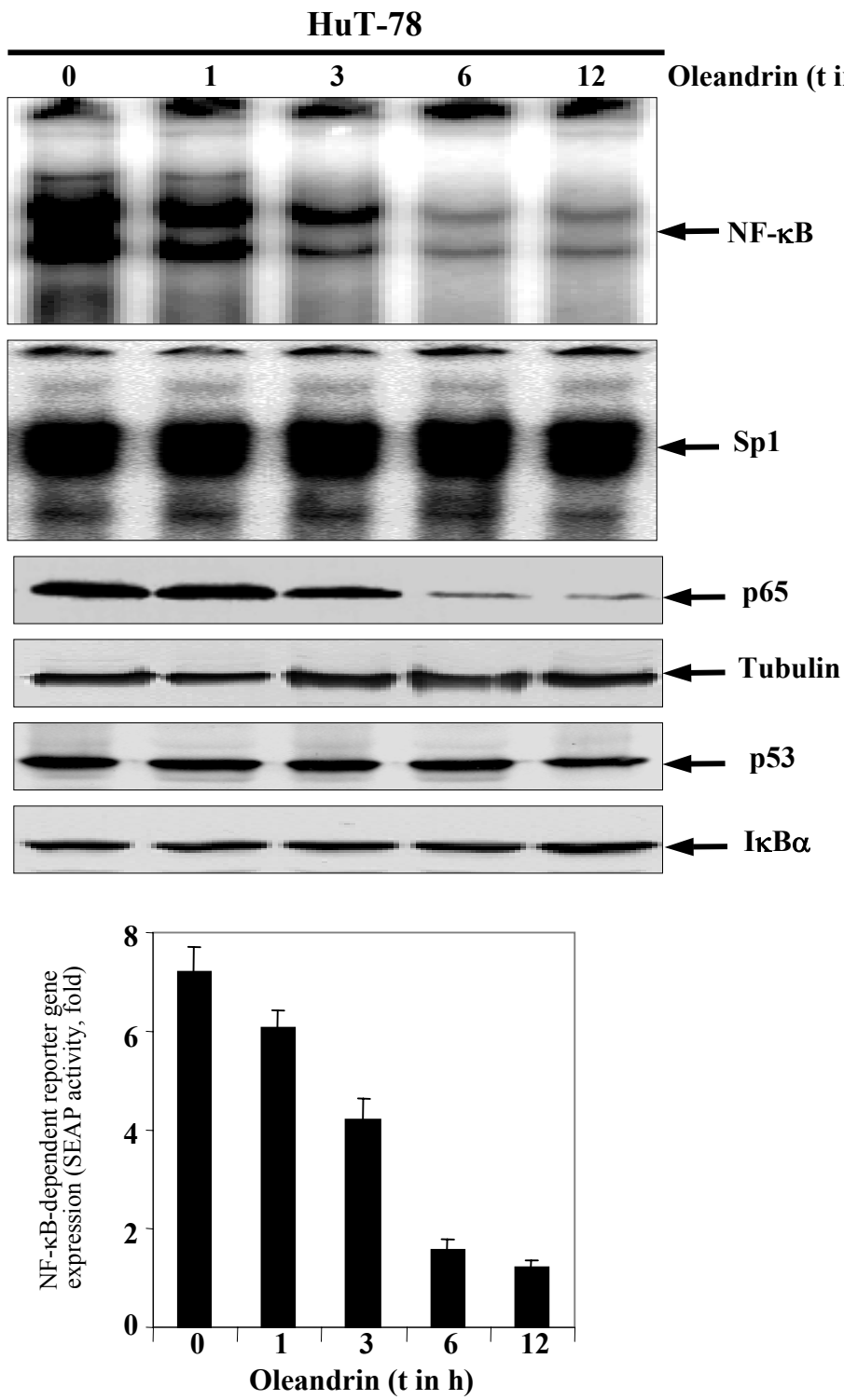


Fig 4.8 Effect of oleandrin on the levels of NF-κB, Sp1, p65, p53, an IκBα in HuT-78 cells. HuT-78 cells were treated with 100 ng/ml oleandrin for different times. Nuclear and whole cell extracts were prepared. NF-κB and Sp1 were assayed from 6 μg NE by EMSA. The p65, p53, and IκBα were detected from 100 μg of whole cell extract proteins by Western blot. The p65 blot was re-probed with anti-tubulin antibody. HuT-78 cells were co-transfected with NF-κB dependent SEAP reporter and β-galactosidase constructs for 6 h followed by 12 h of culture. Then cells were treated with 100 ng/ml oleandrin for different times. The culture supernatant was used to assay SEAP activity and cell extract was used to detect β-galactosidase.

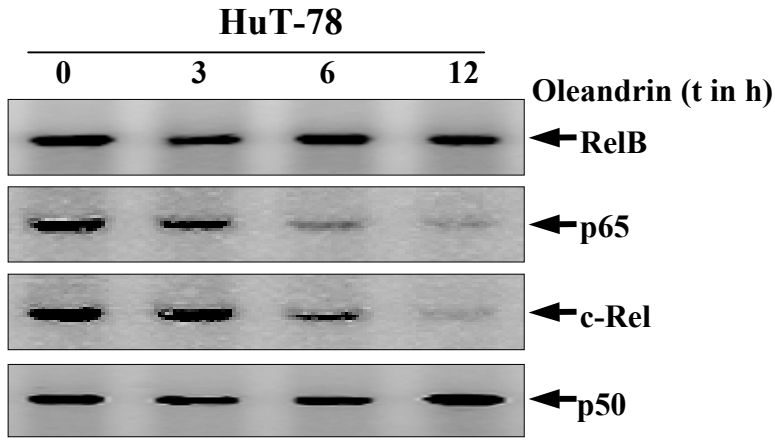


Fig 4.9 Effect of oleandrin on RelB, p65, c-Rel, and p50 levels. HuT-78 cells Were treated with 100 ng/ml oleandrin for different times and cell extracts (100 μg) Were used to detect RelB, p65, c-Rel, and p50 by Western blot.

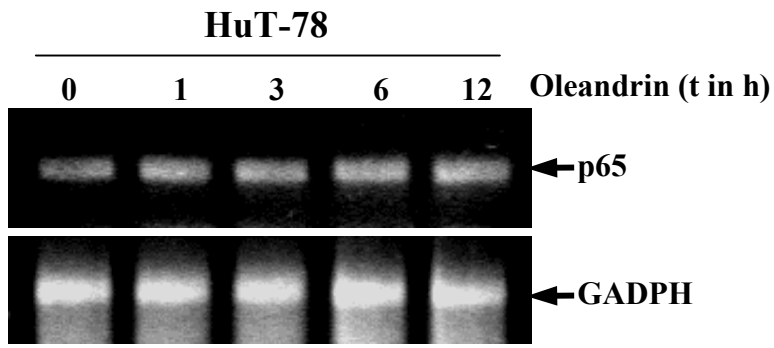


Fig 4.10 Effect of oleandrin on p65 mRNA level in HuT-78 cells. HuT-78 cells were treated with oleandrin (0.1 μg/ml) for different times and then RNA was isolated. The isolated RNA was used to detect p65 and GAPDH using specific primers by RT-PCR and the products were detected in agarose gel.

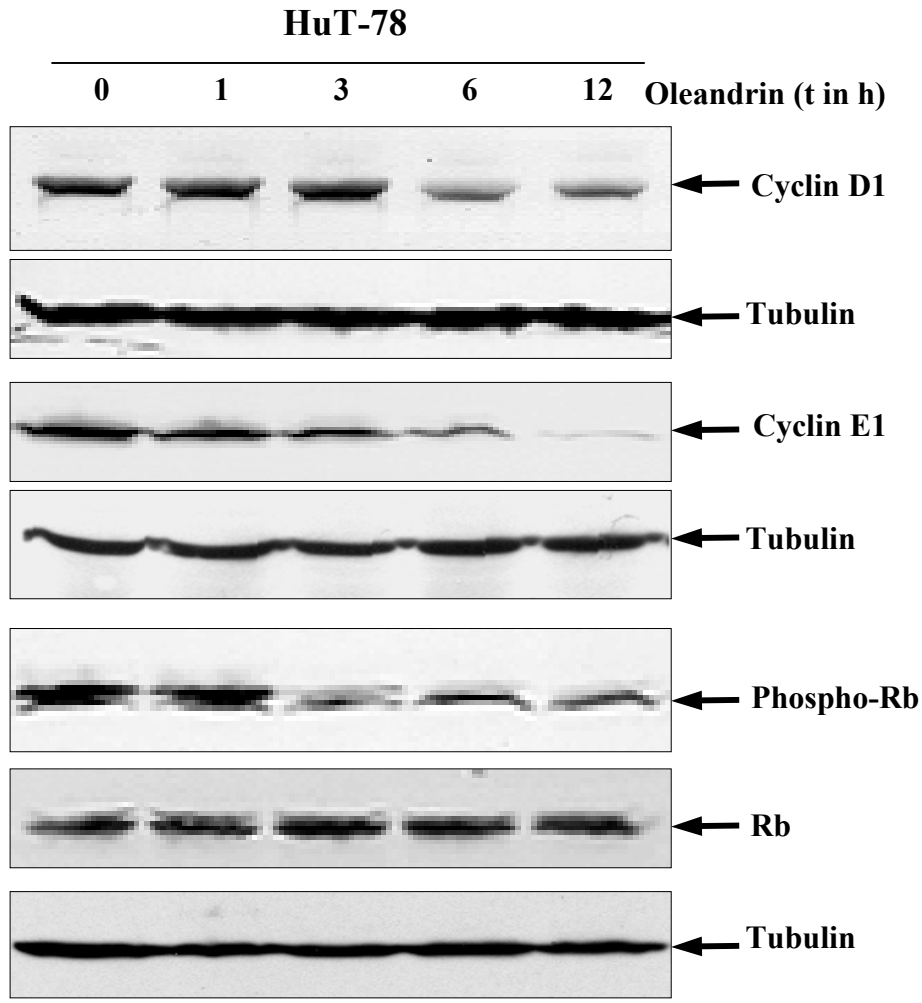


Fig 4.11 Effect of oleandrin on cyclin D1, cyclin E1, phospho-Rb, and Rb levels. HuT-78 cells were treated with oleandrin (100 ng/ml) for different times and the levels of cyclin D1, cyclin E1, phospho-Rb, and Rb were detected by Western blot. All those blots were re-probed with anti-tubulin antibody.

extracted proteins. These results suggest that oleandrin downregulates cell cycle-related proteins.

4.2.10 Oleandrin induces cytotoxicity in NF-κB-expressing cells.

As NF-κB has been shown to block cell death and oleandrin downregulated NF-κB and also p65 level, the effect of oleandrin on cell viability was tested in NF-κB-expressing cells. Jurkat, SA-LPS/Jkt, and HuT-78 cells were incubated with different concentrations of oleandrin for 72 h and then cytotoxicity was assayed by MTT assay. As shown in Figure 4.12, oleandrin induced cytotoxicity in a dose-dependent manner in Jurkat cells. In NF-κB-expressing cells, the cytotoxicity was also observed in a dose-dependent manner similar to Jurkat cells. Oleandrin (100 ng/ml) showed 50-60% cell death as detected by 'Live & Dead' cell assay at 72 h in Jurkat and SA-LPS/Jkt cells (Fig.4.13).

4.2.11 Oleandrin induces PARP, procaspase 3, and Bcl2 cleavage and DNA fragmentation in NF-κB-expressing cells.

Cell viability is reflected by caspase activation, which cleaves a lot of proteins including PARP, Bcl-2 etc. and thereby induces DNA fragmentation. To detect the effect of oleandrin in NF-κB-expressing cells, HuT-78 cells were treated with 100 ng/ml oleandrin for different times and cell extracts were used to detect PARP, procaspase 3, and Bcl2 levels. Oleandrin induced PARP cleavage in a time dependent manner. The procaspase and Bcl2 levels also decreased with increased time of oleandrin treatment (Fig.4.14). The blots were re-probed with anti-tubulin antibody and the levels of tubulin were equal in each lane indicating loading control. Oleandrin enhanced DNA fragmentation in HuT-78 cells in a time dependent manner as shown by DNA laddering (Fig.4.15). Oleandrin-mediated cell death in NF-κB-expressing cells is evident from the results seen.

4.2.12 Oleandrin decreases p65 level and cell viability in IκBα-DN, p65- and IKK-overexpressed cells.

To detect the role of IκBα or IKK on oleandrin-mediated p65 downregulation, Jurkat cells were transfected with IκBα-DN, p65, or IKK constructs. The cells, transfected with IκBα-DN and/or p65, were incubated either TNF (100 pM) for 1 h to detect IκBα level or oleandrin for 6 h to detect p65 and IκBα levels. In vector transfected cells TNF induced IκBα degradation,

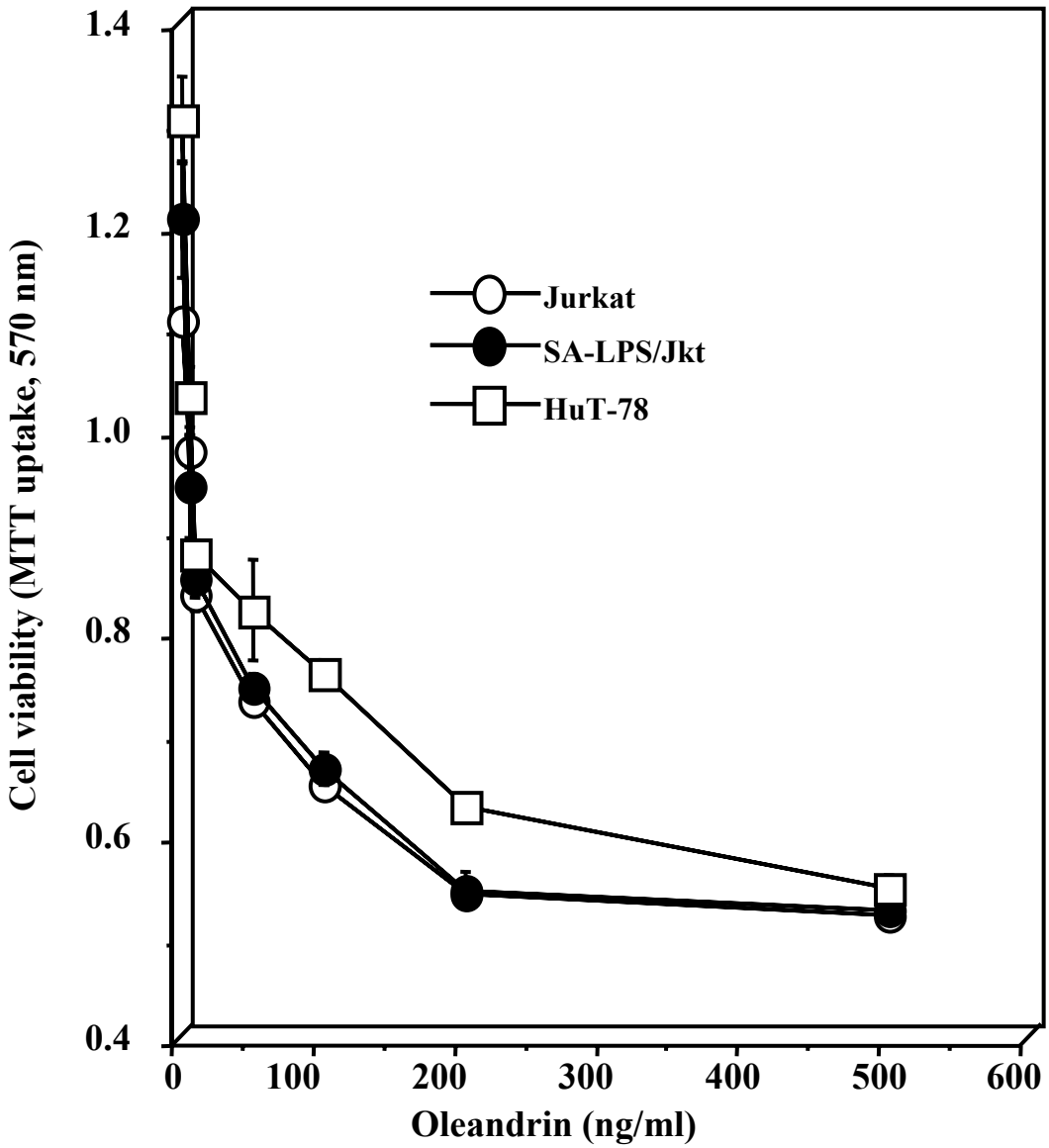


Fig 4.12 Effect of oleandrin on cell viability in Jurkat, HuT-78 and SA-LPS/Jkt cells. Jurkat, HuT-78, and SA-LPS/Jkt cells (10^4/0.1 ml) were incubated with different concentrations of oleandrin for 72 h. Cell viability was assayed by MTT dye uptake. The results are indicated as mean OD of triplicate assays. The result indicated is the representative of three independent experiments.

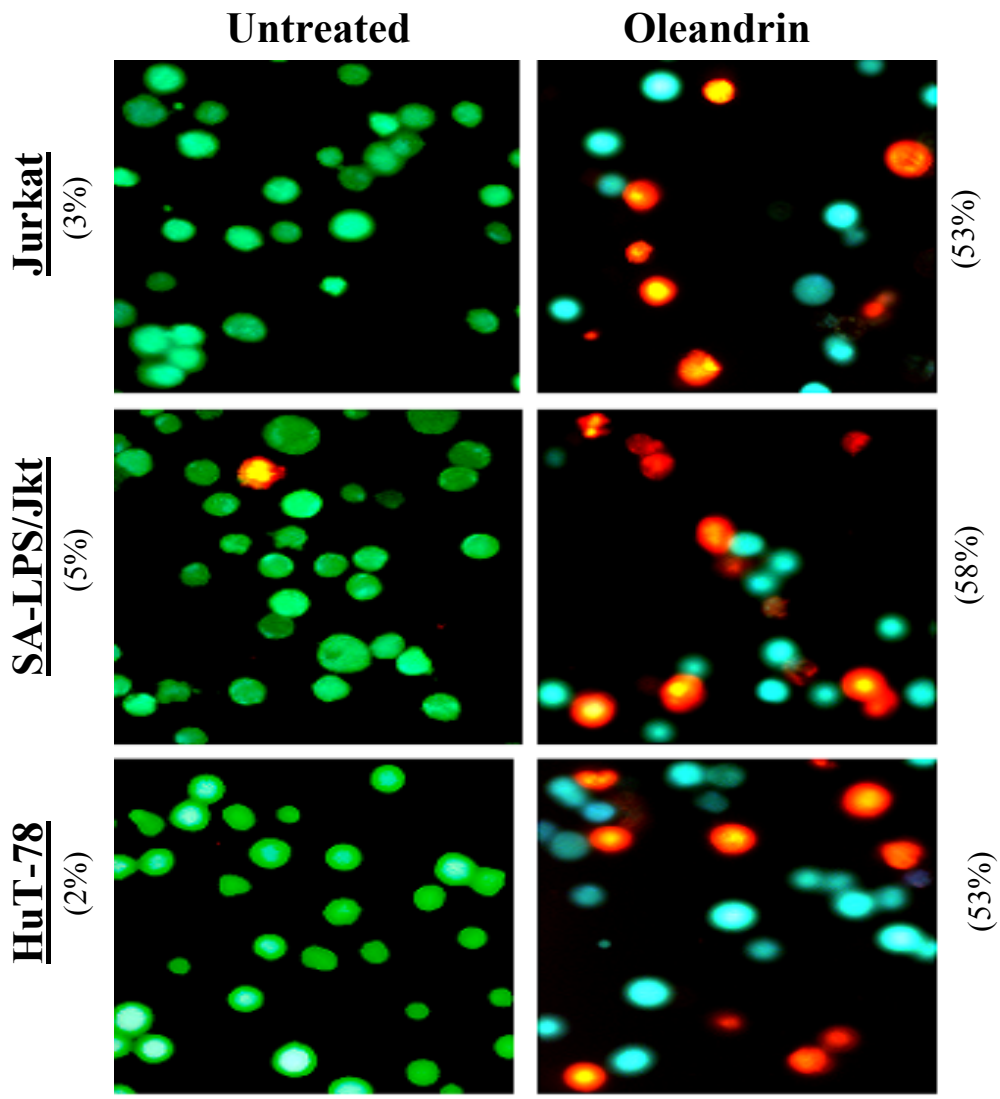


Fig 4.13 Effect of oleandrin on cell viability in Jurkat, HuT-78 and SA-LPS/Jkt cells. Jurkat, HuT-78, and SA-LPS/Jkt cells (0.1 x 10^6) were treated with oleandrin (100 ng/ml) for 72 h at 37^0C CO_2 incubator. The cell viability was assayed by 'Live & Dead' cells assay kit as described in Materials and methods.

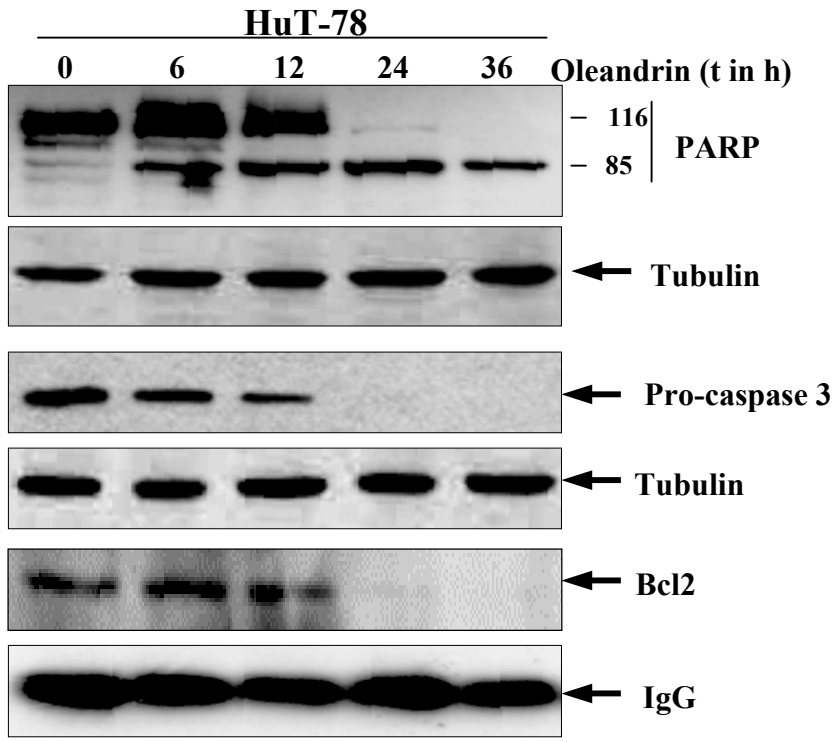


Fig 4.14 Effect of oleandrin on cleavage of PARP, procaspase, and Bcl2. HuT-78 cells were treated with 100 ng/ml oleandrin for different times and then 100 μg cell extract proteins were used to detect PARP and procaspase by Western blot. Blots were re-probed with anti-tubulin antibody. Three hundred μg of extract proteins were incubated with 1 μg of anti-Bcl2 antibody followed by immunoprecipitated by protein A/G sepharose beads. The beads were used to analyze Bcl2 proteins. The IgG band also showed in the figure.

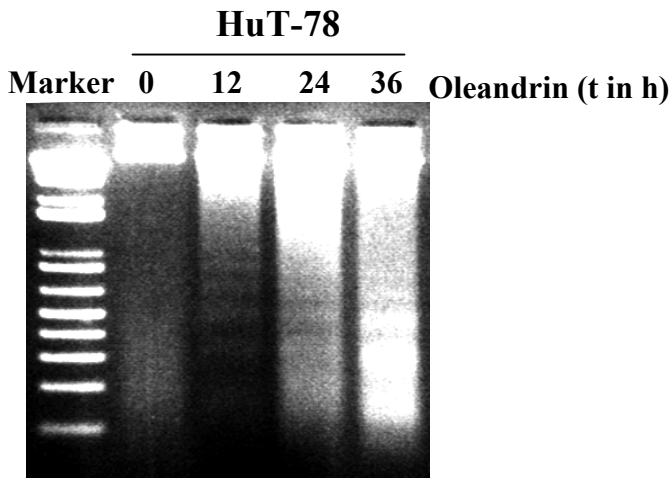


Fig 4.15 Effect of oleandrin on DNA fragmentation in HuT-78 cells. HuT-78 cells were treated with 100 ng/ml oleandrin for different times and then DNA was isolated and analyzed in agarose gel.

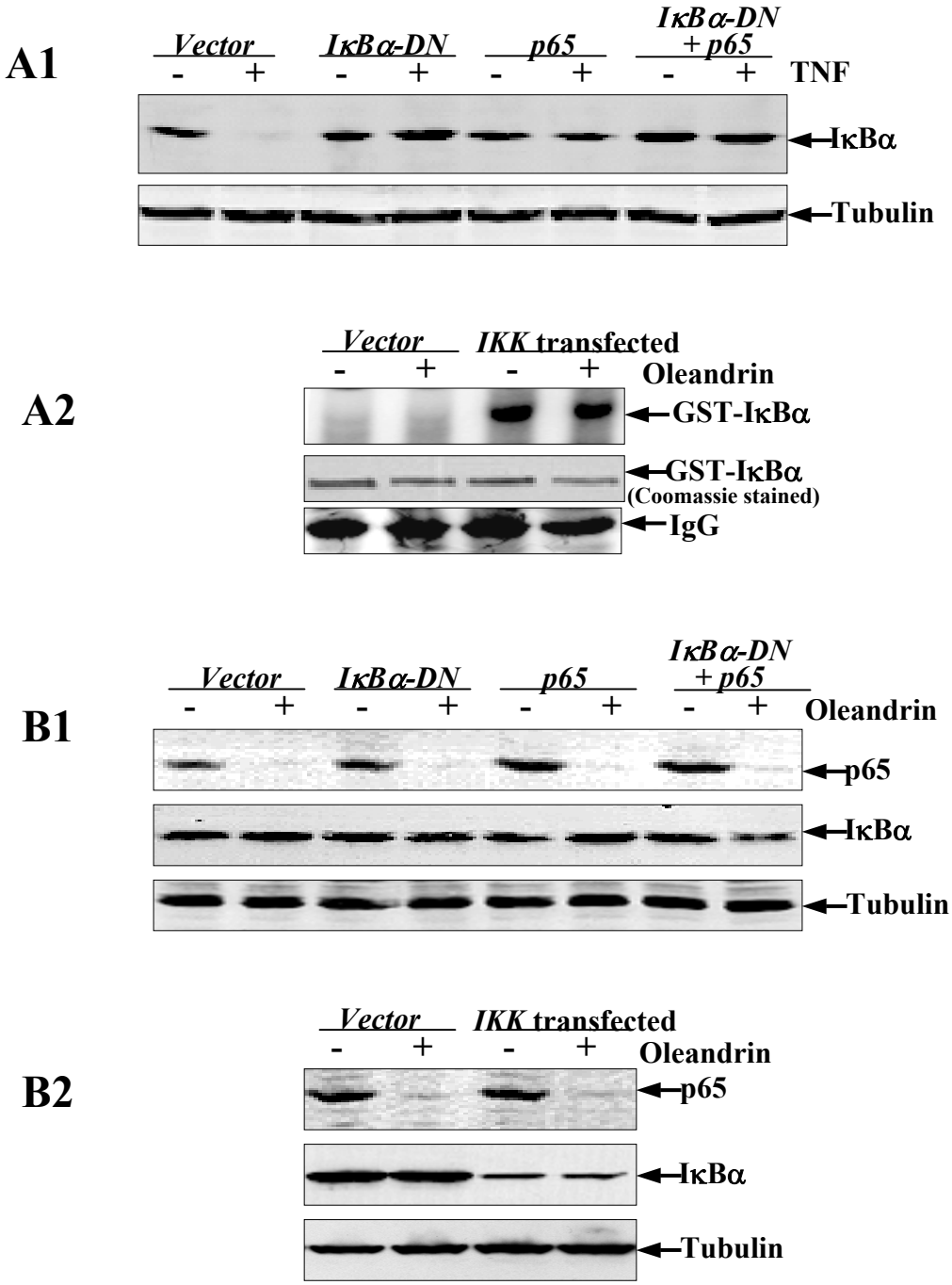


Fig 4.16 Effect of oleandrin on p65 and IκBα levels and cell viability in IκBα-DN, p65- or IKK-overexpressed cells. Jurkat cells, co-transfected with IκBα-DN, p65 or IKK and β-galactosidase for 6 h and then culture for 12 h. Cells, transfected with IκBα-DN and/or p65 were treated with 100 pM TNF for 1 h or 100 ng/ml oleandrin for 6 h. The level of IκBα was detected in TNF-induced cells extracts (A1). The levels of p65 and IκBα were detected from oleandrin treated cell extracts (B1). All these blots were re-probed with anti-tubulin Ab. Cells, transfected with IKK construct were treated with oleandrin (100 ng/ml) for 6 h and the levels of p65 and IκBα (B2) and IKK activity (A2) were detected from cell extracts.

but not in other transfected cells (Fig.4.16 A1). Oleandrin mediated degradation of p65, but not IκBα in all those transfected cells (Fig.4.16 B1), suggesting irrespective of cytoplasmic arrest of IκBα (by IκBα-DN) oleandrin downregulates p65 level. In IKK transfected cells oleandrin did not downregulate IKK activity (Fig.4.16 A2), but decreased the level of p65 (Fig.4.16 B2). All these transfected cells were treated with oleandrin and cell viability was detected. The cell viability was decreased in oleandrin treated cells as detected by MTT uptake (Fig.4.17) or thymidine uptake (Fig.4.18) suggesting that irrespective of the IκBα level (sustainable level by IκBα-DN or decreased level by IKK transfection), oleandrin downregulates p65 levels.

4.2.13 Oleandrin induces caspase 3, 8, and 9 activity.

As different proteins were cleaved by oleandrin treatment, the activity of different caspases was assayed. HuT-78 cells were treated with 100 ng/ml oleandrin for different time and then whole cell extracts were assayed for caspase 3, 8, and 9 as described in Materials and methods using colorimetric paranitroaniline (pNA) substrates. Oleandrin induced all those caspase activities in a time-dependent manner (Fig.4.19).

4.2.14 Oleandrin does not induce p65 downregulation *in vitro.*

To detect the role of caspases activated by oleandrin in the downregulation of p65, HuT-78 cells were treated with oleandrin for different times and extracts were immunoprecipitated with anti-caspase 3, 8, and 9 antibodies (0.5 μg of each/sample). The immune complexes were incubated for 2 h at 37^0C with the pulled down p65 proteins from ^{32}P-orthophosphoric labelled HuT-78 cell extracts and detected after scanning the gel. The level of labeled p65 bands were not decreased at any time of oleandrin treated cells (Fig.4.20) suggesting oleandrin mediated activated caspases have no role in downregulation of p65 *in vitro*. Fifty percent of the beads were used to detect the caspase 3 and the equal intensity of bands in different lanes suggests the equal amount of immunoprecipitation.

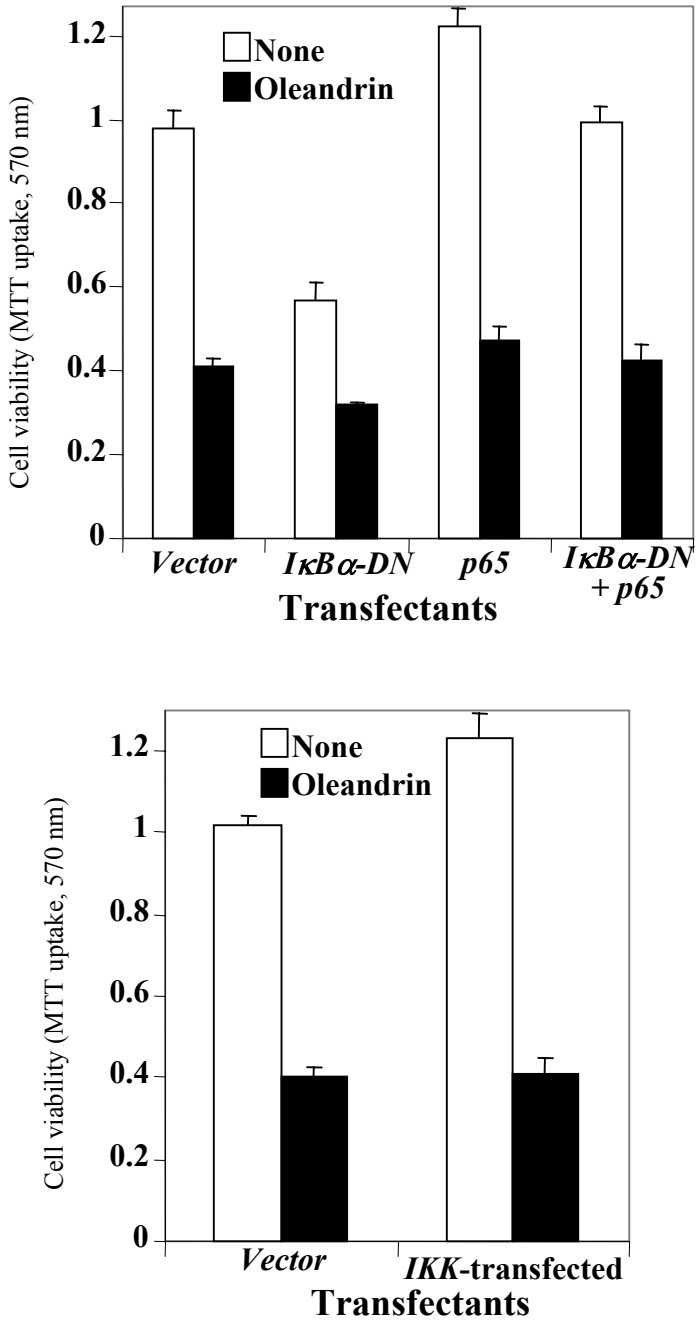


Fig 4.17 Effect of oleandrin on p65 and IκBα levels and cell viability in IκBα-DN, p65- or IKK-overexpressed cells. Jurkat cells, co-transfected with IκBα-DN, p65 or IKK and β-galactosidase for 6 h and then culture for 12 h. Cells, transfected with IκBα-DN and/or p65 were treated with 100 pM TNF for 1 h or 100 ng/ml oleandrin for 6 h. The cell viability was detected from those transfected followed by oleandrin-treated cells for 72 h using MTT.

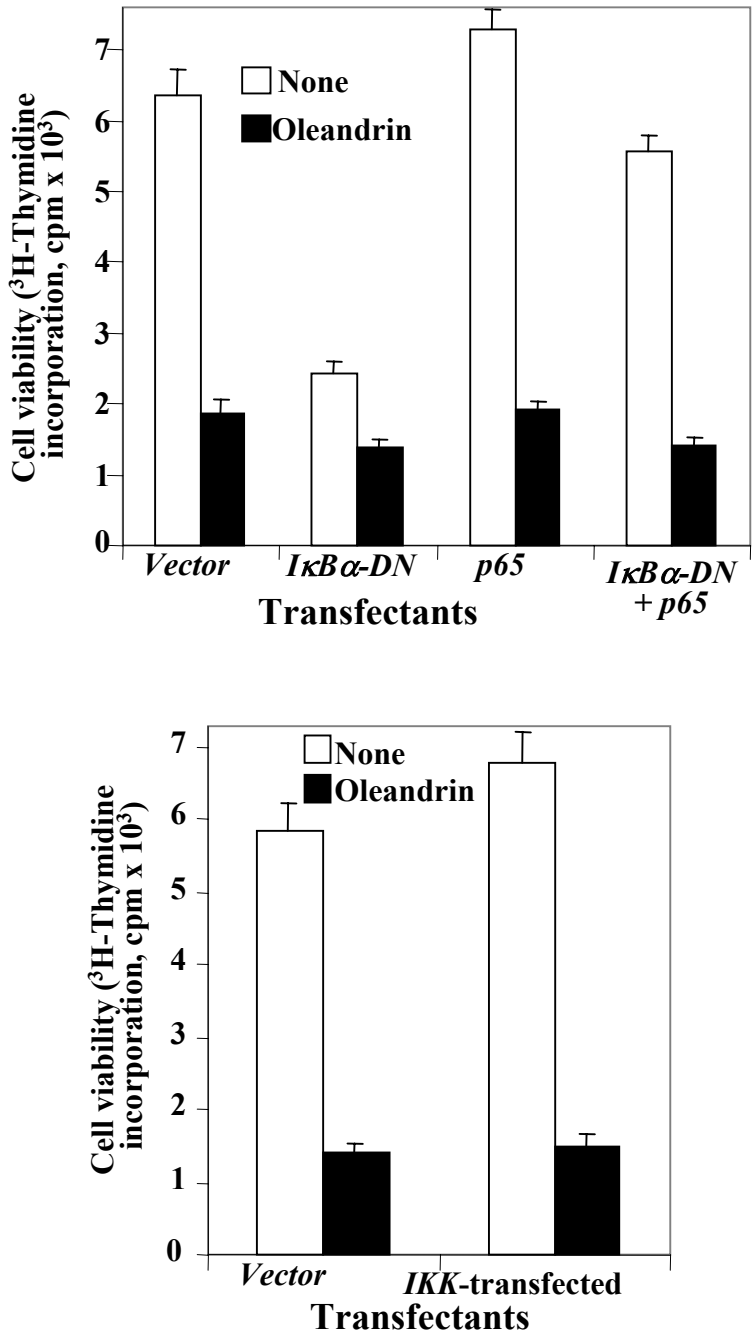


Fig 4.18 Effect of oleandrin on p65 and IκBα levels and cell viability in IκBα-DN, p65- or IKK-overexpressed cells. Jurkat cells, co-transfected with IκBα-DN, p65 or IKK and β-galactosidase for 6 h and then culture for 12 h. Cells, transfected with IκBα-DN and/or p65 were treated with 100 pM TNF for 1 h or 100 ng/ml oleandrin for 6 h. The cell viability was detected from those transfected followed by oleandrin-treated cells for 72 h using thymidine incorporation assays.

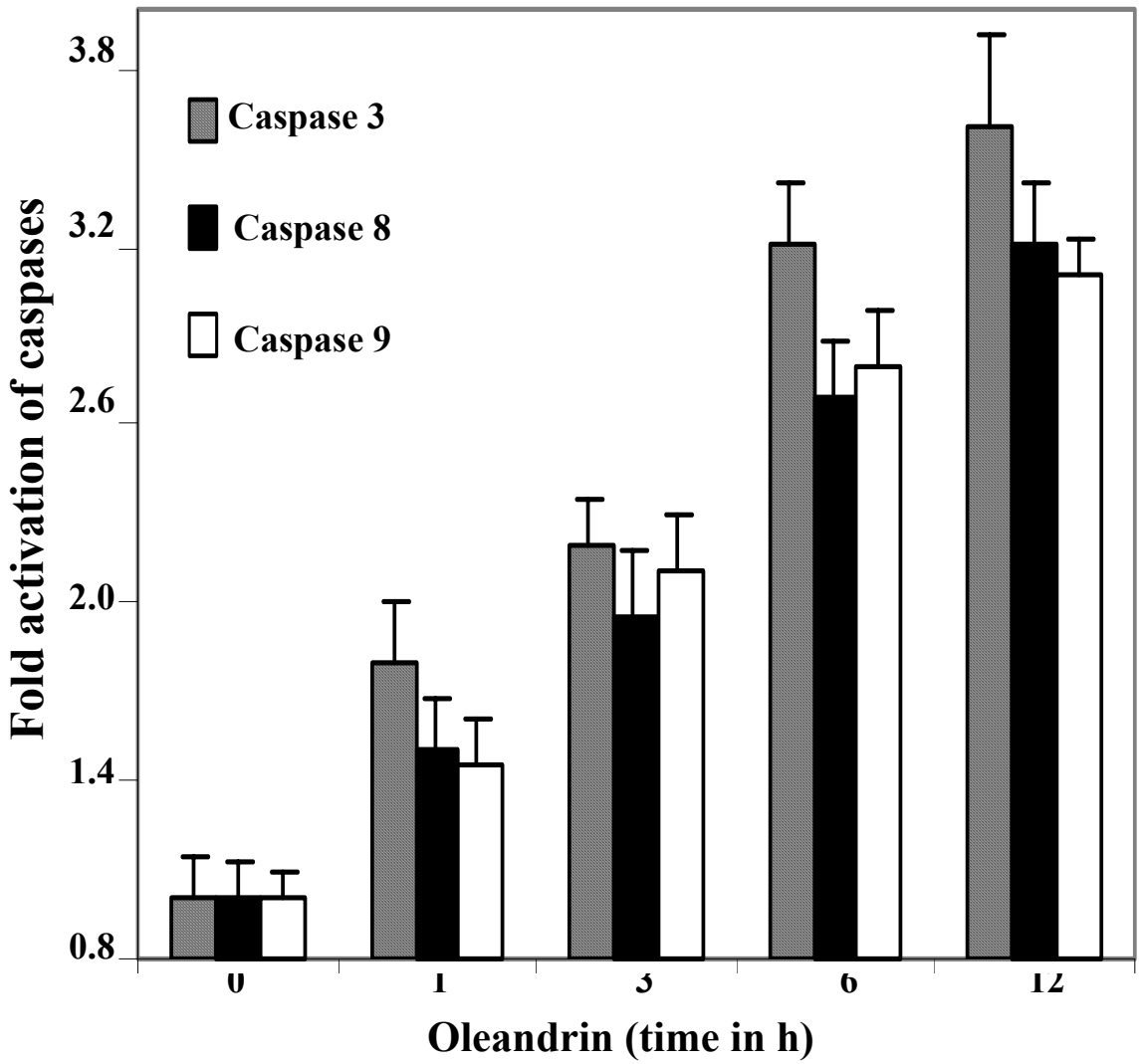


Fig 4.19 Effect of oleandrin on caspase 3, 8, and 9 activation. HuT-78 cells were treated with 100 ng/ml oleandrin for different times as indicated and then cell extracts were used to analyze caspase 3, 8, and 9 activity using colorimetric substrate. The absorbance was taken at 405 nm and results represented in fold activation of caspases.

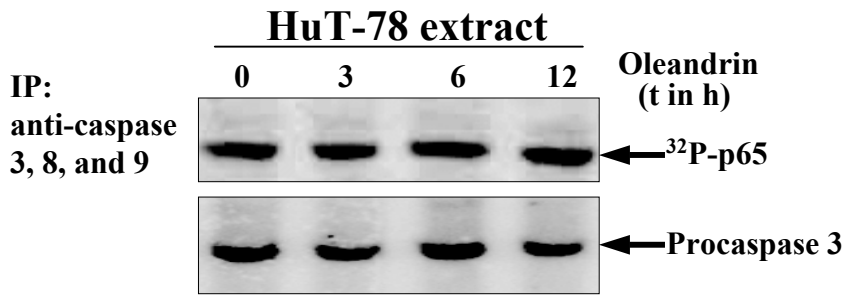


Fig 4.20 Effect of caspases on p65 protein *in vitro*. HuT-78 cells, incubated with ^{32}P-orthophosphoric acid (1 mCi /2 x 10^6 cells) in phosphate-free medium for 6 h and the cell extract was immunoprecipitated with anti-p65 antibody using protein A/G sepharose beads. HuT-78 cells were treated with 100 ng/ml oleandrin for different times and then 400 μg of extract proteins were incubated with anti-caspase 3, 8, and 9 antibodies (1 μg of each/sample) followed by pull down by protein A/G sepharose beads and incubated with equal amount of ^{32}P-labeled p65 protein containing beads for 2 h at 37^0C. The samples were analyzed in 9% SDS-PAGE and detected in Phosphor Imager. Fifty percent beads were used to detect caspase 3 by Western blot.

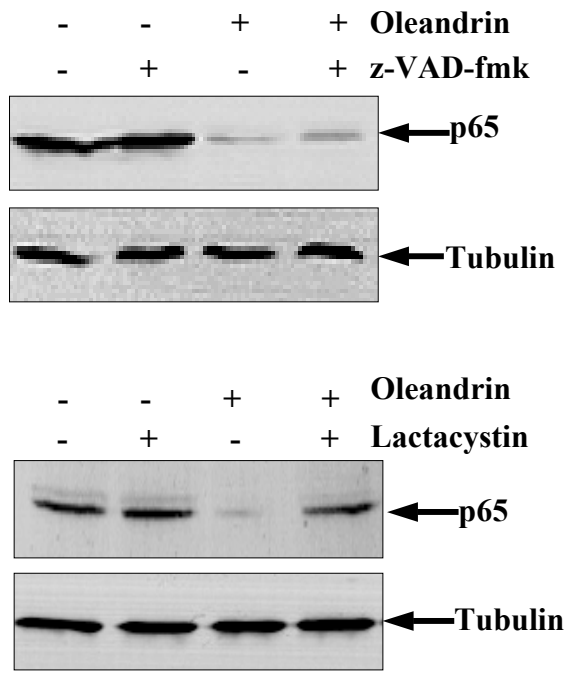


Fig 4.21 Effect of caspase and proteasome inhibitors on oleadrin-mediated downregulation of p65. HuT-78 cells, pretreated with z-VAD-fmk (50 μM) or lactacystin (10 μM) for 2 h were treated with 100 ng/ml oleandrin for 6 h. The level of p65 was detected from cell extracts by Western blot

4.2.15 Lactacystin, but not z-VAD-fmk prevents oleandrin-mediated p65 downregulation.

To detect whether the downregulation of p65 induced by oleandrin is mediated by caspases or proteasome, HuT-78 cells were preincubated with caspases inhibitor, z-VAD-fmk (50 μM) and proteasome inhibitor, lactacystin (10 μM) for 2 h and then treated with oleandrin for 6 h. The lactacystin, but not z-VAD-fmk protected p65 downregulation mediated by oleandrin (Fig.4.21). z-VAD-fmk or lactacystin alone had no effect on the p65 level. These results suggest that proteoasome inhibitor potentially blocks the oleandrin-mediated function. To understand the role of proteasome, HuT-78 cells, pre-treated with ALLN (50 μM) and lactacystin (10 μM) for 2 h were treated with oleandrin (100 ng/ml) for different times. Oleandrin decreased the level of p65 at 6 h of treatment and lactacystin completely protected the downregulation at any given time of oleandrin treatment (Fig.4.22). ALLN pre-treated cells showed almost 50% protection at 6 and 12 h of oleandrin treatment against downregulation of p65 indicating its efficiency. All blots were re-probed with anti-tubulin antibody to show equal loading of proteins.

4.2.16 Oleandrin mediated p65 downregulation is not mediated by ubiquitination.

Proteasome mediated downregulation of proteins is mostly dependend upon the ubiquitination. The ubiquitinated bands are detected by slower mobility in the gel. The result in Figure 4.23 indicates that oleandrin downregulated p65, but lactacystin showed no retarded bands in oleandrin-treated HuT-78 cell extract. The extracts, when immunoprecipitated with anti-ubiquitin antibody followed by detection of p65 by Western blot, did not show any bands (Data not shown) indicating that oleandrin-mediated downregulation of p65 is independent of ubiquitination of p65. The ubiquitinated bands for IκBα were detected from lactacystin-pre-treated followed by TNF stimulated Jurkat cell extracts (Fig.4.23) indicating the control experiment.

4.2.17 Oleandrin does not interact with p65 in vitro.

To detect the direct interaction if any, of oleandrin with p65 protein, different amounts of oleandrin was incubated with 5 μg of GST-p65 protein (full-length p65 with GST cloned, expressed in *E. coli*, and purified) for 6 h. Then p65 was detected by Western blot. The

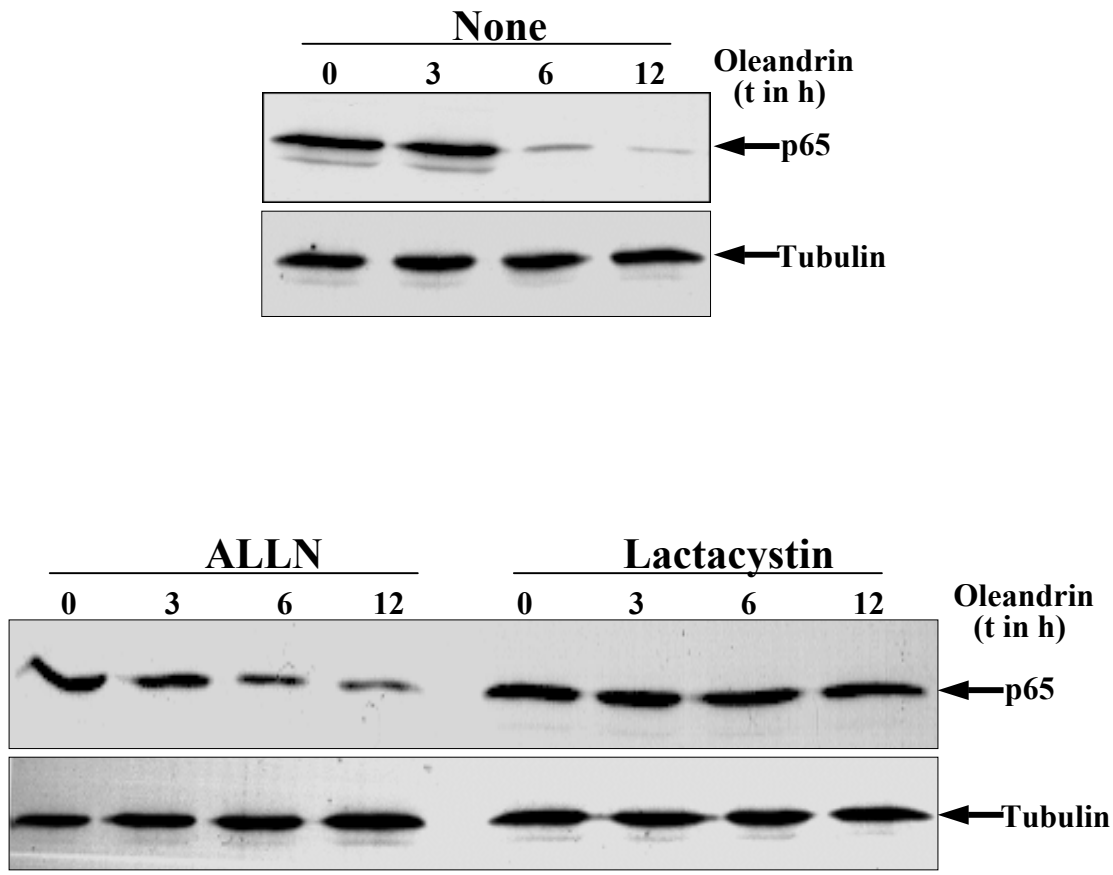


Fig 4.22 Effect of protease inhibitors on oleandrin mediated p65 degradation. HuT-78 cells, pretreated with ALLN (50 μM) and lactacystin (10 μM) for 2 h were treated with 100 ng/ml oleandrin for different times. The level of p65 was detected from extracts by Western blot

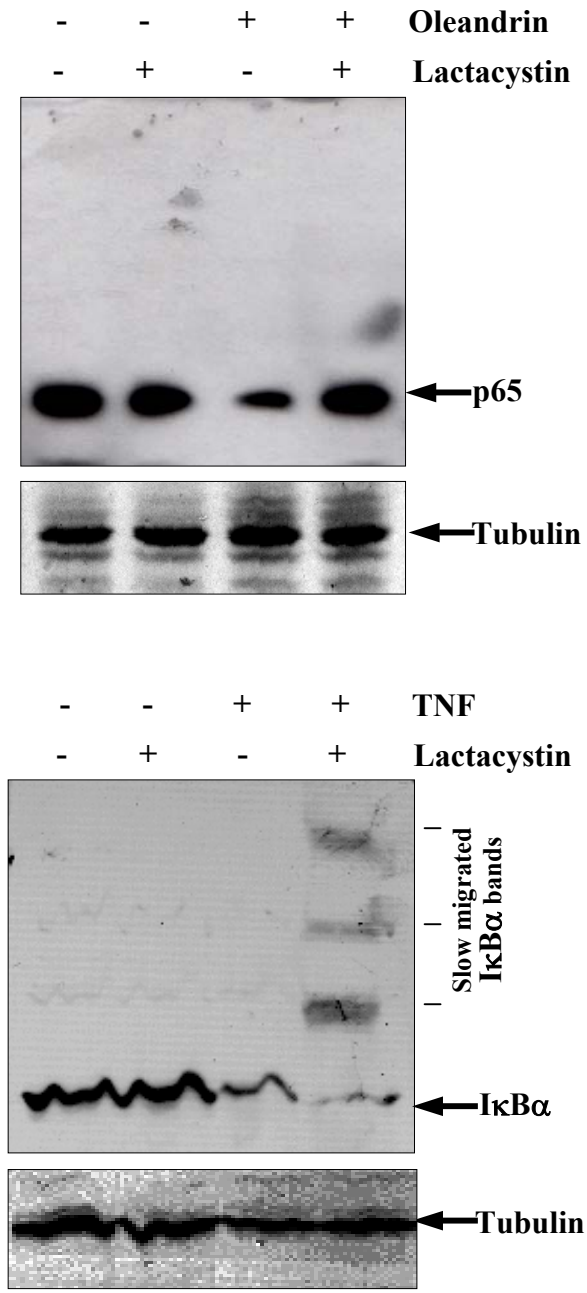


Fig 4.23 HuT-78 cells were pretreated with lactacystin (10 μM) for 2 h followed by treated with 100 ng/ml oleandrin for 6 h. The level of p65 was detected from 100 μg cell extracts by Western blot (**C**). Jurkat cells, pretreated with lactacystin (10 μM) for 2 h were incubated with TNF (1 nM) for 30 min or oleandrin (100 ng/ml) for 6 h. The level of IκBα was detected using 100 μg of cell extracts. All membranes were re-probed with anti-tubulin Ab.

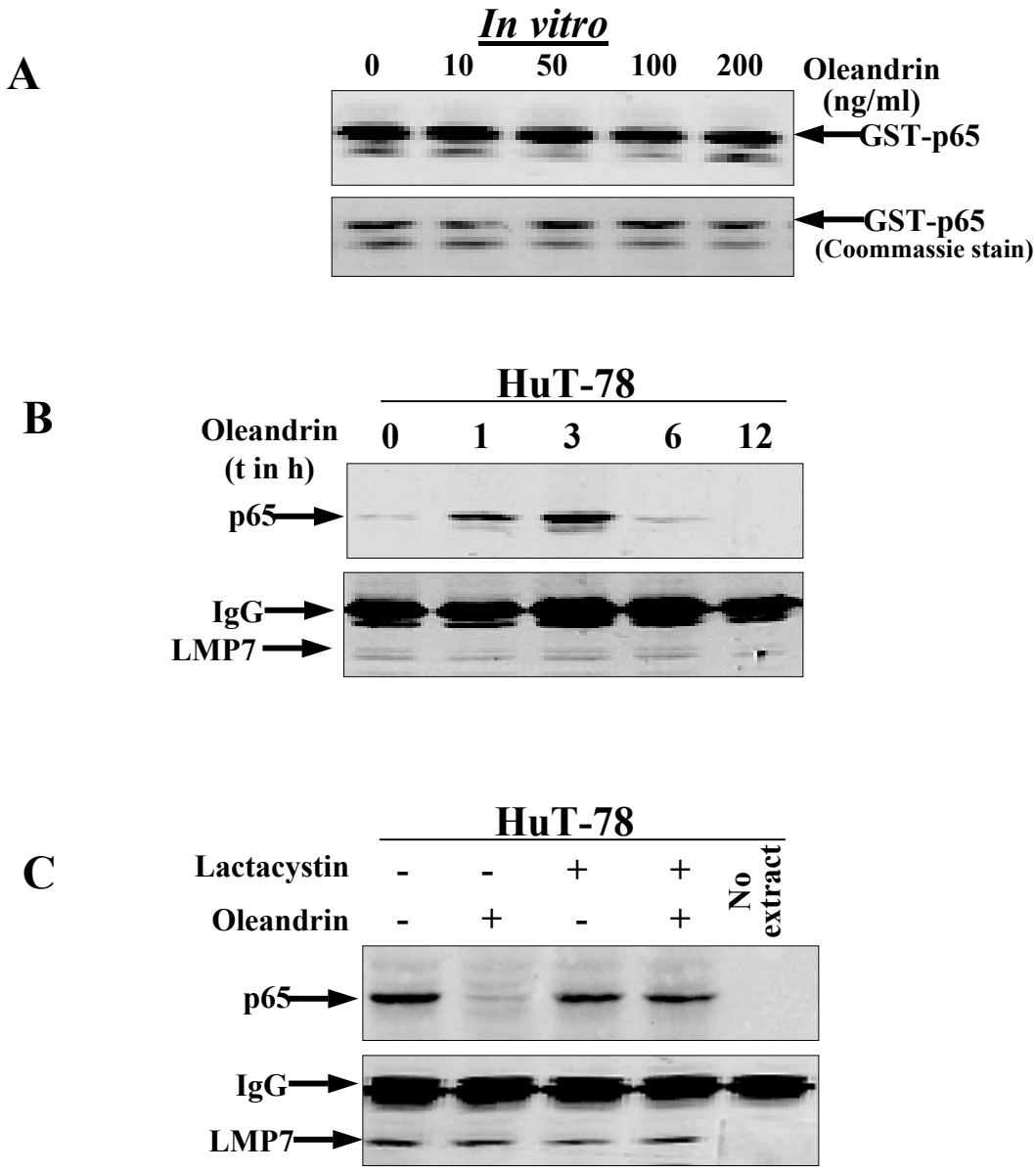


Fig 4.24 A Effect of oleandrin in p65 *in vitro*. Different amount of oleandrin was taken in tubes and incubated with 5 μg of GST-p65 for 6 h. The level of p65 was detected by Western blot. The blot was stripped and stained with Coommassie-blue dye. **B**. **Effect of oleandrin on p65 level.** HuT-78 cells were treated with 100 ng/ml oleandrin for different times and 400 μg cells extract proteins were immunoprecipitated with 5 μg of anti-LMP7 Ab and levels of p65 and LMP7 were detected by Western blot. **C**. **Effect of lactacystin on oleandrin-mediated downregulation of p65.** HuT-78 cells, incubated with lactacystin (10 μM) for 2 h were treated with 100 ng/ml oleandrin for 6 h and 400 μg cells extract proteins were immunoprecipitated with 1 μg of anti-LMP7 Ab and levels of p65 and LMP7 were detected by Western blot.

intensity of bands remained un-altered with different concentrations of oleandrin treatment (Fig.4.24 A), suggesting its inability to downregulate p65 *in vitro*.

4.2.18 Following oleandrin treatment p65 associates with proteasome for its degradation and proteasome inhibitors blocks this degradation.

To detect the possible association of p65 with proteasome following oleandrin treatment, HuT-78 cells were treated with oleandrin for different time intervals and the whole cell extracts were immunopreicipitated with 1 μg of anti-LMP Ab followed by detection of p65 and LMP by Western blot. The levels of p65 were increased till 3 h and then decreased abruptly (Fig.4.24 B) suggesting the association of p65 with proteasome complex upon oleandrin treatment followed by its degradation. The level of LMP (one of the component of proteasome) and IgG remained equal showing immunoprecipitation control. Oleandrin-mediated downregulation of p65 was protected by lactacystin as detected by immunoprecipitation with anti-LMP Ab (Fig.4.24 C). These results suggest that oleandrin-mediated downregulation of p65 occurs via the proteasome.

4.2.19 Lactacystin prevents oleandrin-mediated p65 and NF-κB downregulation.

To detect the role of proteasome inhibitor in oleandrin-mediated p65 downregulation, HuT-78 cells, pre-treated with z-VAD-fmk and/or lactacystin were treated with oleandrin for 6 h. The p65 level was detected from cytoplasmic extracts and NF-κB was assayed from NE. Z-VAD-fmk pre-treated cells did not show any protection of p65 level or NF-κB activity but lactacystin protected the level of p65 or activity of NF-κB DNA binding (Fig.4.25). These results suggest that proteasome, but not caspases inhibitor can protect oleandrin-mediated downregulation of p65 level and NF-κB DNA binding activity.

4.2.20 Proteasome inhibitor partially protects the oleandrin-mediated cytotoxicity.

To understand the role of proteasome on oleandrin mediated apoptosis, HuT-78 cells were pre-incubated with z-VAD-fmk, lactacystin, or in combination for 2 h and then treated with oleandrin for 72 h. The cytotoxicity was assayed by MTT uptake and 'Live & Dead' cell assay. Oleandrin alone induced 60-70% cytotoxicity. Z-VAD-fmk or lactacystin alone did not show significant cytotoxicity, but pre-treated, followed by oleandrin treated cells showed 30-40% cytotoxicity. However, a combination of both completely inhibited oleandrin-induced

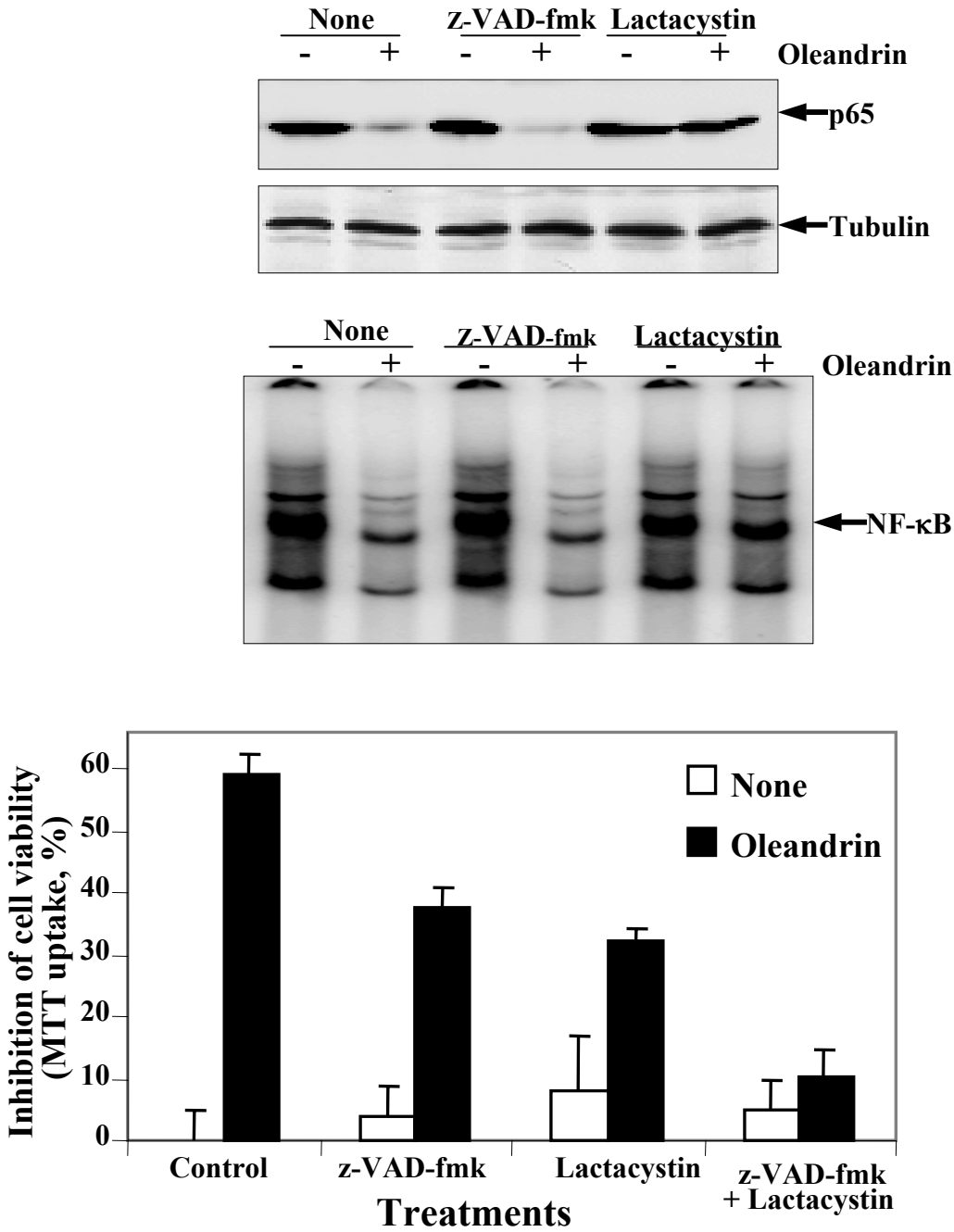


Fig 4.25 Effect of caspase and proteasome inhibitors on oleandrin-mediated downregulation of p65, NF-κB, and cytotoxicity. HuT-78 cells, treated with z-VAD-fmk (50 μM) and lactacystin (10 μM) for 2 h were treated with oleandrin (100 ng/ml) for 6 h. The p65 was detected from cytoplasmic extracts (**A**). The same blot was re-probed with anti-tubulin antibody. Nuclear extracts were used to detect NF-κB by EMSA (**B**). HuT-78 cells were treated with either z-VAD-fmk or lactacystin, or in combination for 2 h and then incubated with oleandrin (100 ng/ml) for 72 h at 37^0C, CO_2 incubator. The cytotoxicity was assayed by MTT method (**C**). The cytotoxicity was represented in percentage in the figure and represented one of the three independent experiments.

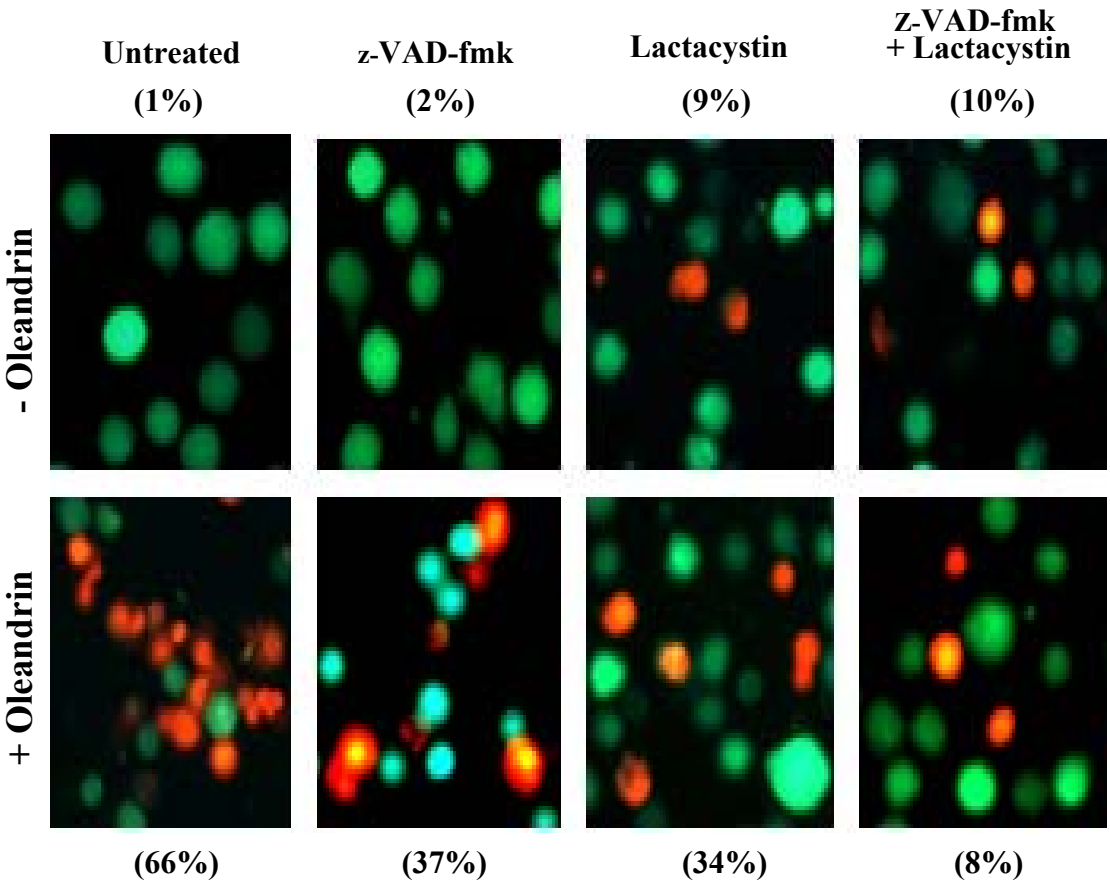


Fig 4.26 Effect of caspase and proteasome inhibitors on oleandrin-mediated cytotoxicity. HuT-78 cells were treated with either z-VAD-fmk or lactacystin, or in combination for 2 h and then incubated with oleandrin (100 ng/ml) for 72 h at 37^0C, CO_2 incubator. The cytotoxicity was assayed by the 'Live & Dead' assay kit.

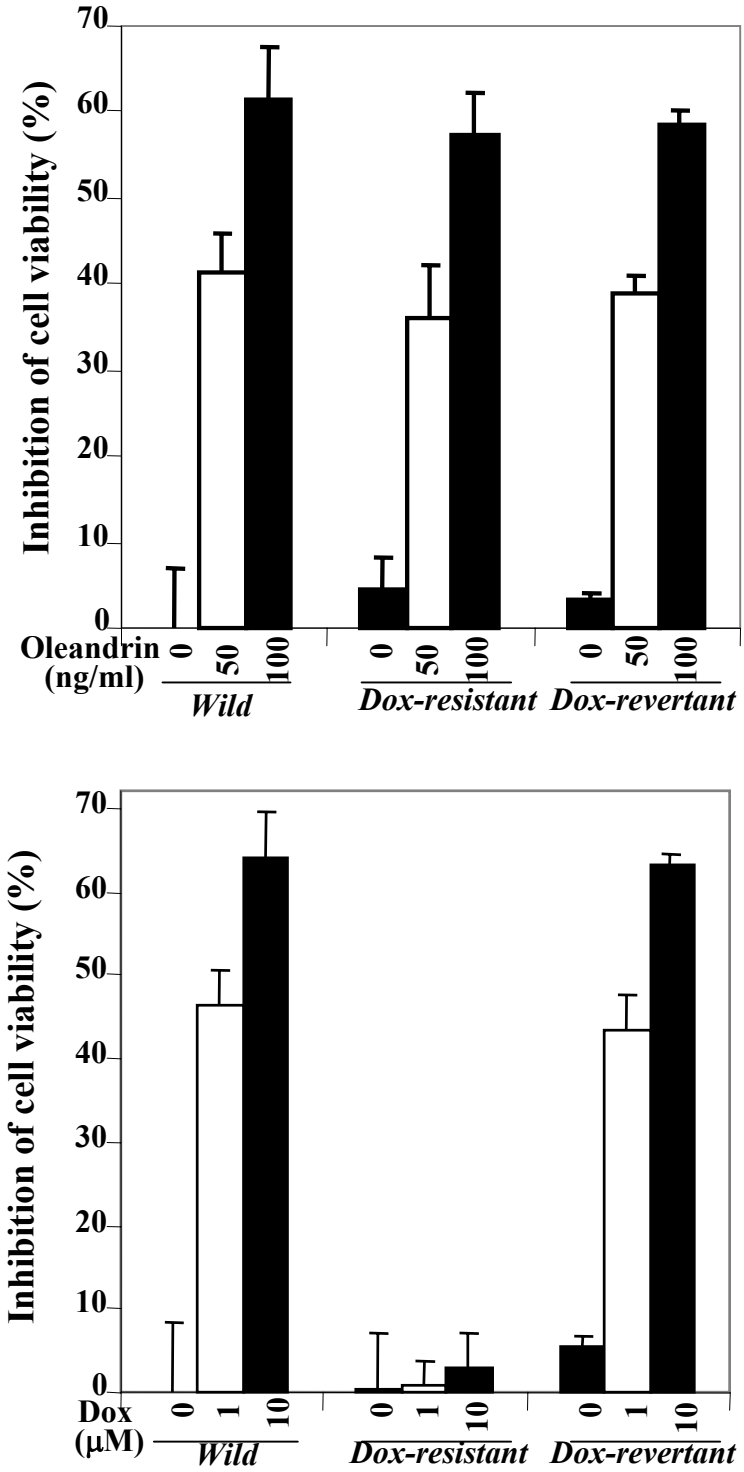


Fig 4.27 Effect of oleandrin on cell viability in doxorubicin-resistant and –revertant cells. MCF-7 (wild), doxorubicin-resistant (Dox.-resistant), and doxorubicin-revertant (Dox-revertant) cells were treated with different concentrations of oleandrin or doxorubicin for 72 h and cell viability was assayed by MTT dye uptake. Results represented as inhibition of cell viability in percentage.

cytotoxicity (Fig. 4.25 & 4.26). The results suggest that oleandrin mediated cytotoxicity is depend on the caspases activation and proteoasome degradation.

4.2.21 Oleandrin mediates p65 downregulation and cytotoxicity in doxorubicin-resistant MCF-7 cells.

To detect the sensitivity of oleandrin in chemotherapeutic drug resistant cells, Dox-resistant, Dox-revertant MCF-7 cells were treated with oleandrin for 72 h and cell viability was detected by MTT uptake. Oleandrin and doxorubicin inhibited cell viability in MCF-7 or Dox-revertant cells (Fig.4.27) Whereas, oleandrin, but not doxorubicin induced cell death in Dox-resistant cells (Fig.4.27). In Dox-revertant cells, oleandrin showed additive cell death with doxorubicin at any concentrations (Fig.4.28). In Dox-resistant cells, oleandrin induced cell death in a dose-dependent manner, to revertant cells (Fig.4.28), suggesting the sensitivity of oleandrin. Dox-resistant cells showed high basal activity of NF-κB and oleandrin downregulated this activity (Fig.4.29). Oleandrin downregulated the level of p65 in wild, Dox-resistant, or Dox-revertant MCF-7 cells (Fig.4.29), suggesting the potency of oleandrin in drug resistant cells.

4.3 Discussion

The induction of apoptosis mediated by oleandrin has been shown via the activation of caspases subsequently leading to the cleavage of several cellular proteins and nuclear fragmentation. Pre-treatment of oleandrin has been shown to inhibit NF-κB activation in diverse cell types (Manna *et al*., 2000b; Sreenivasan *et al*., 2003a) and is implicated to be cytotoxic to various tumour cells (Pathak *et al*., 2000; McConkey *et al*., 2000). NF-κB plays an important role in cell proliferation and cancer progression. Furthermore several reports indicate that constitutive or induced NF-κB expression leads to resistance of apoptosis in tumour cells stimulated by a wide variety of agents (Wang *et al*., 1999; Waddick and Uckun, 1999; Pathak *et al*., 2000; McConkey *et al*., 2000). Hence in this report we have investigated the means by which oleandrin mediates cell death in NF-κB-expressing cells.

Further in this study we demonstrate that oleandrin blocks NF-κB activation and also induces cell death in NF-κB expressing cells (SA-LPS/Jkt, which shows sustainable induction of NF-κB and HuT-78, which has constitutive expression of NF-κB)(Fig 4.1.1, 4.2, 4.12 & 4.13). As NF-κB-expressing cells have been reported to be resistant to different apoptosis inducers-

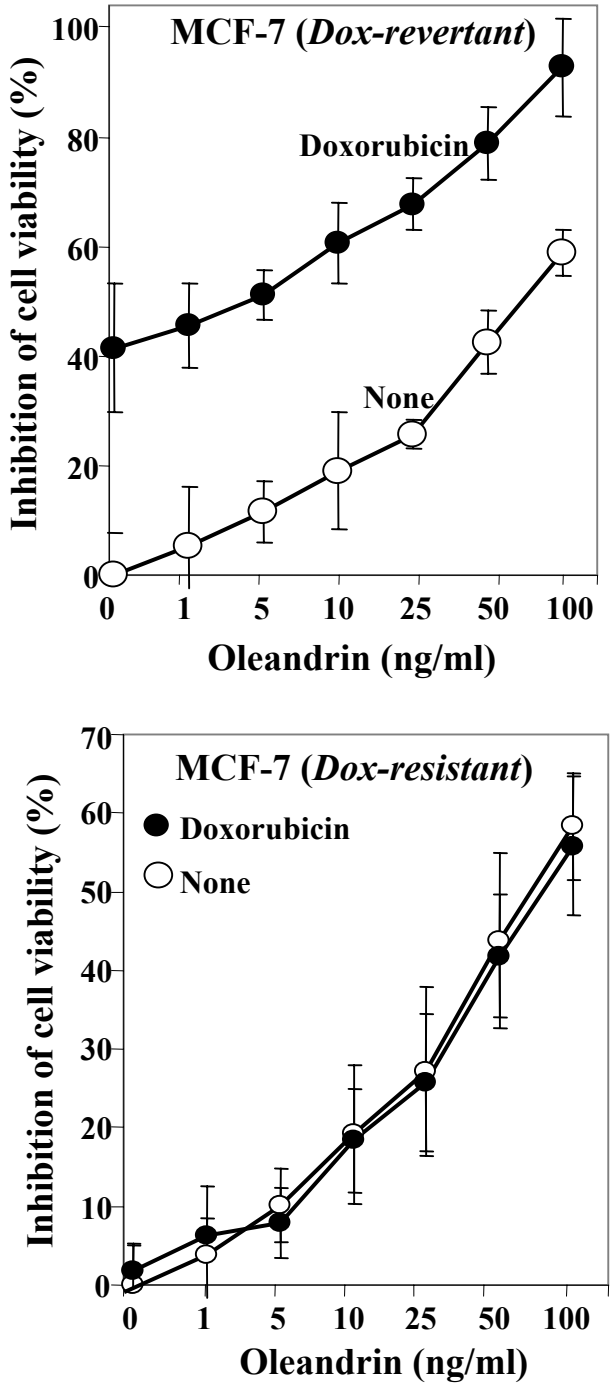


Fig 4.28 Effect of oleandrin on cell viability in doxorubicin-resistant and –revertant cells. Dox-revertant and Dox-resistant cells were treated with different concentrations of oleandrin for 72 h and then MTT was assayed. Results represented as inhibition of cell viability considering 100 % absorbance for un-stimulated cells.

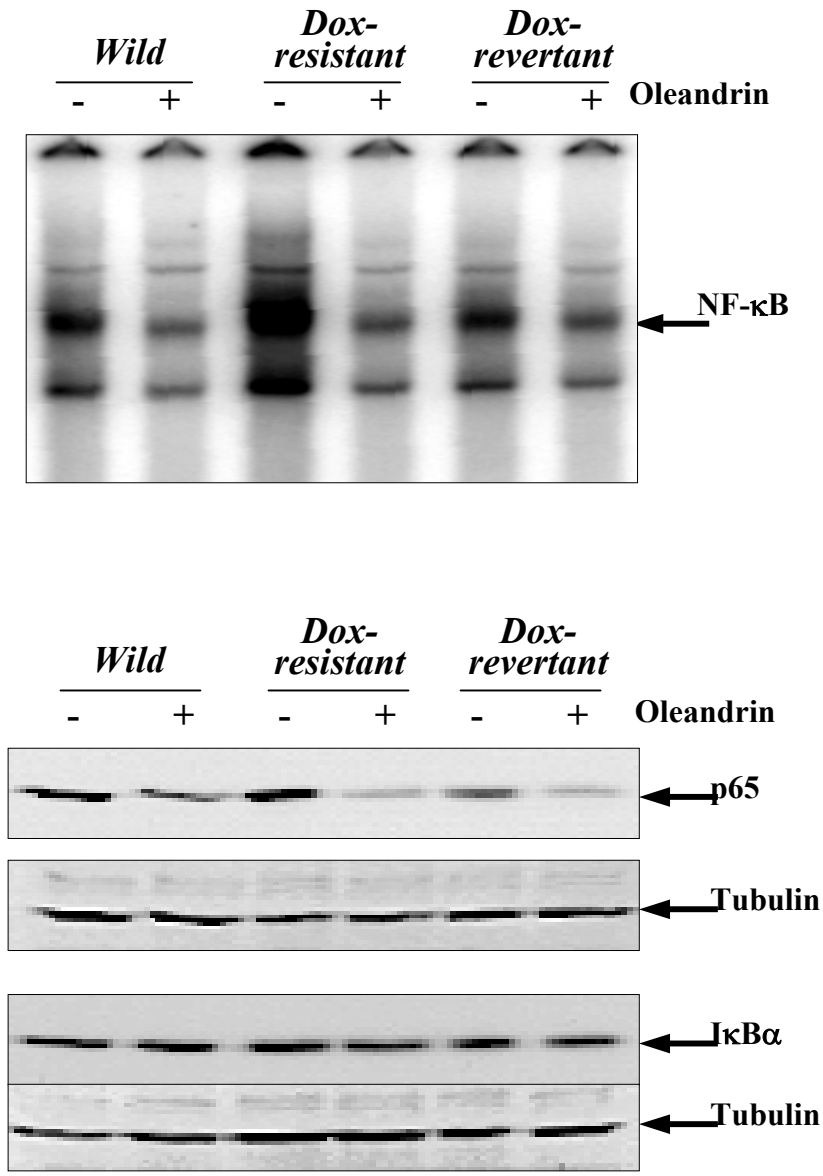


Fig 4.29 Effect of oleandrin on the levels of p65, IκBα, and NF-κB in Dox-resistant and Dox-revertant cells. Wild, Dox-resistant, and Dox-revertant cells were treated with oleandrin for 6 h and then NF-κB DNA binding and levels of p65 and IκBα and were assayed.

mediated cell death, these observations might have interest (Manna and Aggarwal, 1999; Sreenivasan *et al*., 2003b). Oleandrin decreases the levels of p65, a constituent subunit of NF-κB without changing IκBα or p50 levels in the cells suggesting the possibility that oleandrin induced cytotoxicity might be mediated through the suppression of NF-κB activity, which is confirmed by the observation showing inhibition of NF-κB reporter gene activity (Fig 4.2). According to the results it is noted that oleandrin fails to alter the DNA binding activity of housekeeping transcription factor Sp1 and also p53 (Fig 4.8) in HuT-78 cells. Further oleandrin down regulated c-Rel but not Rel B or p50 (Fig 4.9), also there was a decrease in the levels of cyclin D1, cyclin E1 and phospho-Rb in oleandrin treated cells (Fig 4.11). The decrease in cyclin D1 and E1 could be directly correlated to the decrease in p65 levels, as the latter are NF-κB dependent gene products. The possible involvement of cellular phosphatases or kinases in lowering the levels of phospho-Rb remains elusive. Also the ability of oleandrin to induce a decrease in c-Rel levels has to be further investigated. There are various inhibitors reported to down regulate NF-κB activation either by inhibiting IKK activity, IκB Phosphorylation and subsequent degradation or p65 phosphorylation.(Manna *et al*., 2000a; Epinat and Gilmore 1999; Egan *et al*., 1999) Surprisingly, oleandrin inhibits p65 levels (Fig.4.3 & 4.4) and it seems that the down regulation is targeted on the protein perse and not on its expression pattern as evident from the unchanged levels of p65 mRNA taken from cells treated with oleandrin for various time intervals. (Fig.4.11). Also the increased levels of p65 as seen both in the cytoplasm and nucleus of NF-κB expressing cells were decreased upon oleandrin treatment (Fig.4.6 & 4.4). Pretreatment of oleandrin showed inhibition of IKK activity and IκBα degradation induced by SA-LPS (Fig.4.7) which is similar to results earlier reported (Manna *et al*., 2000b) obtained by using TNF as an inducer. Surprisingly, oleandrin did not inhibit IKK activity or IkBα level (Fig 4.7) in NF-κB expressing cells where higher levels of IkBα and higher activity of IKK were observed which is further proved in IKK transfected cells. The increased levels of IkBα and activity of IKK can be correlated to the constitutive and induced activation of NF-κB in NF-κB expressing cells also IκBα expression is NF-κB dependent. As mentioned earlier IκBα sequesters the p50-p65 complex in the cytoplasm, thereby making NF-κB non-avaliable for transcription regulation. Oleandrin is shown to down regulate p65 level in IkBα-DN cells (Fig 4.4 & 4.16 B1) proving that irrespective of the masking of p50-p65 complex by IκBα oleandrin is able to down regulate

p65, which explains for the decreased p65 level in the cytoplasm. Further oleandrin induces cell death in Jurkat cells and also in SA-LPS/Jkt or HuT-78 cells as shown by MTT based cytotoxicity assay. Oleandrin activates caspase-3 subsequently inducing PARP cleavage and DNA fragmentation in NF-κB expressing cells confirming the induction of apoptosis in these cells. Also oleandrin was able to mediate p65 down regulation and cell death in p65 or IKK over expressed cells.

Results so far indicate that oleandrin is able to down regulate p65 levels in cells processing high NF-κB activity and also mediate cell death unlike TNF or ceramide which fail to exert their action in NF-κB expressing cells as reported earlier. (Sreenivasan *et al*., 2003) Hence the question asked was what mediates oleandrin-induced down regulation of p65? Oleandrin did not induce p65 down regulation *in vitro* proving that oleandrin does not interact directly with p65. The induction of caspase 3,8 and 9 is seen in oleandrin treated cells, however z-VAD-fmk a broad-spectrum caspases inhibitor was unable to protect oleandrin mediated p65 degradation. This shows that caspases have no role in oleandrin-induced action on p65. But lactacystin, the proteosome inhibitor was able to fully protect oleandrin mediated p65 degradation also ALLN, a weak proteosome inhibitor was able to protect p65 degradation partially. (Fig 4.21 & 4.22) The proteosome inhibitor, lactacystin alone induced cell death by 9, 28 and 46% at 10, 50 and 100 μM concentrations respectively at 72 hours post treatment, which is similar to observations reported earlier (Dong *et al*., 1999; Cui *et al*., 1997). Hence a concentration of 10 μM of lactacystin was used to detect oleandrin mediated p65 down regulation. As the proteasome mediated protein cleavage is known to occur in the cytoplasm, nuclear p65 shuttling and subsequent degradation in the cytoplasm might explain for the decreased levels of p65 both in the nucleus and cytoplasm. (Fig.4.4). Proteasome mediated protein degradation is usually preceded by ubiquitination on p65 as the ubiquitinated (slow migrated) bands were not seen in lactacystin pretreated and oleandrin treated cells (Fig.4.23) as seen in the case of TNF-induced IkBα ubiquitination (Fig.4.23). Further oleandrin mediates the association of p65 with the proteosome as indicated by immunoprecipitation experiments wherein the inducible subunit of the proteosome, LMP7 is co-immunoprecipitated with p65 subsequently leading to p65 degradation at greater time intervals. However the proteosome inhibitor is able to only partially protect oleandrin mediated cell death. But a combination of both caspases and proteosome inhibitors completely inhibited oleandrin induced cytotoxicity (Fig.4.25 & 4.26) in NF-κB expressing cells. Several observations suggest that high NF-κB activity in tumor cells

could be a major cause for chemo resistance (Wang *et al*., 1999; Waddick and Uckun, 1999; Pathak *et al*., 2000; McConkey *et al*., 2000). Hence in an attempt to address this doxorubicin resistant or revertant (similar to parental MCF-7) cells where challenged with oleandrin. It is shown that oleandrin mediates cell death in the above-mentioned cells unlike doxorubicin, which was able to do so only in the revertant MCF-7 cells (Fig 4.27 & 4.28). Doxorubicin resistant cells showed high NF-κB activity and oleandrin countered this activity also there was a decrease in the p65 level, suggesting the potency of oleandrin in drug resistant cells which may be attributed to the lowering of NF-κB activity.

We report here for the first time that degradation of p65 is one of the key mechanism of oleandrin mediated down regulation of NF-κB activity and cell death in NF-κB expressing cells. The ubiquitin independent proteosome mediated p65 degradation is an interesting observation made, which has not been reported aprior. However, the mechanism by which oleandrin is able to induce the proteosome remains elusive. Interestingly, both caspases and the proteosome are activated upon oleandrin treatment, which collectively contribute to the cell death. Hence activation of the proteosome leading to the degradation of p65 could be visualized as an important advantage conferred by oleandrin in mediating cell death in addition to apoptosis mediated by the caspases thereby, neutralizing the proliferative drive induced by increased NF-κB activity in NF-κB expressing cells. As most of the tumor cells have high NF-κB activity, oleandrin may serve as a potential chemotherapeutic agent especially in rapidly proliferating and progressive tumors or in chemoresistant cells. Further this study may find immense application in underlining strategies for the design of pharmaceutical interventions for cancer.

Chapter Five

Oleandrin mediates inhibition of IL-8 induced biological responses

5.1 Introduction.

During chronic inflammatory diseases such as rheumatoid arthritis, gout, asthma, or inflammatory bowel disease, uncontrolled accumulation of neutrophils at the site of infection and residential macrophages liberate inflammatory molecules such as cytokines, reactive oxygen intermediates, and proteolytic enzymes which become major contributors to tissue damage (Pillinger and Abranson, 1995; Dallegri and Ottonello, 1997). Thus, regulation of neutrophil and macrophage recruitment into inflammatory sites and their clearance are critical processes assuring effective host defense without tissue injury. Extravasation of neutrophils from blood vessels requires adhesion to vascular endothelial cells and subsequent migration of these cells into the tissue (Feng *et al.*, 1998). These events are mediated mostly by chemokines such as IL-8 (Baggiolini *et al.*, 1994; Baggiolini *et al.*, 1997). Interleukin (IL)-8, a cytokine of the CXC chemokine family is widely recognized for its capacity to rapidly induce leukocyte migration and recruitment during the inflammatory response generated due to infection or following tissue damage (Gangur *et al.*, 2002). IL-8 binds to two distinct receptors, IL-8R1 and IL-8R2 with a high affinity (Holmes *et al*, 2000; Murphy and Tiffany, 1991). They consist of 350 to 360 aminoacids and are membrane bound molecules composed of seven-transmembrane domains coupled to G-proteins at the C-terminal portion and possibily the third intracellular loop, hence called G-protein coupled receptor (GPCR) (Murdoch, 2000; Murphy, 1994). After ligand binding, human IL-8 receptors are internalized and subsequently recycled and reappear on the cell surface rapidly within 60 minutes. IL-8 is also known as one of the earliest growth factors expressed in wound repair during the early stages of angiogenesis, resulting in neutrophil, macrophage and endothelial cell chemotaxis (Belperio *et al.*, 2000). These different cell types in turn release protease, increase expression of adhesion molecules, express crucial angiogenic growth factors such as FGF and VEGF for the continuation of vascular repair (Klagsbrun and Moses, 1999). IL-8 was originally classified as a neutrophil chemoattractant, is now reported to play an important role in tumor progression, angiogenesis and metastasis in a variety of human cancers (Itoh *et al*., 2005) IL-8 is also highly expressed in several cancers (Inoue *et al*., 2000; Shi *et al.*, 1999; Singh and Varney, 2000) where it serves as an autocrine growth factor, a chemoattractant, or a principal angiogenic component. The biological activity of IL-8 in tumors and the tumor microenvironment may contribute to tumor progression through its potential function in the regulation of angiogenesis, cancer cell growth

and survival, tumor cell motion, leukocyte infiltration and modification of immune responses. (Itoh *et al*., 20050 IL-8 is a transcriptional target of Ras signaling. Extracellular signal related kinase (MEK) also induces IL-8 expression. [Sparman and Bar-Sagi, 2004] It is shown that stimulation of GPCR can induce shedding of epidermal growth factor (EGF) ligands via activatio of a disintegrin and metalloprotease with subsequent transactivation of the EGF receptor(EGR) thus aiding cell proliferation. Evidence shows that cancer cell and stromal cell interaction can stimulate boths the cells in the expression of IL-8 and other growth factors. (Yuan *et al.*, 2005) A strong correlation exits between the metastatic potential of breast carcinoma cell lines and their ectopic expression of IL-8. The undifferenciated highly metastatic cell lines with high metastatic potential produce much more IL-8 than their differenciated lower metastatic counterparts (De Larco *et al*., 2003). Anti-IL-8 antibody administered mice showed decrease in implanted lung cancer or melanoma tumors, which correlated with reduced tumor angiogenesis (Yatsunami *et al.*, 1997; Huang *et al.*, 2002).

IL-8 interacts with its receptors, IL-8Rs and internalize rapidly by endocytosis. Endocytosed receptors are recycled back to the cell surface. The IL-8 receptors are of two types (type A and B). The type A receptor (IL-8R1) binds IL-8 with high affinity but shows low affinity to MGSA, whereas type B receptor (IL-8R2) binds to all the three chemokine with high affinity. The cyclic process of endocytosis and subsequent recycling of the receptor are intimately associated with the IL-8 mediated biological responses **(**Samanta ***et al.*****,** 1990; Murphy and Tiffany, 1991; Holmes ***et al.*****,** 1991; Lee ***et al.*****,** 1992). Since the cytokine-mediated response is related to the expression of functionally active IL-8R, modulation of the receptor at the ligand interacting domain may be a viable strategy for the regulation of IL-8-induced inflammatory responses and angiogenesis.

Nuclear transcription factor kappa B (NF-κB) regulates the expression of various genes that play critical roles in inflammation, viral replication, tumorigenesis, and apoptosis (Sen and Baltimore, 1986; Baeuerie and Baichwal, 1997; Baichwal and Baeuerie, 1997) and therefore, this factor is an ideal target of pharmaceutical interest (Lee and Burckart, 1998). IL-8 production from different cells is regulated by NF-κB (Message and Johnston, 2004; Brasier ***et al.***, 1998; Yang ***et al.***, 2001). Recently, we reported that IL-8 induces NF-κB through recruitment of TRAF6 and IRAK (Manna and Ramesh, 2005). The possible strategies for ameliorating the inflammatory distress may be either by prevention of excessive neutrophil

migration by reducing the interaction of neutrophils with the inflammatory cytokine or by regulating IL-8 production. As NF-κB expression is the key feature of all cancerous cells, it is important to regulate this factor and to understand their mechanism of action. Different plant products exhibit chemopreventive effects (Wattenberg, 1990). Oleandrin, a polyphenolic cardiac glycoside derived from the leaves of *Nerium oleander* has been used to treat congestive heart failure and shown to be toxic to a wide variety of tumor cells (Gupta and Chopra, 1985; Gupta ***et al.***, 1986; Gupta and Chopra, 1987). The cardiac glycosides, especially oleandrin and oleandrigenin, inhibit fibroblast growth factor (FGF)-2 export through membrane interaction with the Na^+, K^+-ATPase pump (Smith ***et al.***, 2001). Oleandrin has been shown to downregulate TNF- or ceramide-induced NF-κB activation in different tumor cells, but not in primary cells (Manna ***et al.***, 2000; Sreenivasan ***et al.***, 2003). The effect of oleandrin on IL-8-mediated responses has not been detected in the primary cells such as macrophages and neutrophils so far.

In this study it is demonstrated, for the first time, that oleandrin inhibits IL-8 mediated biological responses by downregulating IL-8Rs in neutrophils and macrophages. Downregulation of IL-8Rs by oleandrin is mediated through alteration of membrane fluidity and microviscosity, which may modulate the surface IL-8R for IL-8 binding without changing the affinity. Downregulation of IL-8Rs was protected by phospholipids when pre-incubated with oleandrin. Overall, the data suggest the downmodulation of IL-8Rs by oleandrin through altering fluidity of plasma membrane followed by inhibition of IL-8-mediated biological responses in macrophages, monocytes, T- and B-cells, and neutrophils, which might have potential therapeutic approaches for neutrophil- and/or macrophage-driven inflammatory diseases as well as neovascularization in cancer metastasis. The effects of oleandrin to downregulate various receptors in macrophages are also important findings of this study.

5.2 Results.

In this study, the effect of oleandrin on IL-8-induced biological responses in different cell types was examined. THP1 cells were converted into macrophages induced with 10 ng/ml PMA for 16 h and HL-60 cells were converted into neutrophils by 1.3% DMSO in 2 days. These cells were used in most of the experiments. After conversion, macrophages were cultured for 48 h without decreasing cell viability (cell viability was 99.66 ±4.42, 98.44 ± 5.46,

97.66 ± 5.08, and 94.26 ± 5.52 % at 0, 24, 36, and 48 h of incubation respectively) and for neutrophils the cell viability was 99.02 ±2.24, 97.34 ± 3.36, 94.78 ± 3.24, and 85.46 ± 5.52 % at 0, 24, 36, and 48 h of incubation respectively.

5.2.1 Oleandrin inhibits IL-8-, FMLP-, EGF-, or NGF-, but not IL-1-, or TNF-induced NF-κB activation.

To detect the role of oleandrin on IL-8-, IL-1-, FMLP-, TNF-, EGF-, or NGF-induced NF-κB activation in macrophages, cells were incubated with different concentrations of oleandrin for 3 h and then stimulated with IL-8 (100 ng/ml), FMLP (100 pM), IL-1 (100 ng/ml), EGF (100 pM), NGF (100 pM)), or TNF (100 pM) for 2 h at 37^0C. Nuclear extract (NE) was prepared and assayed for NF-κB by gel shift assay as described in Materials and methods. NF-κB activation was observed in macrophages by all these inducers and oleandrin inhibited NF-κB activation induced by IL-8 (Fig. 5.1), FMLP (Fig.5.2), EGF (Fig.5.4), or NGF (Fig.5.3), but not significantly by IL-1 (Fig.5.2) or TNF (Fig.5.3). The data suggest that oleandrin inhibited IL-8-, FMLP-, EGF-, or NGF-induced, but not TNF- or IL-1-induced NF-κB activation on macrophages.

To detect the IL-8-mediated activation by binding of IL-8-to-IL-8 receptor, macrophages were pretreated with anti-IL-8R antibody and then stimulated with IL-8 for 2 h to rule out possibility of NF-κB activation due to endotoxin contamination. In other sets, cells were stimulated with anti-IL-8 antibody (1 μg) or polymixin B-sulphate (10 μg) with IL-8 (100 ng/ml) mixture (incubated for 1 h at 37^0C). Nuclear extracts were then used to detect NF-κB. The results showed that polymixin B-sulphate-IL-8 mixture treated cells did not inhibit IL-8-induced NF-κB. Either anti-IL-8 antibody-IL-8 mixture or anti-IL-8R antibody followed by IL-8 stimulation completely inhibited NF-κB activation (Fig.5.4) suggesting that IL-8 is free of endotoxin contamination, NF-κB activation is due to IL-8, and IL-8 mediates NF-κB activation through its receptors in macrophages.

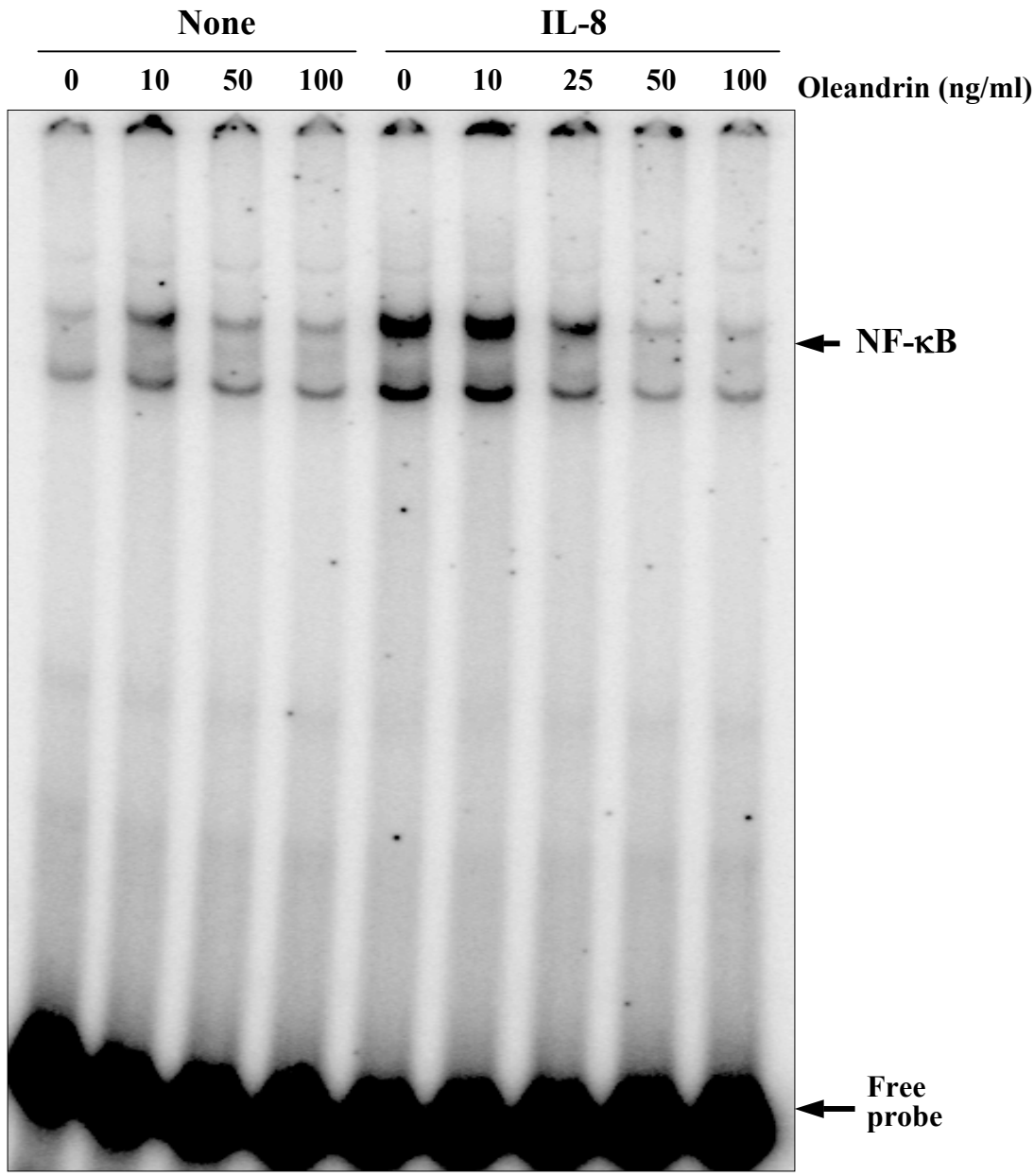


Fig 5.1 Effect of oleandrin on IL-8-induced NF-κB activation. THP1 cells at 50% confluency treated with 10 ng/ml PMA for 16 h and then cells were washed with fresh medium. Cells were then treated with different concentrations of oleandrin for 3 h followed by stimulation with 100 ng/ml IL-8 for 2hrs.

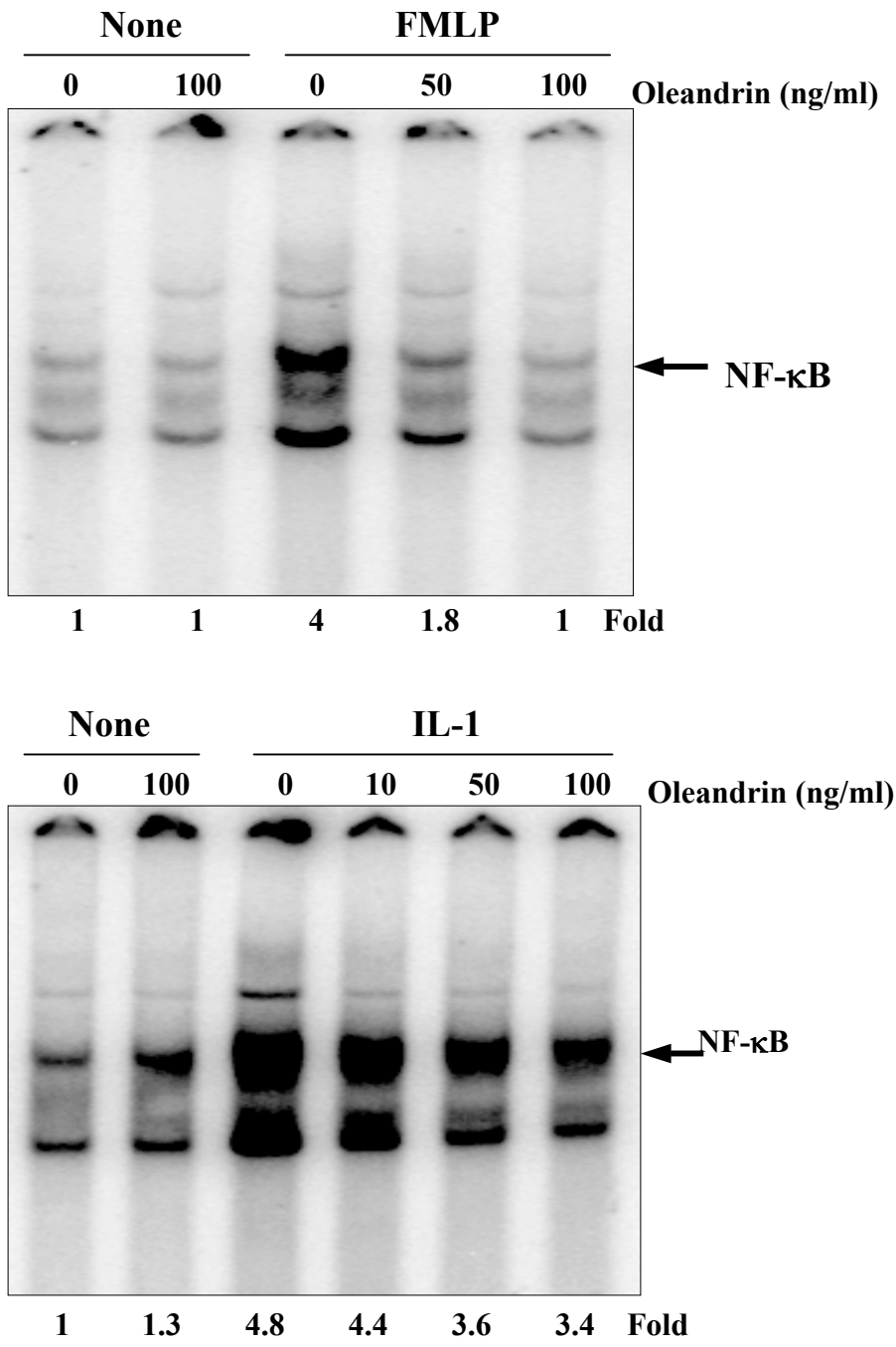


Fig 5.2 Effect of oleandrin on FMLP-or IL-1-induced NF-κB activation. THP1 cells at 50% confluency treated with 10 ng/ml PMA for 16 h and then cells were Washed with fresh medium. Cells were then treated with different concentrations of oleandrin for 3 h followed by stimulation with 100 pM FMLP or 100 ng/ml IL-1 for 2hrs.

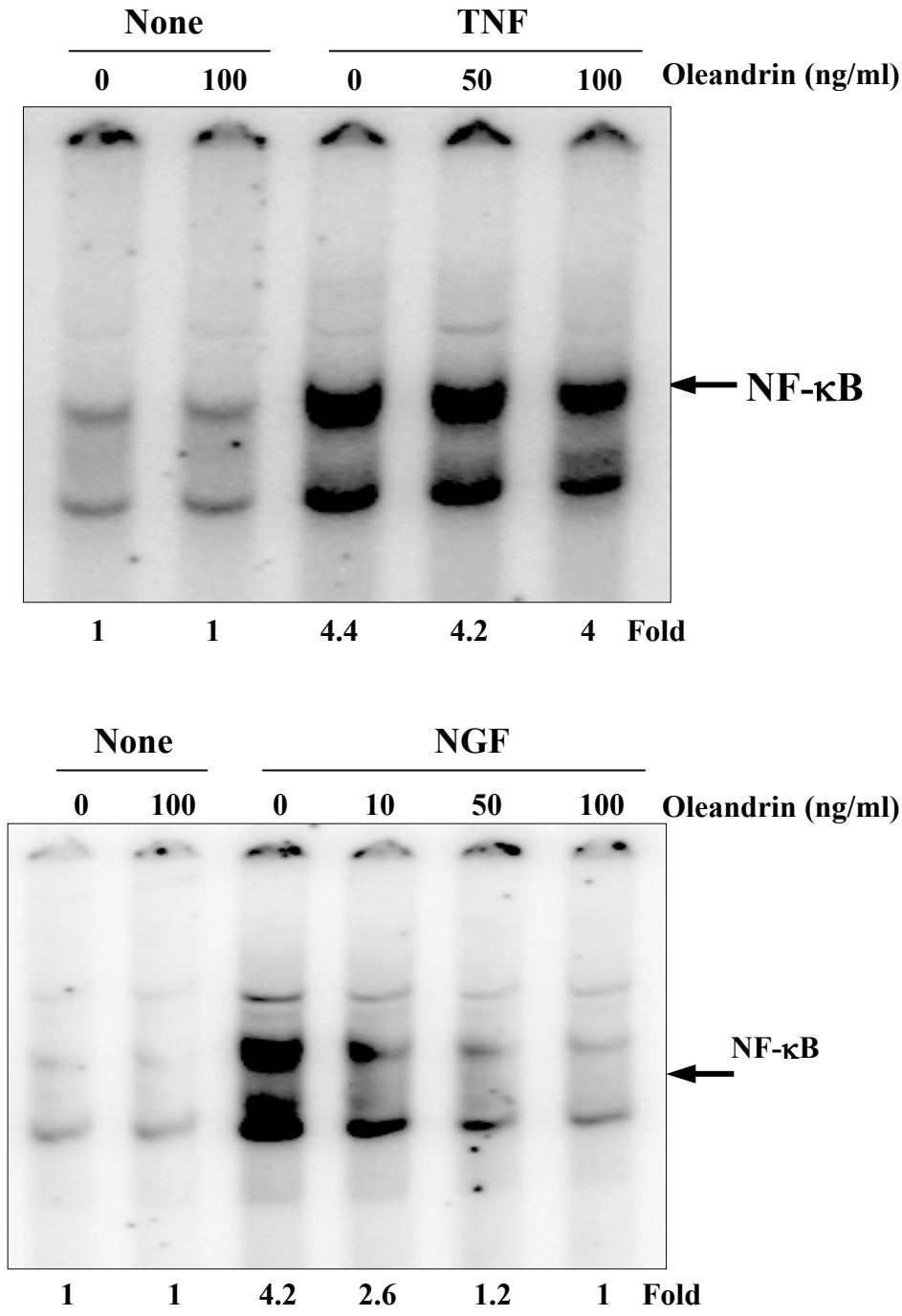


Fig 5.3 Effect of oleandrin on TNF- or NGF-induced NF-κB activation. THP1 cells at 50% confluency treated with 10 ng/ml PMA for 16 h and then cells were washed with fresh medium. Cells were then treated with different concentrations of oleandrin for 3 h followed by stimulation with 100 pM TNF or 100 ng/ml NGF for 2 hrs.

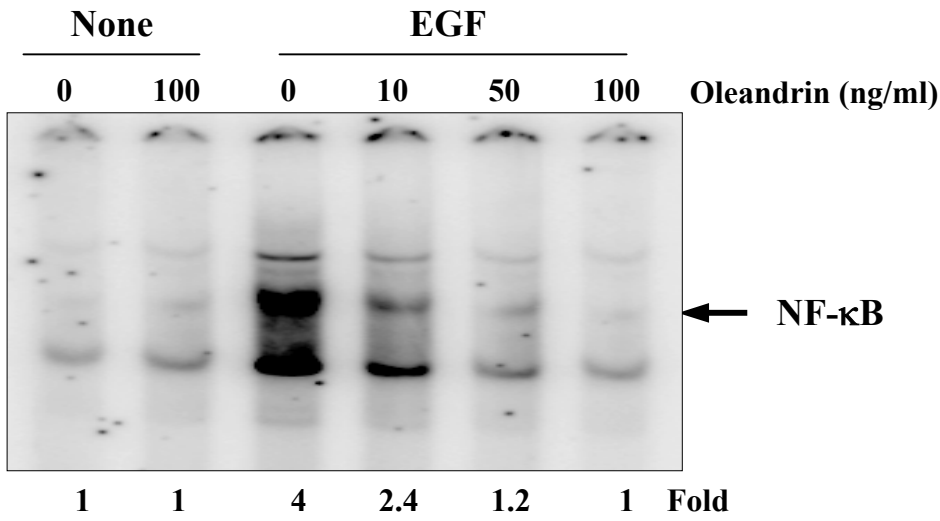


Fig 5.4 Effect of oleandrin on EGF-induced NF-κB activation. THP1 cells at 50% confluency treated with 10 ng/ml PMA for 16 h and then cells were washed with fresh medium. Cells were then treated with different concentrations of oleandrin for 3 h followed by stimulation with 100 pM EGF for 2 hrs.

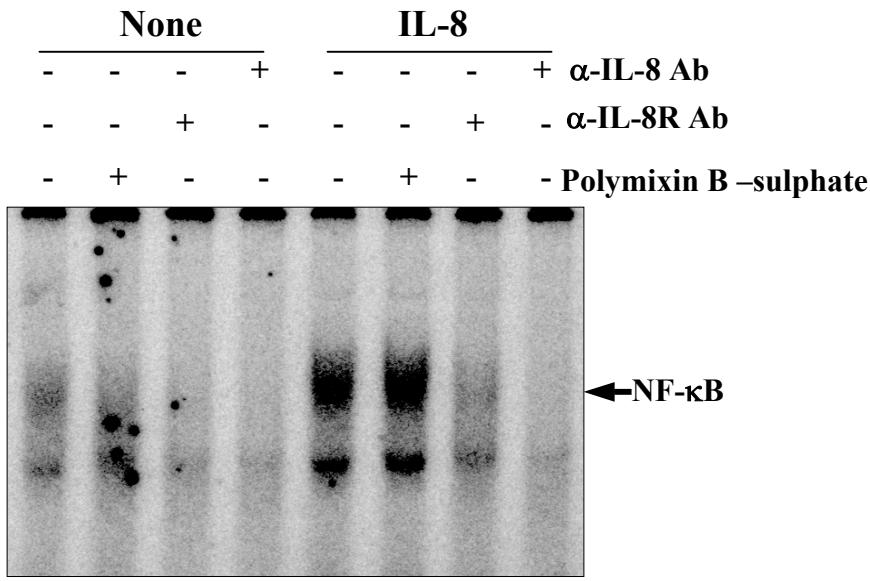


Fig 5.5 Effect of IL-8 antibodies on NF-κB activation induced by IL-8. Macrophages were pretreated with anti-IL-8R antibody and then stimulated with IL-8 for 2 h. In other sets, cells were stimulated with anti-IL-8 antibody or polymixin B-sulphate and IL-8 (100 ng/ml) mixture (incubated for 1 h at 37^0C). Nuclear extracts were prepared and assayed for NF-κB.

5.2.2 Optimum time required for oleandrin-mediated inhibition of IL-8-induced NF-κB activation.

To determine the time it took for oleandrin to inhibit IL-8-induced NF-κB activation, cells were incubated with oleandrin for 240, 180, 120, 60, or 30 min before the addition of IL-8; oleandrin was added at the same time as the IL-8; or cells were incubated with oleandrin 60 min after the addition of IL-8. In all instances the cells were treated with IL-8 for 120 min. NF-κB activation was completely inhibited in cells at least 180 min pre-treatment of oleandrin (Fig.5.6).

5.2.3 Oleandrin inhibits IL-8-induced phosphorylation and degradation of IκBα and IKK activation.

The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα [Sarkar ***et al***, 2004]. To determine whether the inhibitory action of oleandrin was due to an effect on IκBα degradation, the IκBα proteins was detected from cytoplasmic extracts and NF-κB was assayed from nuclear extracts. Oleandrin inhibited IL-8-induced (time-dependent) NF-κB activation (Fig.5.7). IκBα degradation started at 15 min of IL-8 (100 ng/ml) treatment in macrophages and completed at 30 min. The presence of oleandrin inhibited IL-8-induced IκBα degradation (Fig.5.9). To determine whether oleandrin modulates TNF-induced IκBα phosphorylation, Western blot with antibodies against the phosphorylated form of IκBα was performed. Oleandrin was found to inhibit IL-8-induced phosphorylation of IκBα (Fig.5.9). Oleandrin-pretreated cells showed complete inhibition of IL-8-induced IKK activation (Fig.5.10) whereas, oleandrin did not inhibit immunoprecipitated IKKs from IL-8-stimulated cells extract at any concentrations *in vitro* (Fig. 5.11) suggesting oleandrin's effect lies more upstream of IL-8-mediated signaling.

5.2.4 Activated NF-κB inhibited by oleandrin consists of p50 and p65 subunits.

To detect the composition and specificity of the induced band visualized by EMSA, nuclear extracts from IL-8-activated macrophages were incubated with antibodies (Abs) p50 (NF-κBI), p65 (Rel A), or in combination and 50-fold excess of cold or mutant NF-κB. Abs to either subunit of NF-κB shifted the band to a higher molecular weight (Fig. 5.8), thus suggesting that

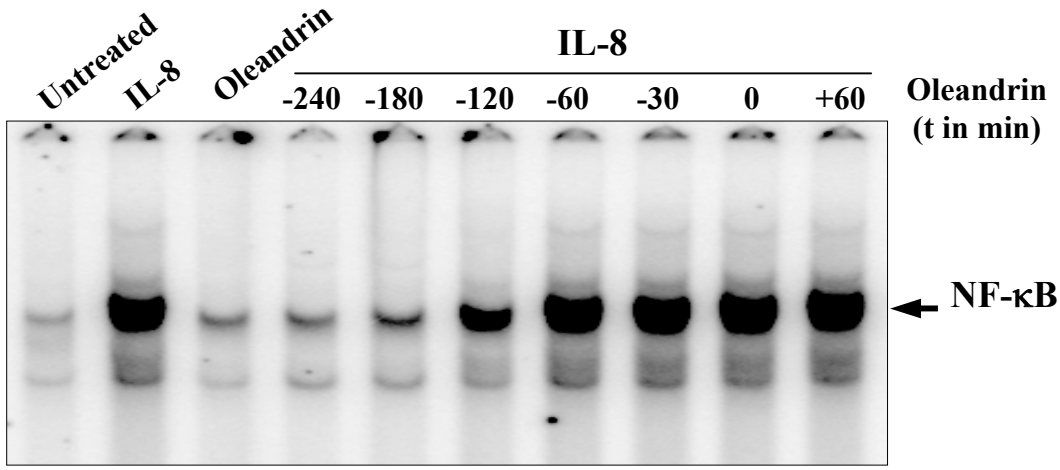


Fig 5.6 Time kinetics of oleandrin-mediated NF-κB activation. Macrophages, either pre-, co-, or post-incubated with 100 ng/ml oleandrin at 37°C for the indicated times were stimulated with 100 ng/ml IL-8 at 37°C for 2 h. After these treatments nuclear extracts were prepared and then assayed for NF-κB.

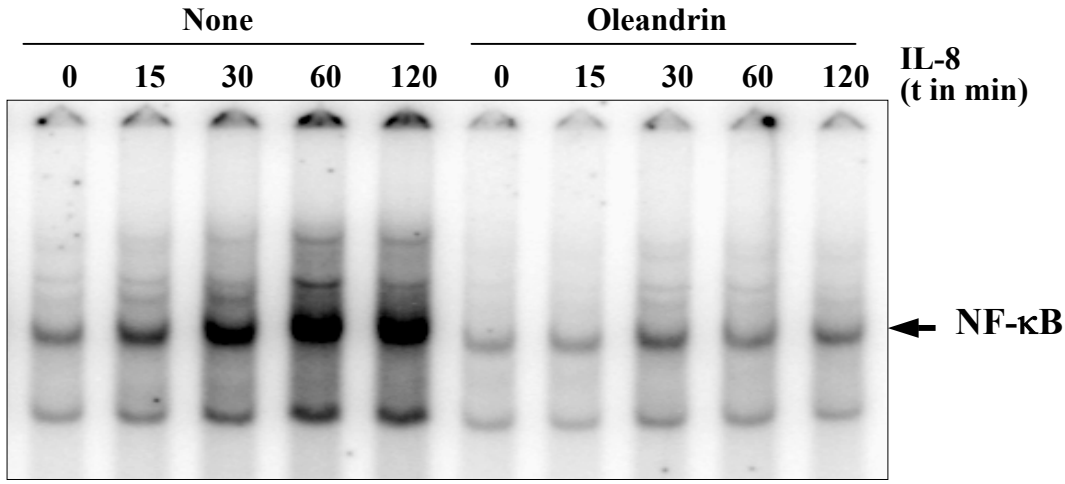


Fig 5.7 Time course of inhibition of IL-8-dependent NF-κB activation by oleandrin. Macrophages were incubated at 37^0C either with or without 100 ng/ml of oleandrin for 3 h and then stimulated with 100 ng/ml IL-8 for different times. After these treatments nuclear extracts were prepared and then assayed for NF-κB.

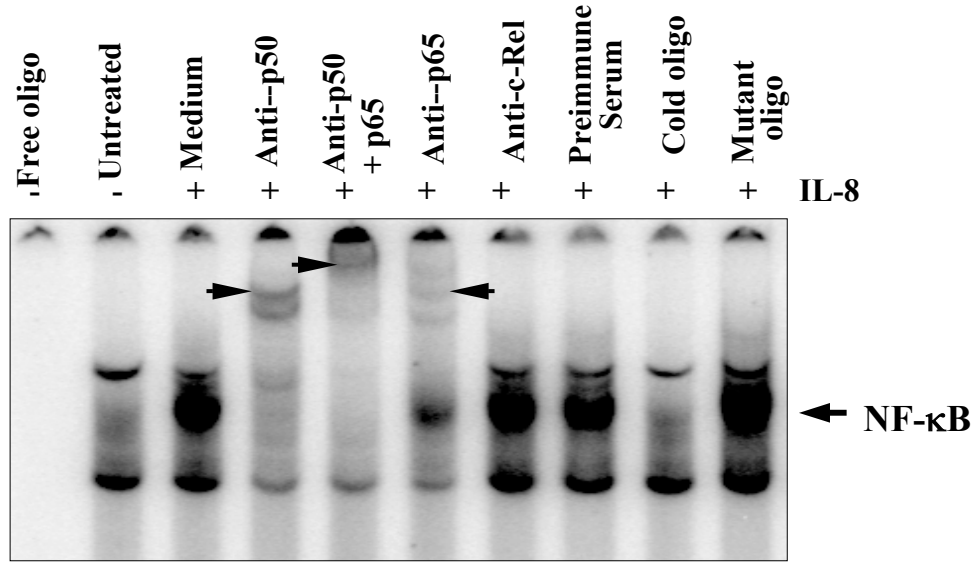


Fig 5.8 Supershift and specificity of NF-κB activation. Nuclear extracts, prepared from untreated or IL-8-treated macrophages were incubated for 15 min with different Abs and cold or mutant NF-κB oligonucleotides, and then assayed for NF-κB, as described in materials and methods.

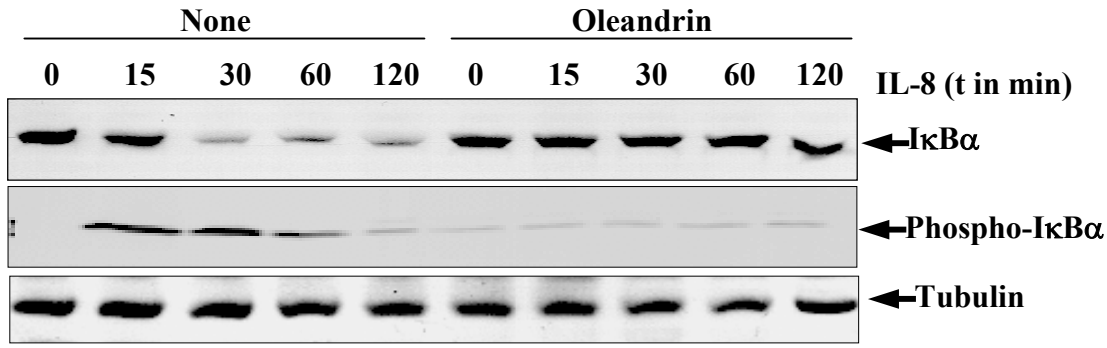


Fig 5.9 Effect of oleandrin on IL-8-induced degradation of IκBα and on levels of phospho-IκBα. Macrophages, either untreated or pretreated with 100 ng/ml oleandrin for 3 h were stimulated with IL-8 (100 ng/ml) for different times. Cytoplasmic extracts were prepared and assayed for IκBα and phospho-IκBα by Western blot and the blot was reprobed with anti-tubulin Ab

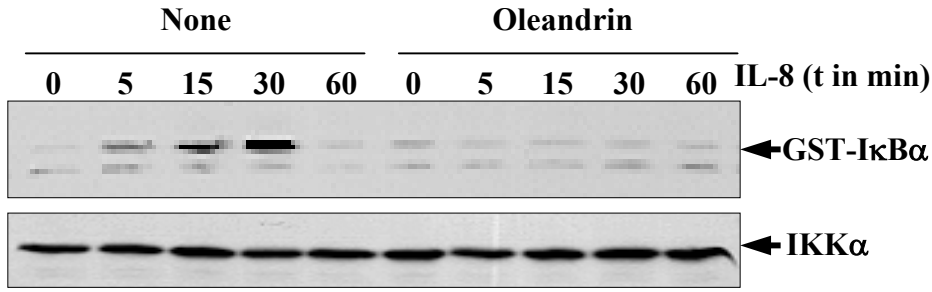


Fig 5.10 Effect of oleandrin on IL-8-induced activation of IKKα. Cells were pretreated with 100 ng/ml oleandrin for 3 h and then activated with 100 ng/ml IL-8 for the indicated times. Whole-cell extracts were prepared and IKK was assayed as described under Materials and Methods.

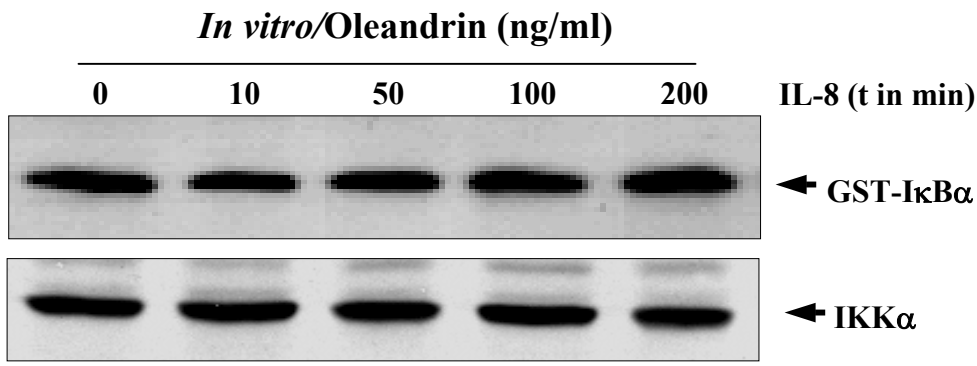


Fig 5.11 Effect of oleandrin on IL-8-induced activation of IKKα. The level of IKKα was detected from 50 μg cells extract proteins by Western blot. To detect the direct effect of oleandrin on the IL-8-induced activation of IKKα, cells were stimulated with IL-8 for 1 h and then whole-cell extracts were prepared. Then 250 μg extract proteins were immunoprecipitated with anti-IKKα antibody. The immune complexes were incubated with different concentrations of oleandrin for 3 h and then IKK assayed

the IL-8-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor irrelevant Ab such as anti-c-Rel had any effect on the mobility of NF-κB. The complex completely disappeared in presence of cold NF-κB indicating the specificity of NF-κB. Mutant NF-κB was unable to bind with the IL-8-induced protein complex further suggesting its specificity.

5.2.5 Oleandrin inhibits IL-8-induced myeloperoxidase, alkaline phosphatase, or β-D glucuronidase activity.

IL-8 interacts with cells surface receptors to secrete different proteolytic enzymes. To detect IL-8-induced biological responses, macrophages were treated with different concentrations of oleandrin for 3 h and then stimulated with IL-8 (100 ng/ml) for 2 h. Culture supernatant was then used to analyze myeloperoxidase, alkaline phosphatase, and β-D-glucuronidase. Oleandrin inhibited IL-8-induced proteases release in a dose-dependent manner indicating its effect on IL-8-mediated biological response in macrophages (Fig.5.12).

5.2.6 Oleandrin inhibits IL-8-, FMLP-, or NGF-, but not TNF-induced oxidative burst response. Different inducers induce macrophages to generate reactive oxygen species (ROS) is well established (Manna ***et al.***, 1997). The generation of ROS is detected by NBT (nitroblue tetrazolium) test. Does oleandrin modulate oxidative burst response mediated by different inducers as seen in case of NF-κB activation? To address this question, macrophages were treated with different concentrations of oleandrin for 3 h and then stimulated with 100 ng/ml IL-8, 100 nM FMLP, 100 pM TNF, or 100 nM NGF for 2 h at 37^0C in presence of NBT solution (0.1%). NBT positive cells were counted under microscope and presented in percentage above unstimulated cells. Oleandrin did not increase the number of NBT positive cells alone than the unstimulated cells. IL-8, FMLP, TNF, or NGF induced 74, 87, 58, or 72% NBT positive cells respectively. Oleandrin decreased IL-8-, FMLP-, or NGF-, but not TNF-induced NBT positive cell numbers in a dose-dependent manner. These data (Fig.5.13) indicate that oleandrin downregulates IL-8-, FMLP-, or NGF-, but not TNF-induced oxidative burst response suggesting the specific activity towards chemoattractants and growth factors mediated responses.

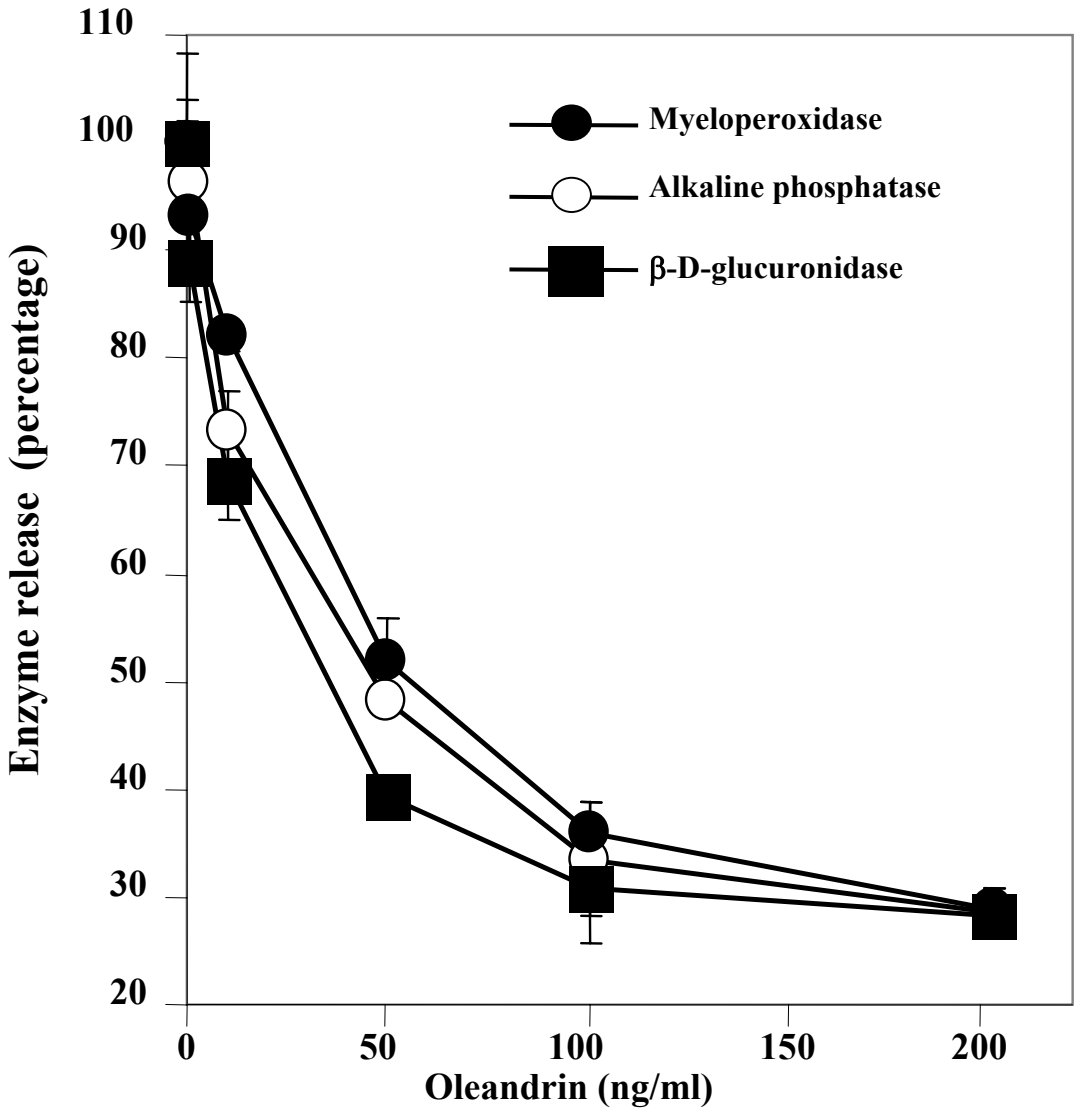


Fig. 5.12 Effect of oleandrin on IL-8-induced enzymes release. Macrophages were treated with different concentrations of oleandrin for 3 h in phenol red-free medium and then stimulated with 100 ng/ml of IL-8 for 2 h. The supernatant was collected and analyzed for myeloperoxidase, alkaline phosphatase, and β-D-glucuronidase.

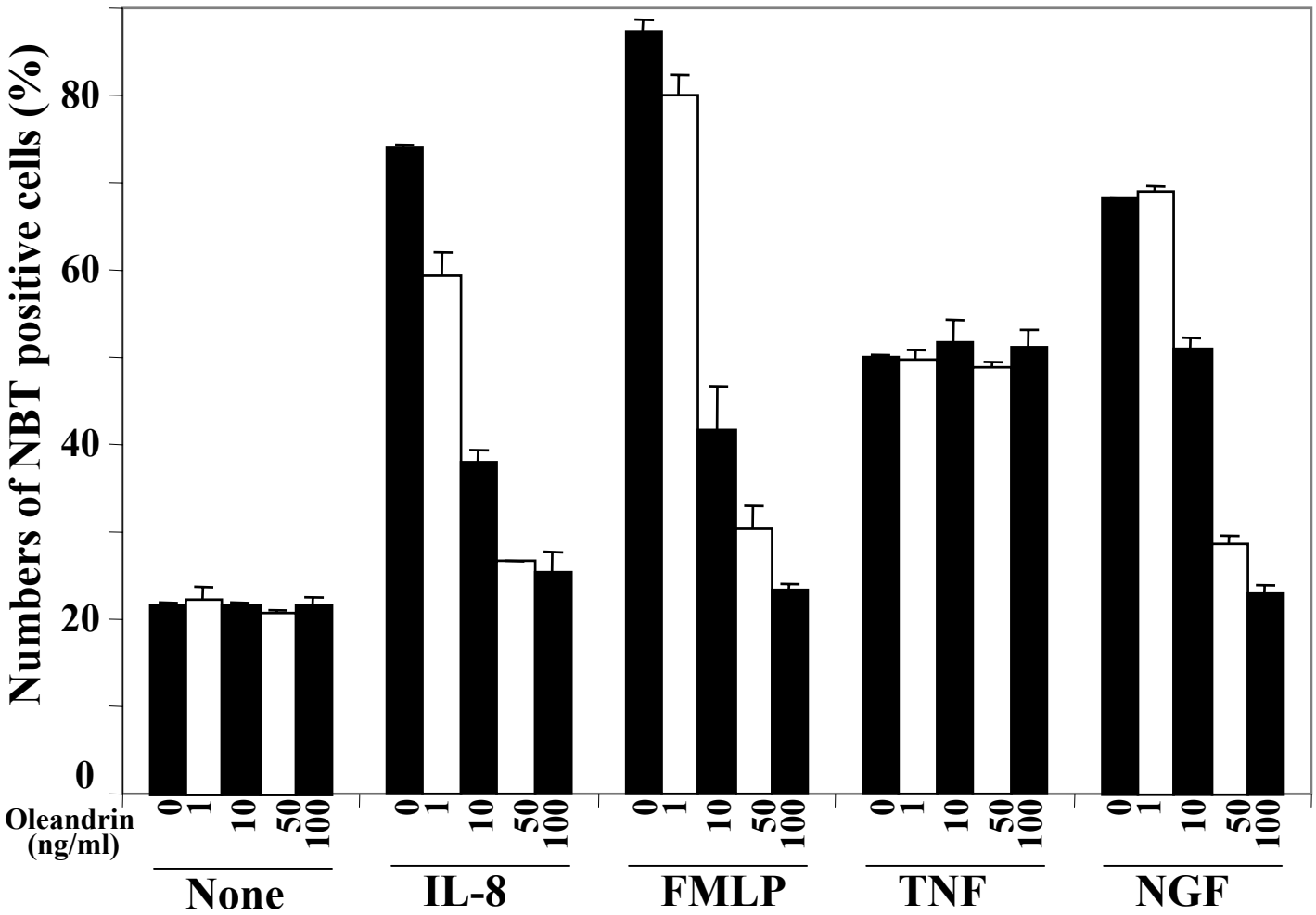


Fig 5.13 Effect of oleandrin on IL-8-, FMLP, TNF-, and NGF-induced oxidative burst response. Macrophages were treated with different concentrations of oleandrin for 3 h and then stimulated with 100 ng/ml IL-8, 100 pM FMLP, 100 pM TNF, or 100 pM NGF for 2 h. Then nitroblue tetrazolium (NBT) test was carried out. NBT positive and negative cells were counted under microscope and NBT positive cells were presented in percentage.

5.2.7 Oleandrin inhibits IL-8-induced NF-κB-dependent SEAP, ICAM1, Cox2, and Cox2-dependent luciferase activation.

As IL-8-induced NF-κB activation was blocked by oleandrin, the NF-κB regulated genes, adhesion molecule ICAM1 and Cox2 expressions were carried out. THP-1 cells were transfected with NF-κB reporter plasmid containing SEAP gene with Qiagen superfect reagent for 6 h and then stimulated with PMA for differentiation. Differentiated macrophages were treated with oleandrin, stimulated with different concentrations of IL-8 or TNF for 12 h and the culture supernatant was used to assay secretory alkaline phosphatase (SEAP) activity. The result in Figure 5.14 indicated that IL-8 or TNF induced SEAP activity in a dose-dependent manner and oleandrin completely inhibited IL-8-, but not TNF-induced SEAP activity at any concentrations. The results suggest that oleandrin inhibits IL-8-, but not TNF-induced NF-κB activation. Macrophages were treated with different concentrations of oleandrin for 3 h and then stimulated with IL-8 (100 ng/ml) or TNF (100 pM) for 12 h. We analyzed ICAM1 and Cox2 in the cell extract proteins by Western blot. IL-8- but not TNF-induced ICAM1 (Fig.5.14 A & B) and Cox2 (Fig.5.14 C & D) expression were decreased with increase concentrations of oleandrin treatment. Oleandrin alone did not induce ICAM1 or Cox2 expression at any concentrations. Upon re-probing the blots with anti-tubulin antibody, we found that the band intensities in all lanes were uniform indicating equal loading of proteins in the lanes.

Although it was shown that oleandrin blocks the Cox2 expression, the dependent gene transcription was also assayed. THP1 cells, transiently transfected with the *Cox2-Luciferase* reporter construct for 6 h were stimulated with PMA (10 ng/ml) for 16 h. Cells were then treated with different concentrations of oleandrin for 3 h followed by stimulation with IL-8 (100 ng/ml) or TNF (0.1 nM) for 12 h. An almost 7.5-or 9.2-fold increase in luciferase activity over the vector transfected cells was noted upon stimulation with IL-8 or TNF respectively (Fig.5.16). IL-8-, but not TNF-induced luciferase activity was inhibited by oleandrin in a dose-dependent manner. These results demonstrate that oleandrin selectively inhibits IL-8-induced NF-κB and dependent several genes activation.

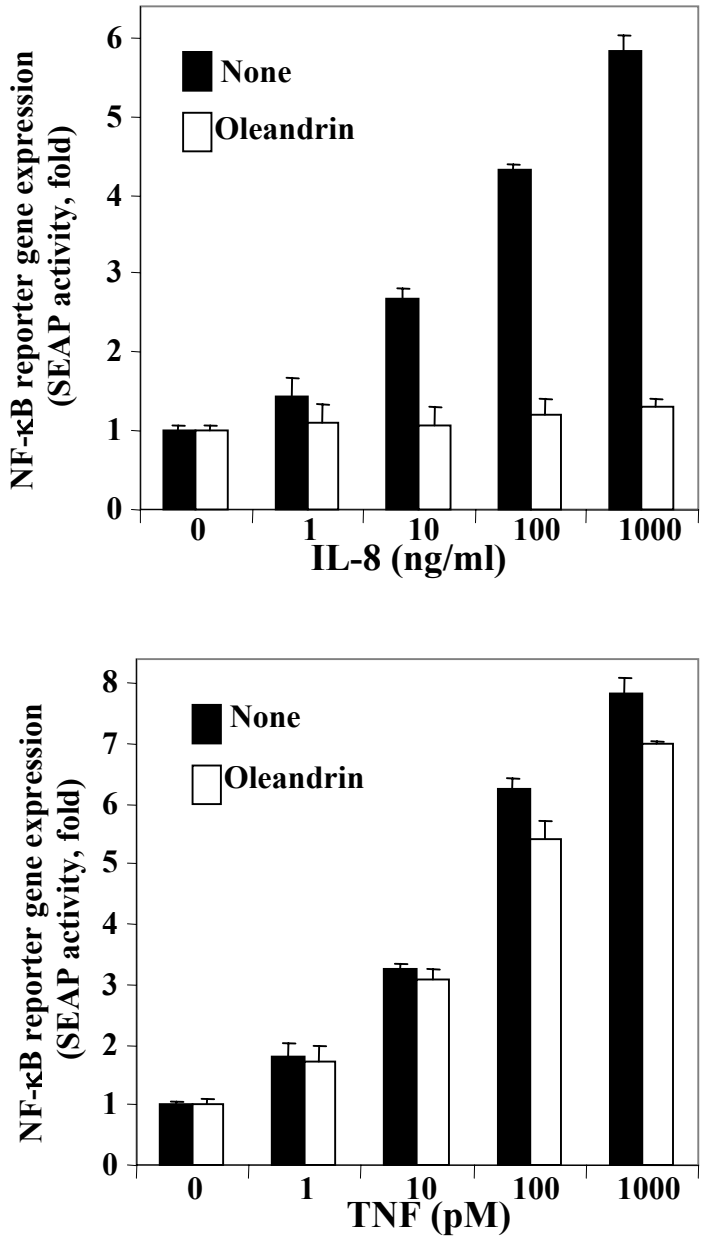


Fig 5.14 Effect of oleandrin on IL-8- or TNF-induced NF-κB-dependent SEAP expression. THP1 cells were transfected with NF-κB-containing plasmid linked to the SEAP gene for 6 h and then cultured in presence of 10 ng/ml PMA for 16 h. Cells were treated with 100 ng/ml oleandrin for 3 h and then stimulated with different concentrations IL-8 (A1) or TNF (A2) for 12 h. Then culture supernatant was taken and assayed for SEAP.

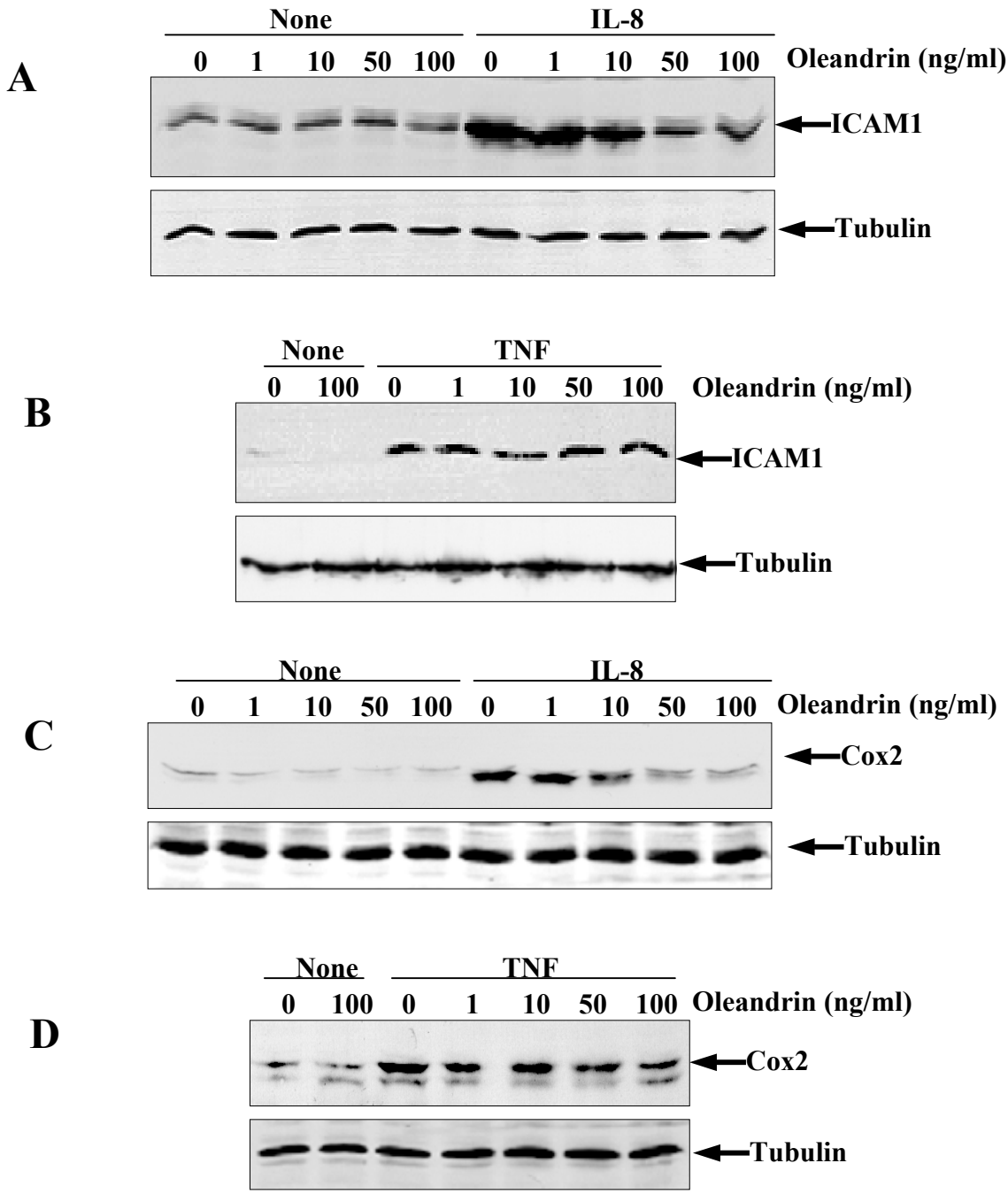


Fig 5.15 Effect of oleandrin on IL-8- or TNF-induced NF-κB-dependent ICAM1 and Cox2 expression. Cells were stimulated with different concentrations of oleandrin for 3 h and then treated with IL-8 (100 ng/ml) or TNF (100 pM) for 12 h at 37^0C in a CO_2 incubator. ICAM1 (A & B) and Cox2 (C & D) were detected from cell extract proteins (100 μg) by Western blots. As loading control, Tubulin was detected by re-probing same blots.

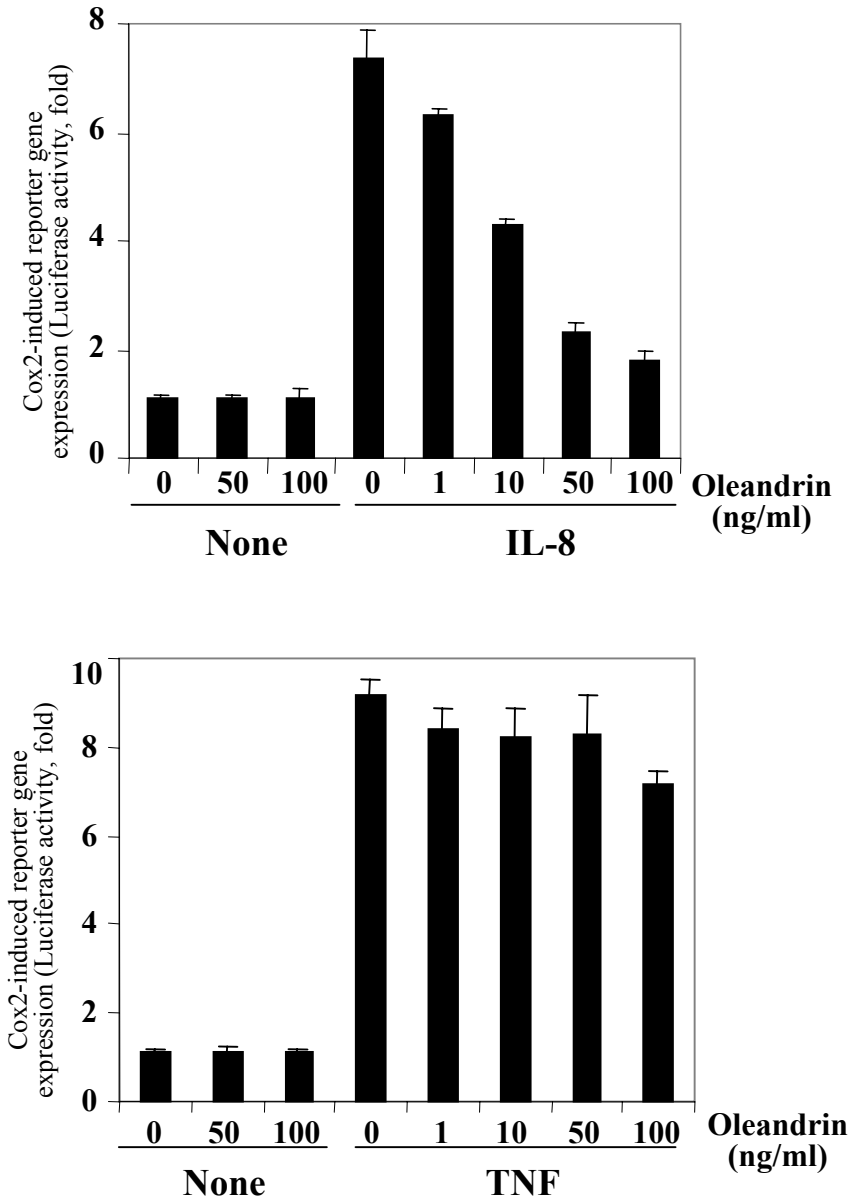


Fig 5.16 Effect of oleandrin on IL-8- or TNF-induced NF-κB-dependent Cox-2-dependent luciferase expression. THP1 cells, transfected with the *Cox2-Luciferase* expression vector and *β-galactosidase* gene for 6 h were stimulated with 10 ng/ml PMA for 16 h. Cells were treated with different concentrations of oleandrin for 3 h and then stimulated with IL-8 (100 ng/ml) or TNF (100 pM) for 12 h. The luciferase and β-galactosidase enzymes activity was measured

5.2.8 Oleandrin downregulates IL-8, EGF, or NGF receptors, but not TNF or IL-1 receptors.

As oleandrin inhibited IL-8-induced biological responses and IL-8 exerts its effect through surface IL-8Rs, the effect of oleandrin on expression of different receptors in macrophages were detected. Macrophages (1 x 10^6/well of 12-well plate) were incubated with different concentrations of oleandrin for 3 h at 37^0C, CO_2 incubator in triplicate samples. Then radiolabeled ligands binding was assayed using ^{125}I-labeled TNF, IL-8, IL-1, EGF, and NGF (5 x 10^4 cpm/sample) for 2 h at 4^0C. The results indicated in Figure 5.17 that ^{125}I-labeled IL-8, EGF, or NGF binding was decreased with increase concentrations of oleandrin but not for the binding of TNF or IL-1. The**se** results indicate that oleandrin down-regulates IL-8R, EGFR, or NGFR, but not TNFR or IL-1R in macrophages in a dose-dependent manner.

To detect the optimum concentration and time of oleandrin treatment to downregulate the IL-8Rs, macrophages were treated with 100 ng/ml oleandrin for different times as indicated in Figure 5.18 at 37^0C, CO_2 incubator. Then ^{125}I-IL-8 binding was assayed at 4^0C. As shown in Figure 5.18, the IL-8 binding was gradually decreased upto 3 h. From this result it is clear that optimum time required for maximum IL-8Rs down-regulation by oleandrin is 3 h. Similarly, cells were treated with different concentrations of oleandrin for 3 h and then ^{125}I-IL-8 binding was assayed and 100 ng/ml concentration is optimum for maximum IL-8R downregulation (Fig.5.19). At higher concentrations further downregulation of IL-8Rs were not observed. Viable receptors were detected by accumulation of labeled ligand inside the cells (total binding) at 37^0C. As shown in Figure 5.20, the accumulated ^{125}I-IL-8 was decreased in oleandrin treated cells about 75-85% at any concentrations of added ^{125}I-IL-8 compared to untreated cells indicating downregulation of IL-8R by oleandrin.

5.2.9 Oleandrin inhibits IL-8 binding.

To estimate the number of IL-8Rs expressed in oleandrin treated macrophages, Scatchard analysis was performed (Manna *et al*., 1997; Manna and Aggarwal, 1998). Cells, either untreated or treated with oleandrin (100 ng/ml) for 3 h were incubated with different concentrations of ^{125}I-IL-8 at 4^0C for 2 h. In another set, same binding was assayed in presence of 50-fold unlabeled IL-8. The specific binding of IL-8 was shown in Figure 5.21 as mean count ± SD of duplicate samples. From specific count, bound/free values were calculated

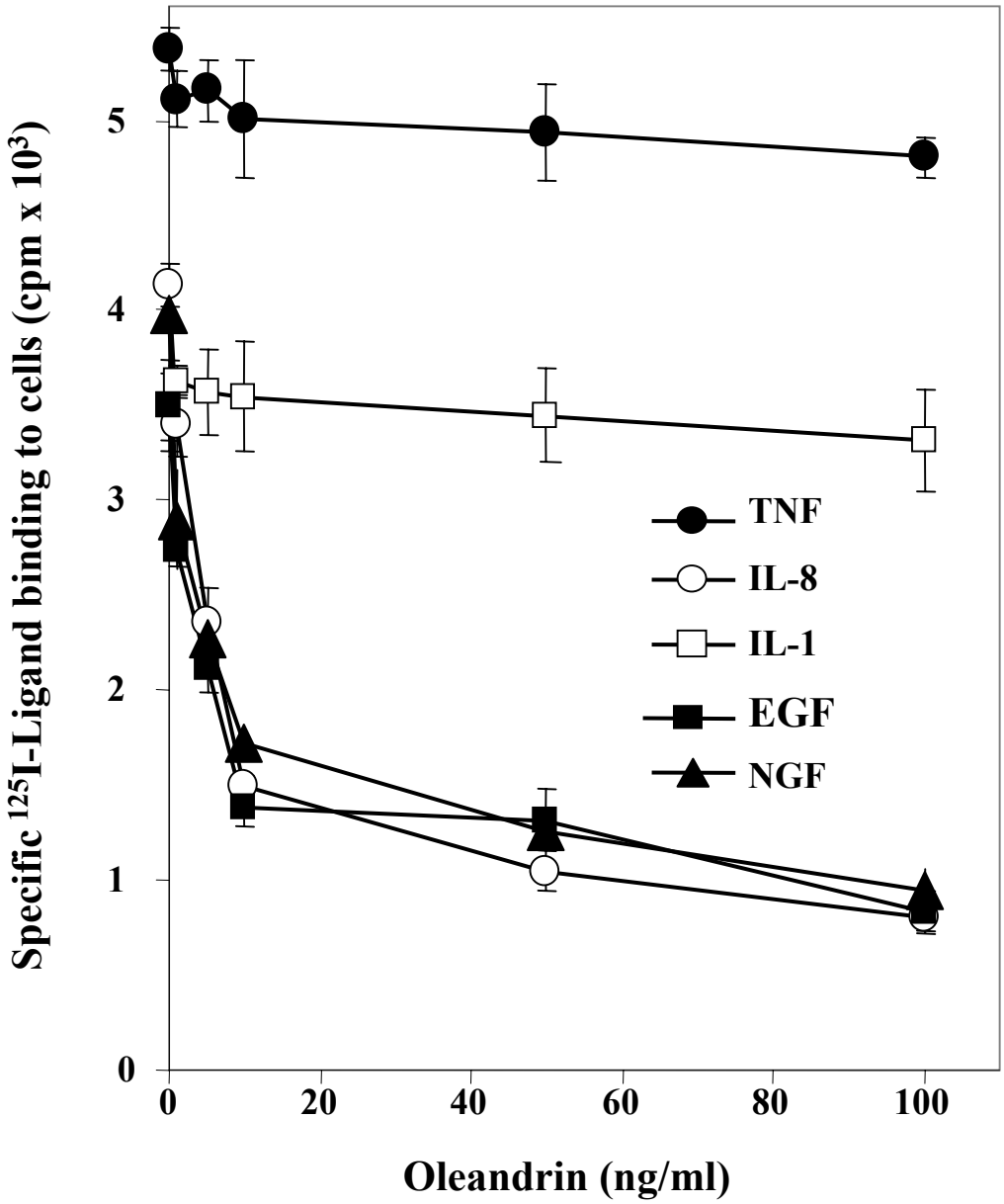


Fig 5.17 Effect of oleandrin on TNF, IL-8, IL-1, EGF, or NGF receptors level. Macrophages (10^6/well of 12 well plate) were incubated with different concentrations of oleandrin for 3 h at 37^0C, CO_2 incubator. Labeled TNF, IL-8, IL-1, EGF, or NGF binding was assayed at 4^0C as described in Materials and Methods.

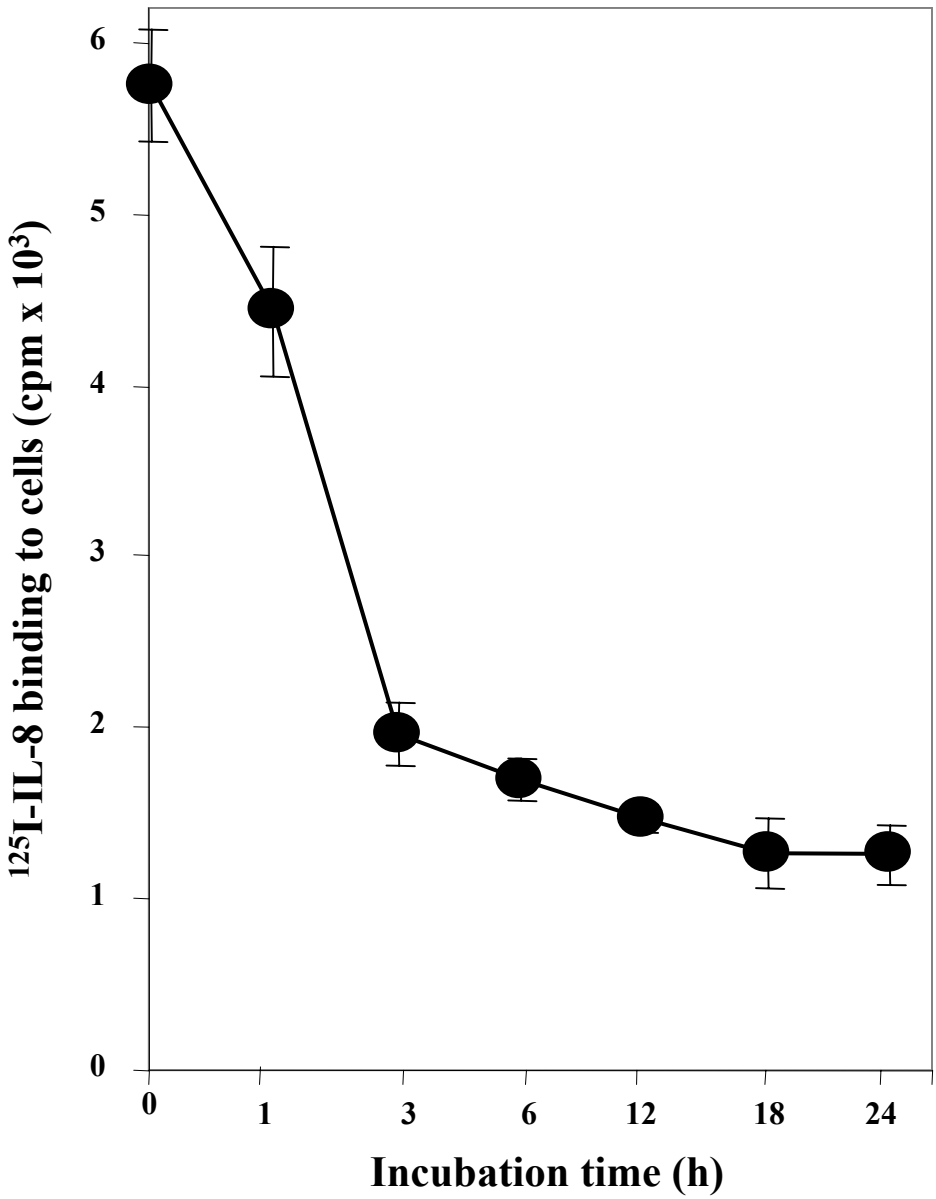


Fig 5.18 Optimum time of oleandrin treatment to downregulate the IL-8Rs. Macrophages were incubated with 100 ng/ml oleandrin for different times as indicated in Figure and then ^{125}I-IL-8 binding was assayed of triplicate samples.

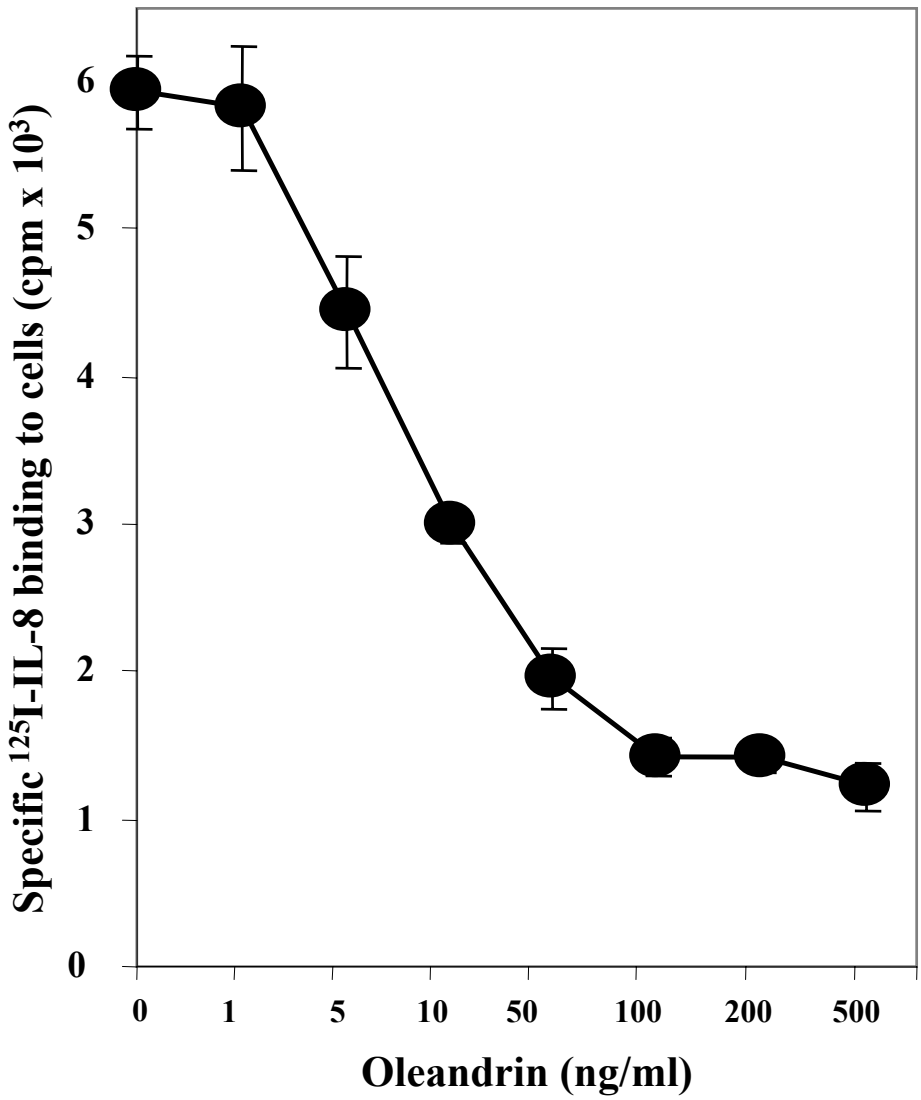


Fig 5.19 Optimum concentrations of oleandrin to downregulate IL-8R. Macrophages treated with different concentrations of oleandrin for 3 h and then IL-8 binding was assayed.

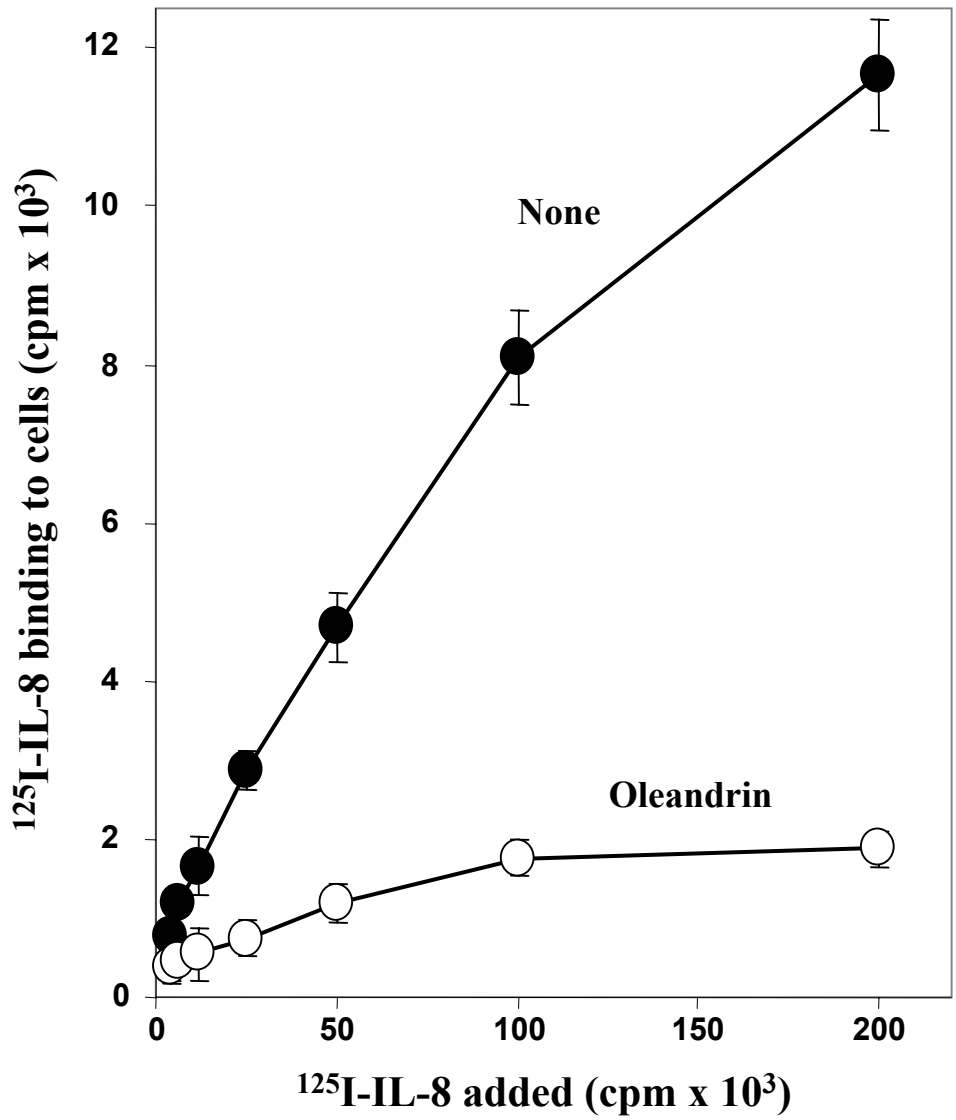


Fig 5.20 Effect of oleandrin on ^{125}I-IL-8 binding. Macrophages were treated with 100 ng/ml oleandrin for 3 h and then cells were washed and incubated with different amount of ^{125}I-IL-8 at 37^{0}C for 1 h. Then IL-8 binding was assayed as described in Materials and Mehtods.

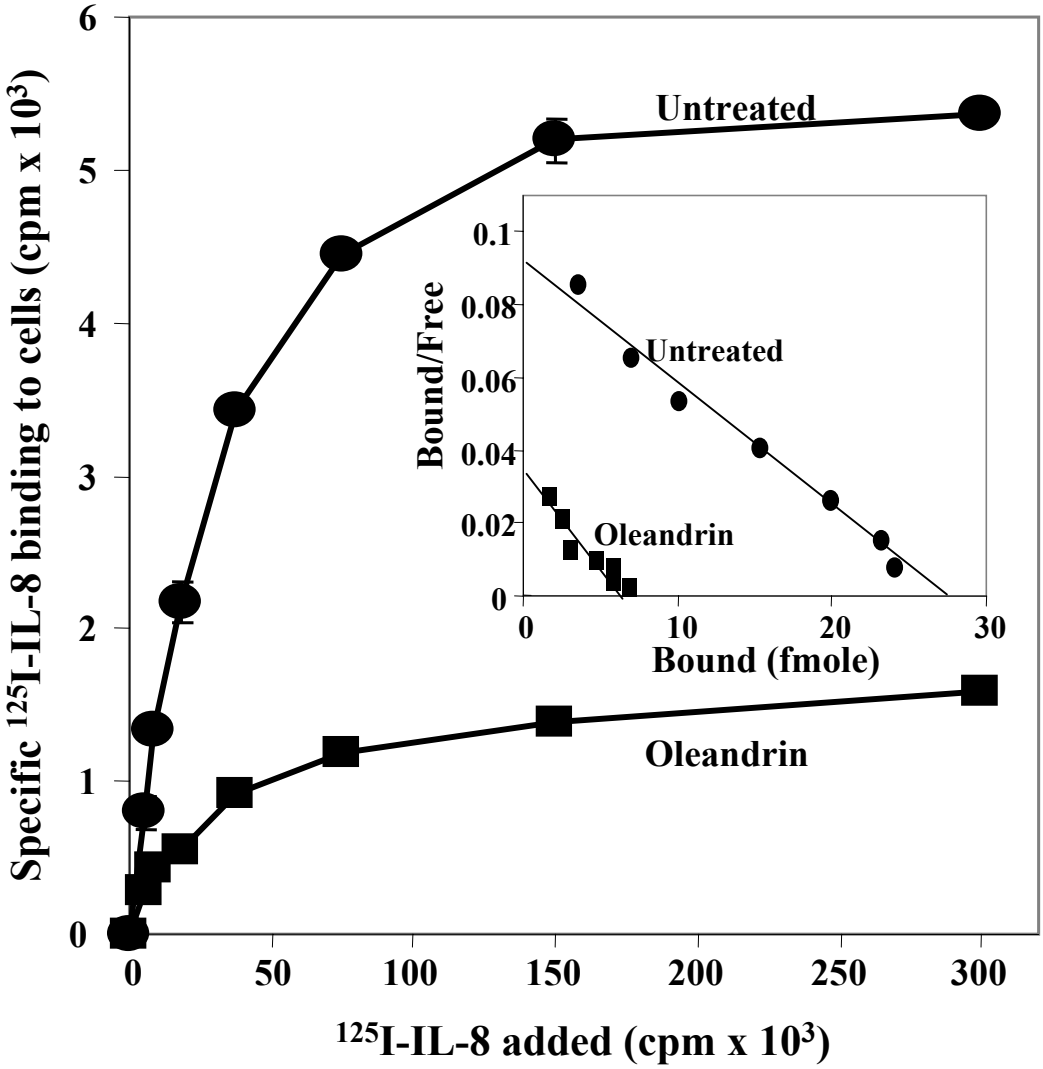


Fig 5.21 Scatchard analysis of ^{125}I-IL-8 binding to oleandrin-treated macrophages. Oleandrin-treated and untreated macrophages (1 x 10^6) were incubated with different amounts of ^{125}I-IL-8 at 4^0C for 2 h. Then IL-8 binding was assayed. To determine the specific binding, nonspecific binding (obtained from a 50-fold excess of cold IL-8 used in binding) was subtracted. From specific binding, ligand bound versus bound/free ratio was indicated (inset).

and plotted against bound values (Fig.5.21, inset). From these data total number of IL-8R was calculated as 11,560 IL-8 receptors per unstimulated neutrophil (Kd, 0.42 nM) versus 2,480 (Kd, 0.48 nM) by oleandrin treatment. Oleandrin downregulates about 79% IL-8R as detected by Scatchard plot that is correlated with IL-8 binding data with out changing affinity towards IL-8.

To demonstrate the effect of oleandrin on IL-8 binding, chemical coupling of ^{125}I-IL-8 to receptors was performed at 4^0C by a bifunctional cross-linker DSS with different concentrations of oleandrin-treated cells as described in Experimental procedures. Figure 5.22 showed that the intensity of 67 and 75 kDa bands were decreased with increasing concentrations of oleandrin, thus indicating downregulation of IL-8Rs. To detect the type of IL-8Rs were downregulated by oleandrin, cells were treated with oleandrin (100 ng/ml) for 3 h and then incubated with 250 ng of IL-8 or MGSA for 2 h at 4^0C and then ^{125}I-IL-8 was assayed. The cold IL-8 suppressed almost 90% labeled IL-8 binding, whereas cold MGSA suppressed about 50% labeled IL-8 binding (Fig.5.23), suggesting the downregulation of both types IL-8Rs by oleandrin.

5.2.10 Oleandrin inhibits IL-8 binding in isolated macrophages membrane, but not in purified IL-8R.

To detect the effect of oleandrin in macrophages membrane and purified IL-8Rs, macrophages, isolated membrane, and affinity purified IL-8Rs were incubated with different concentrations of oleandrin for 3 h at 37^0C. Labeled IL-8 binding was assayed in intact cells, membrane, and purified IL-8Rs. The results shown in Figure 5.24 indicate that ^{125}I-IL-8 binding was decreased in intact cells and isolated membrane, but not in purified IL-8R. These results suggest that down modulation of IL-8R by oleandrin is mediated through its interaction with cells and its membrane but not directly with IL-8R.

5.2.11 IL-8 or anti-IL-8R Ab is unable to protect oleandrin-mediated IL-8Rs downregulation.

The relevant question is whether oleandrin-mediated IL-8R modulation within IL-8 binding domain of the receptor. To address this question ligand protection experiment was performed. Since IL-8R rapidly internalized with IL-8 at 37^0C and monodansyl cadaverine (MDC) can

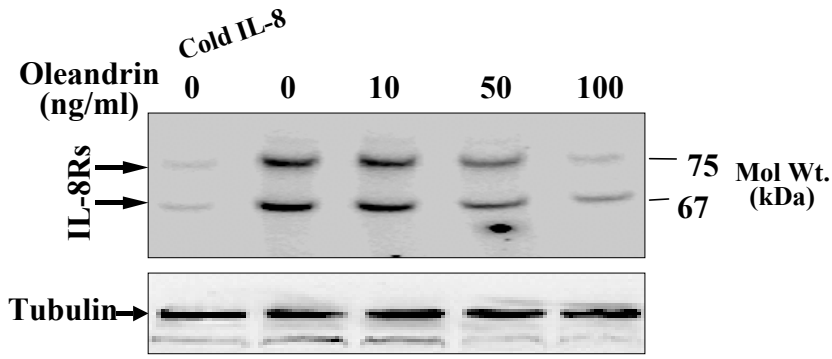


Fig 5.22 Effect of oleandrin on IL-8R downregulation. Macrophages were treated with different concentrations of oleandrin for 3 h and then ^{125}I-IL-8 binding was done. Then cells were scrapped and used to cross-link for ligand–receptor and then extracted and 200 μg proteins were analyzed in 10% SDS-PAGE and the dried gel was scanned in PhosphorImager. 50 μg proteins was used to detect tubulin by Western blot for loading control.

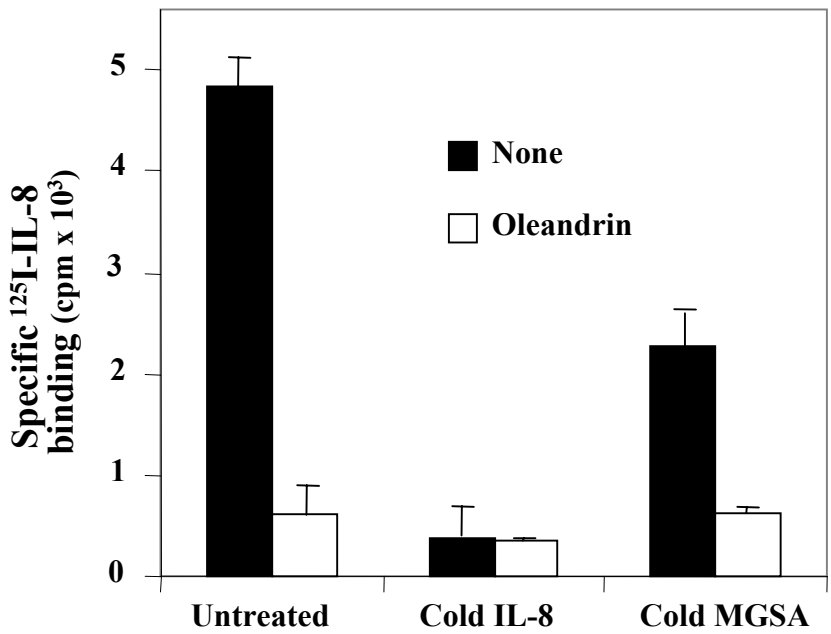


Fig 5.23 Specific IL-8 binding to macrophages. Macrophages treated with 100 ng/ml oleandrin for 3 h and then cells were washed, incubated with 250 ng of unlabeled IL-8 or MGSA for 30 min followed by labeled IL-8 for 2 h at 4^0C and binding was assayed. Results represented one out of three experiments.

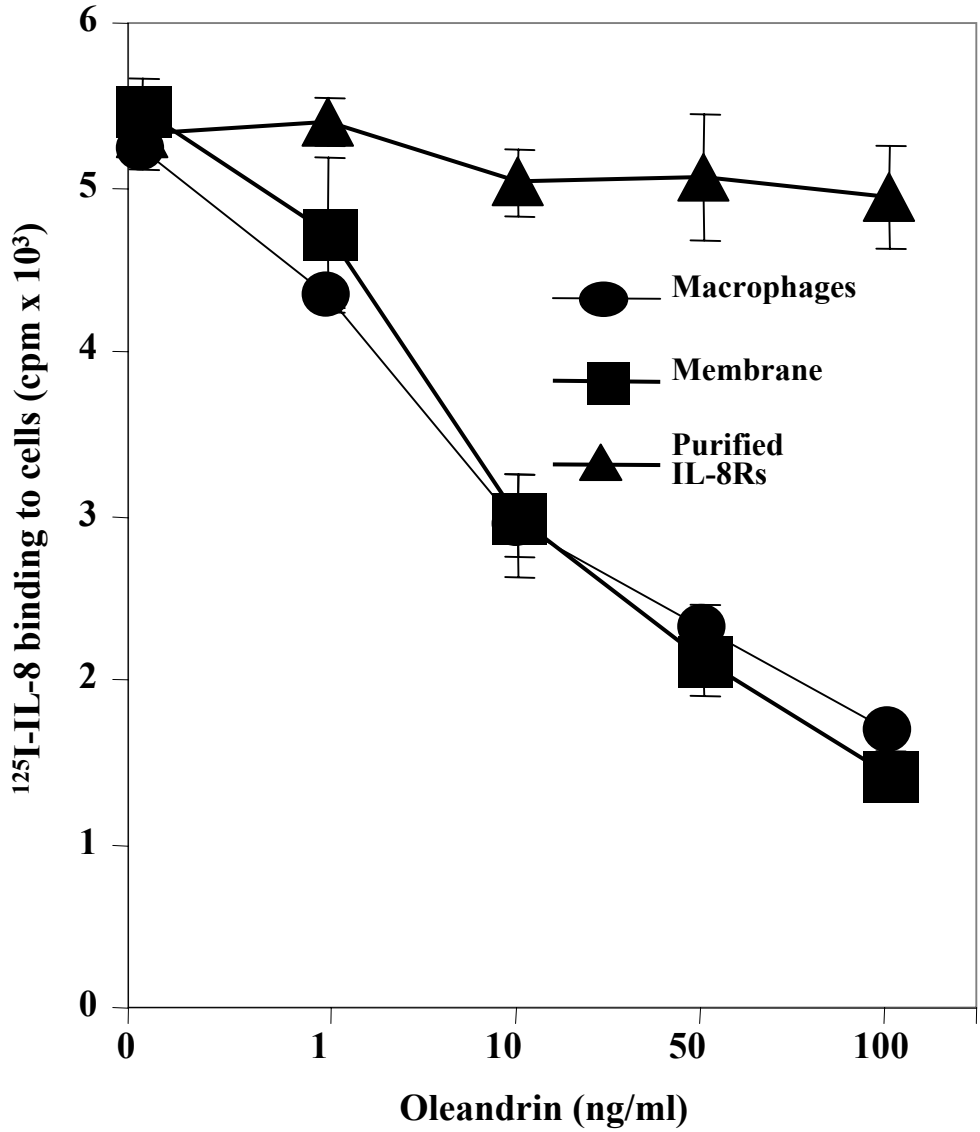


Fig 5.24 Effect of oleandrin on purified macrophages membrane and affinity purified IL-8R. Purified membrane and affinity purified IL-8R, taken in nitrocellulose discs and cells were treated with different concentrations of oleandrin. The nitrocellulose discs and cells were washed and then assayed for ^{125}I-IL-8 binding.

protect endocytosis at 37^0C (Ray and Samanta, 1997), cells were incubated with MDC for 15 min at 37^0C and then incubated with IL-8 for 1 h at 37^0C. The cells were washed, treated with different concentrations of oleandrin for 3 h, and then washed with glycine-HCl (50 mM, pH 3.0). The binding of ^{125}I-IL-8 was assayed at 4^0C immediately after washing the cells. IL-8R was decreased in macrophages by oleandrin in a dose dependent manner and IL-8 preincubated cells showed almost similar decrease in IL-8 binding (Fig.5.25). The results suggest that IL-8 is unable to protect oleandrin-mediated downregulation of IL-8Rs. Oleandrin-mediated downregulation of IL-8Rs were not due to production of IL-8 as oleandrin-treated cells (for 12 h) did not show any change in IL-8 level either in supernatant or pellet extracts as detected by ELISA (Fig.5.26). To examine the protection of IL-8R from the effect of oleandrin by anti-IL-8R antibody, cells were incubated with the 1 μg of each of anti-IL-8R1 and 2 antibodies/well of 12-well plate for 1 h at 37^0C. After washing, different concentrations of oleandrin was added and incubated at 37^0C, CO_2 incubator for 24 h. Cells were then washed with 0.5 M potassium thiocyanate for 10 sec. After immediate washing, the mean binding of ^{125}I-IL-8 (cpm) was assayed at 4^0C. The result indicated in Figure 5.27 that anti-IL-8Rs Abs pre-incubated cells showed decrease in IL-8 binding with increased concentrations similar to oleandrin treated cells indicating anti-IL-8Rs Ab's inability to protect oleandrin-mediated downregulation of IL-8Rs.

5.2.12 Lipid molecules, but not protease inhibitors protect oleandrin-mediated downregulation of IL-8Rs.

As proteases are known to cleave different proteins on the cell surface, it is logical to detect the role of proteases on oleandrin-mediated downregulation of IL-8Rs. Macrophages were preincubated with different protease inhibitors for 1 h and subsequently treated with oleandrin (100 ng/ml) for 3 h. Then ^{125}I-IL-8 binding was assayed. The results indicated in Figure 5.28 that the binding of IL-8 was not decreased by different protease inhibitors alone in macrophages, but also oleandrin-mediated inhibition of IL-8 binding was not protected by pre-treatment of different proteases suggesting proteases have no role in oleandrin-mediated IL-8Rs downregulation. As different lipid molecules interact with modulators to inactivate the modulator (Manna ***et al.***, 1997), we incubated oleandrin with cephalin, cholesterol, lecithin, or sphingosine for 3 h and then cells were incubated with those mixtures for 3 h and ^{125}I-IL-8

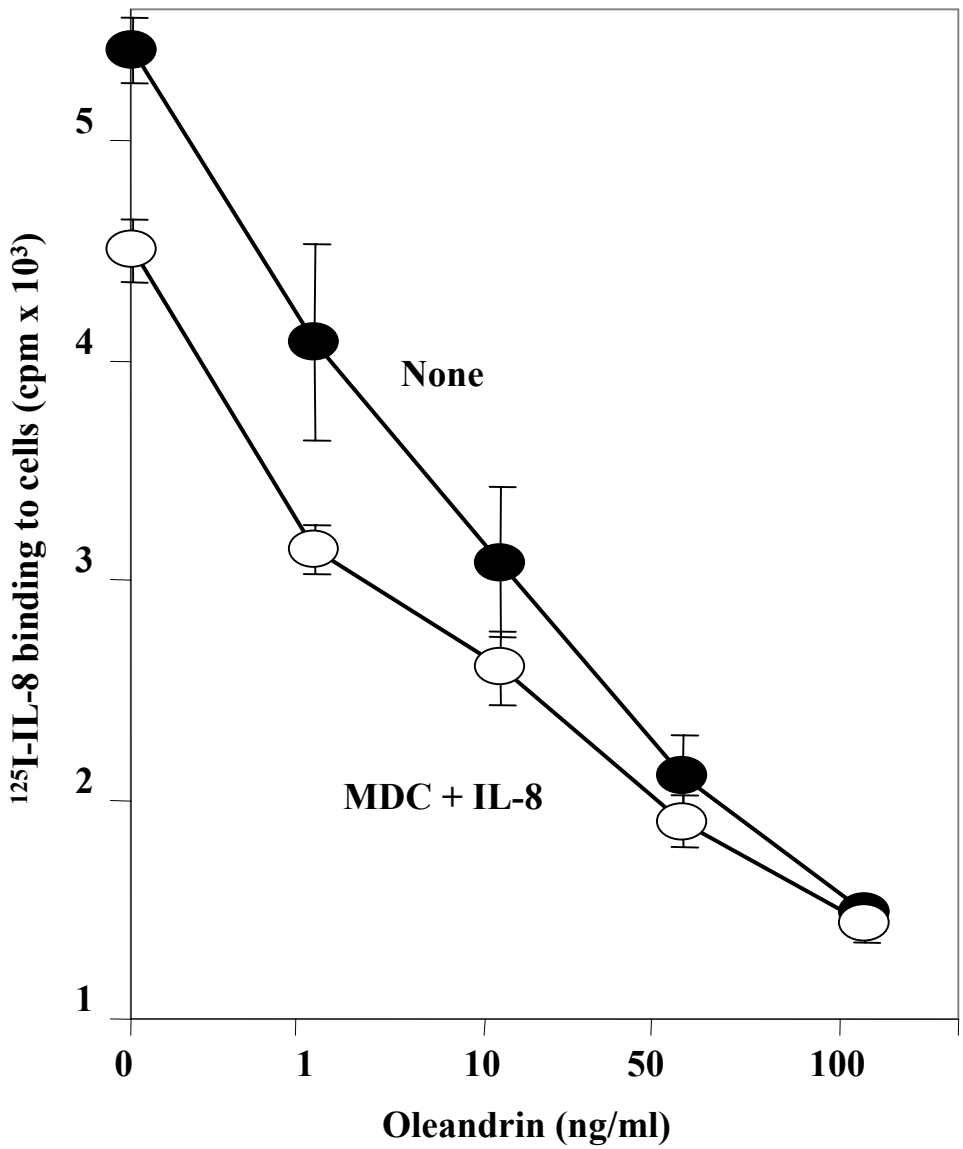


Fig 5.25 IL-8 was unable to protect oleandrin-mediated down-regulation of IL-8R. Macrophages, pre-incubated with MDC for 15 min were incubated with IL-8 (500 ng/ml) for 1 h at 37^{0}C. After washing, the cells were treated with different concentrations of oleandrin for 3 h. Cells were washed with 0.05 M glycine-HCl, pH 3.0 and then assayed for ^{125}I-IL-8 binding.

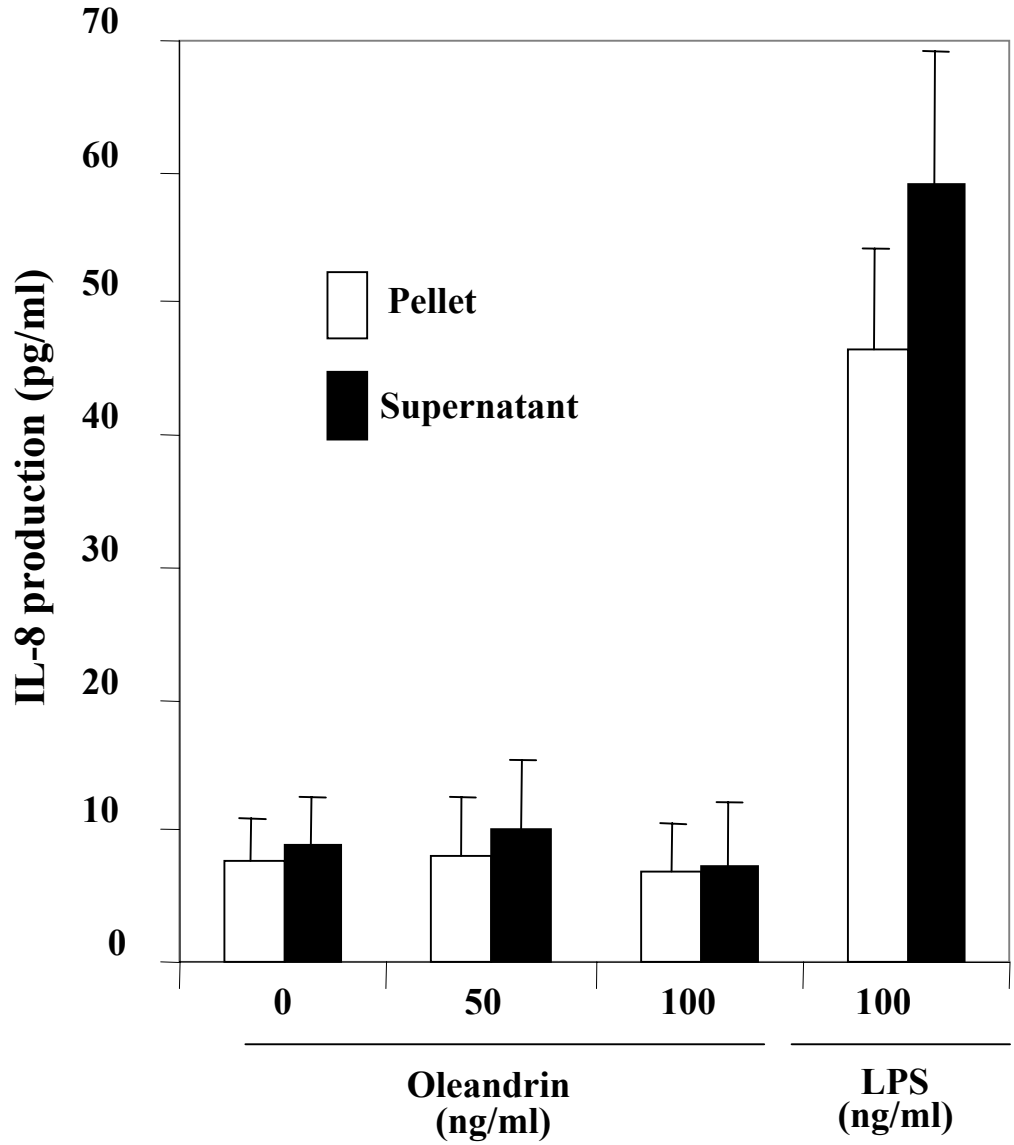


Fig 5.26 Effect of oleandrin on IL-8 expression. Macrophages were treated with different concentrations of oleandrin or 100 ng/ml LPS for 12 h and then supernatant and cell pellet extracts were used to detect IL-8 by ELISA as per manufacturer's instruction. Results indicated in pg/2 x 10^5 cells

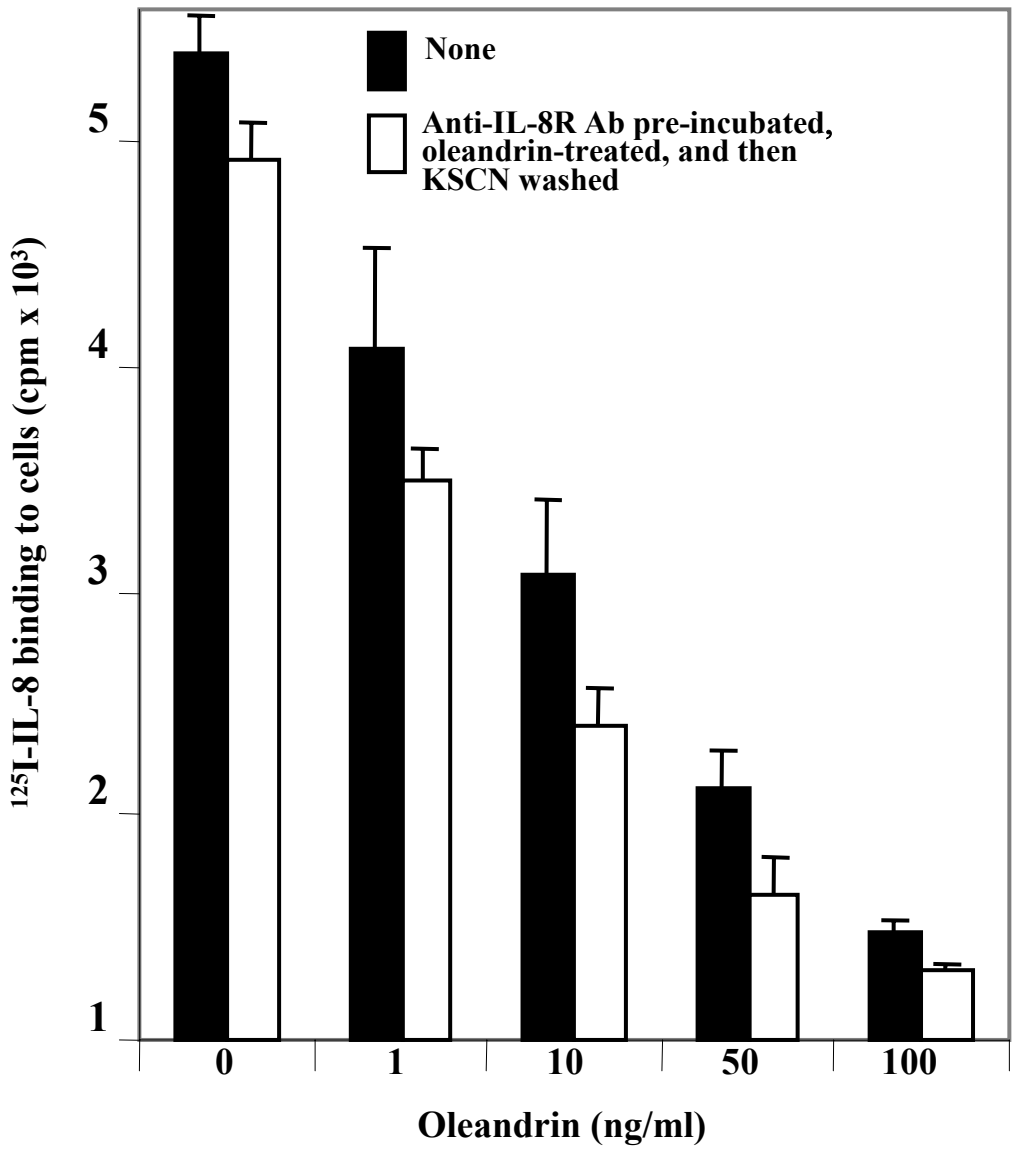


Fig 5.27 Anti-IL-8Rs antibodies were unable to protect oleandrin-mediated downregulation of IL-8Rs. Cells, incubated with anti-IL-8R1 and 2 Abs (1 μg each) for 1 h were treated with different concentrations of oleandrin for 3 h. Cells were washed with 0.5 M potassium thiocyanate and then assayed for ^{125}I-IL-8 binding. The data presented in these figures are representative of three independent experiments.

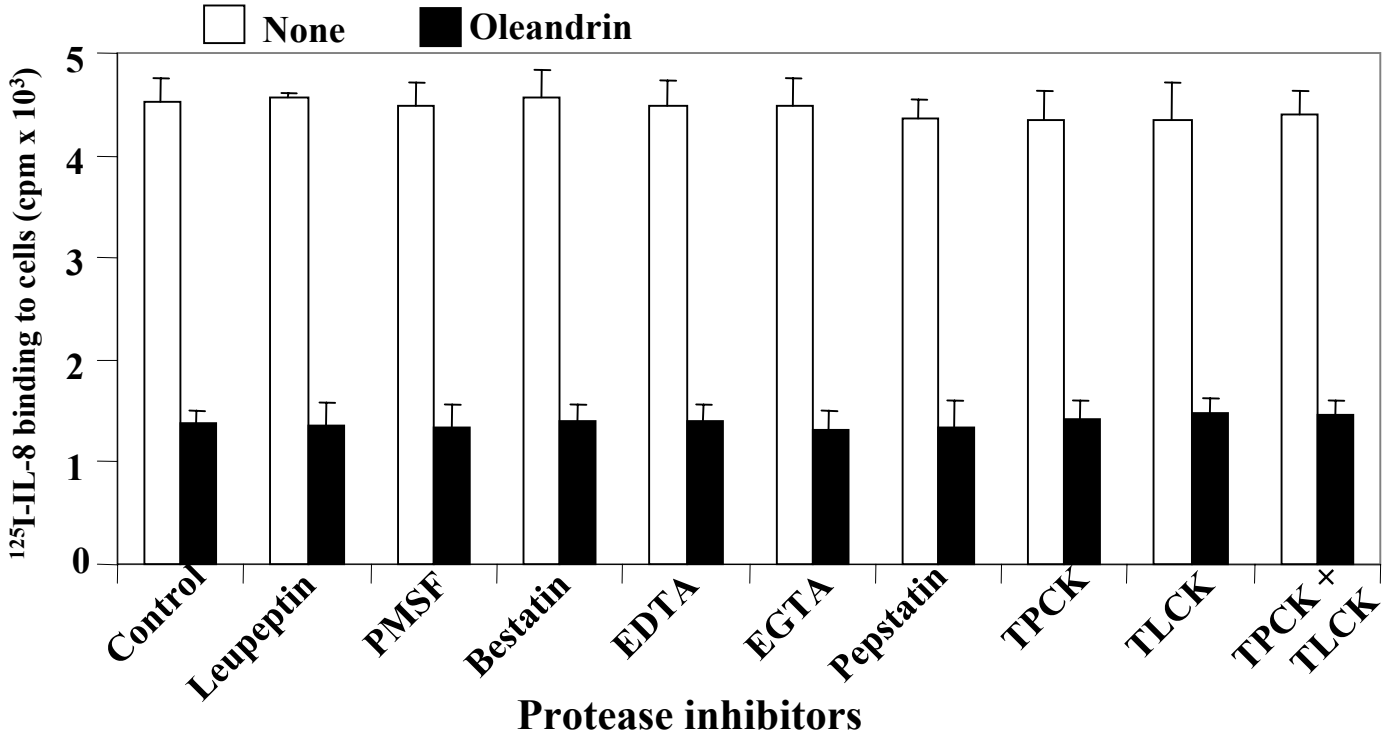


Fig 5.28 Effect of protease inhibitors on oleandrin-mediated downregulation of IL-8R. Macrophages were treated with 100 μM of leupeptin, PMSF, bestatin, EDTA, EGTA, pepstatin, TPCK, TLCK, or TPCK and TLCK for 1 h and then treated with oleandrin for 3 h at 37^0C. Then ^{125}I-IL-8 binding was carried out.

binding was assayed. The result showed that oleandrin-mediated downregulation of IL-8Rs was protected by cholesterol, cephalin, lecithin, or shingosine by 11, 40, 41, or 71% respectively (Fig.5.29) indicating the role of lipid molecules in oleandrin mediated IL-8R downregulation. Oleandrin-mediated downregulation of IL-8Rs and its protection by lipid molecules reflected IL-8-induced NF-κB activation (Fig.5.30).

5.2.13 Oleandrin inhibits diphenylhexatriene (DPH) binding in neutrophils.

DPH is a moderate membrane-perturbing fluorescent probe that is extensively used for measurement of fluidity and microviscosity of the membrane. To monitor the fluorescence of DPH, cells, treated with different concentrations of oleandrin for 3 h were incubated with DPH for 2 h at 37°C. After washing, the fluorescence was measured at 430 nm. Figure 5.31 shows that the DPH binding was decreased with increased concentrations of oleandrin suggesting alteration of microviscosity.

5.2.14 Oleandrin alters membrane fluidity in neutrophils.

The lipid fluidity of neutrophil membrane was determined by fluorescence depolarization measurement. The data presented in Figure 5.32 show that the microviscosity parameter $[(r_0/r) - 1]^{-1}$ was higher in untreated cells. In oleandrin-treated cells the value of the parameter was decreased in a dose-dependent manner and 25% decrease in microviscosity parameter was shown in 100 ng/ml oleandrin. Under identical conditions, 10 μM amphotericin B showed 40% decrease in microviscosity parameter. The results suggest that oleandrin alters membrane fluidity and microviscosity, which inturn leads to down modulation of IL-8Rs.

5.3 Discussion

IL-8 is an important inflammatory cytokine involved in all forms of neutrophil- and macrophage-driven inflammation and tumor progression through angiogenesis, hence the role of oleandrin is important on IL-8-mediated neutrophil and macrophage functions. Even though some reports indicate that oleandrin downregulates TNF- or ceramide-induced NF-κB activation in several tumor cell types, it does not do so in primary cells. Oleandrin induces apoptosis in several cancer cells through inhibition of NF-κB (Sreenivasan *et al.*, 2003). The purpose of this study was to investigate the effect of oleandrin on different growth factors-

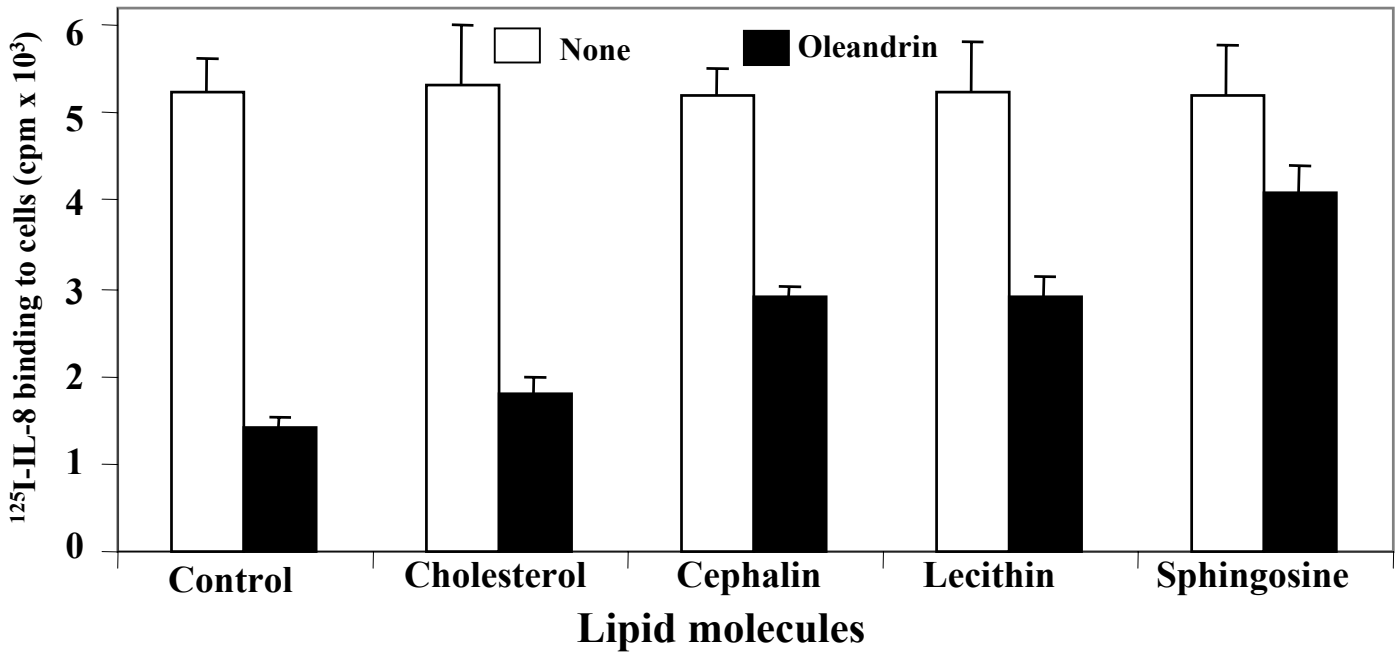


Fig 5.29 Effect of different lipid molecules on oleandrin-mediated downregulation of IL-8R activation. Oleandrin (100 ng/ml) was incubated with 500 μg of cholesterol, cephalin, lecithin, or sphingosine for 3 h and then macrophages were treated with these mixtures for 3 h. ^{125}I-IL-8 binding was carried out at 4^0C as triplicate samples to detect IL-8 binding

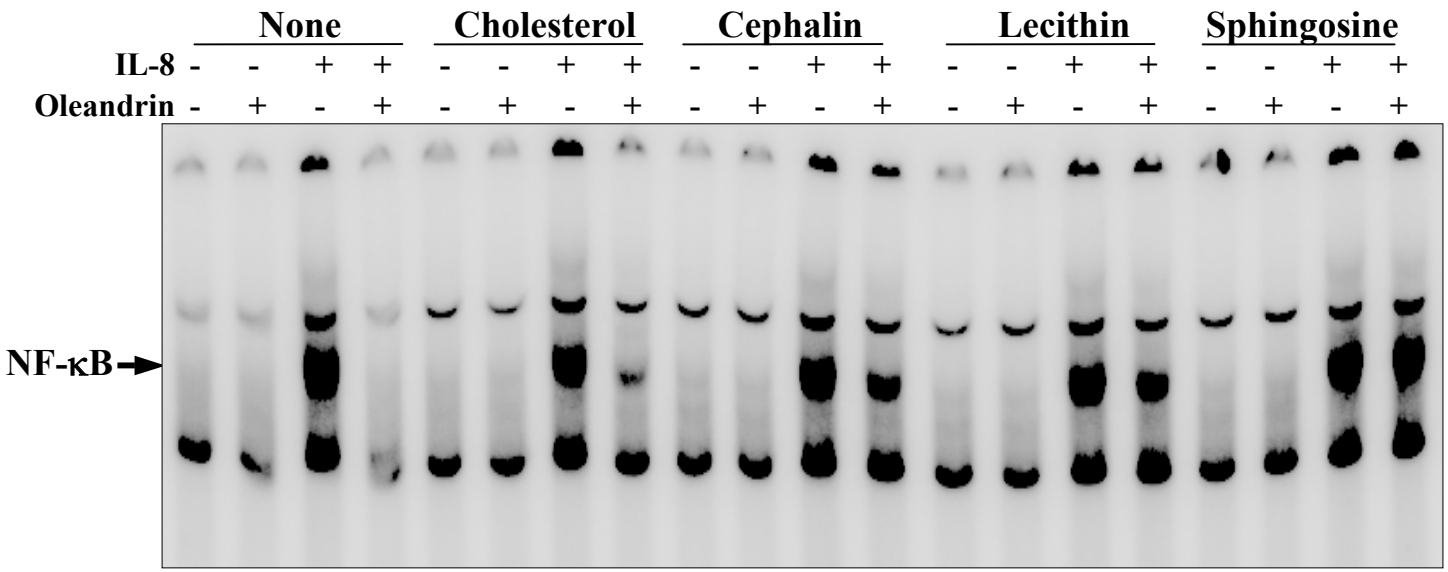


Fig 5.30 Effect of different lipid molecules on oleandrin-mediated downregulation of NF-κB activation. Oleandrin (100 ng/ml) was incubated with 500 μg of cholesterol, cephalin, lecithin, or sphingosine for 3 h and then macrophages were treated with these mixtures for 3 h. Nuclear extracts were prepared from same treated cells and assayed for NF-κB

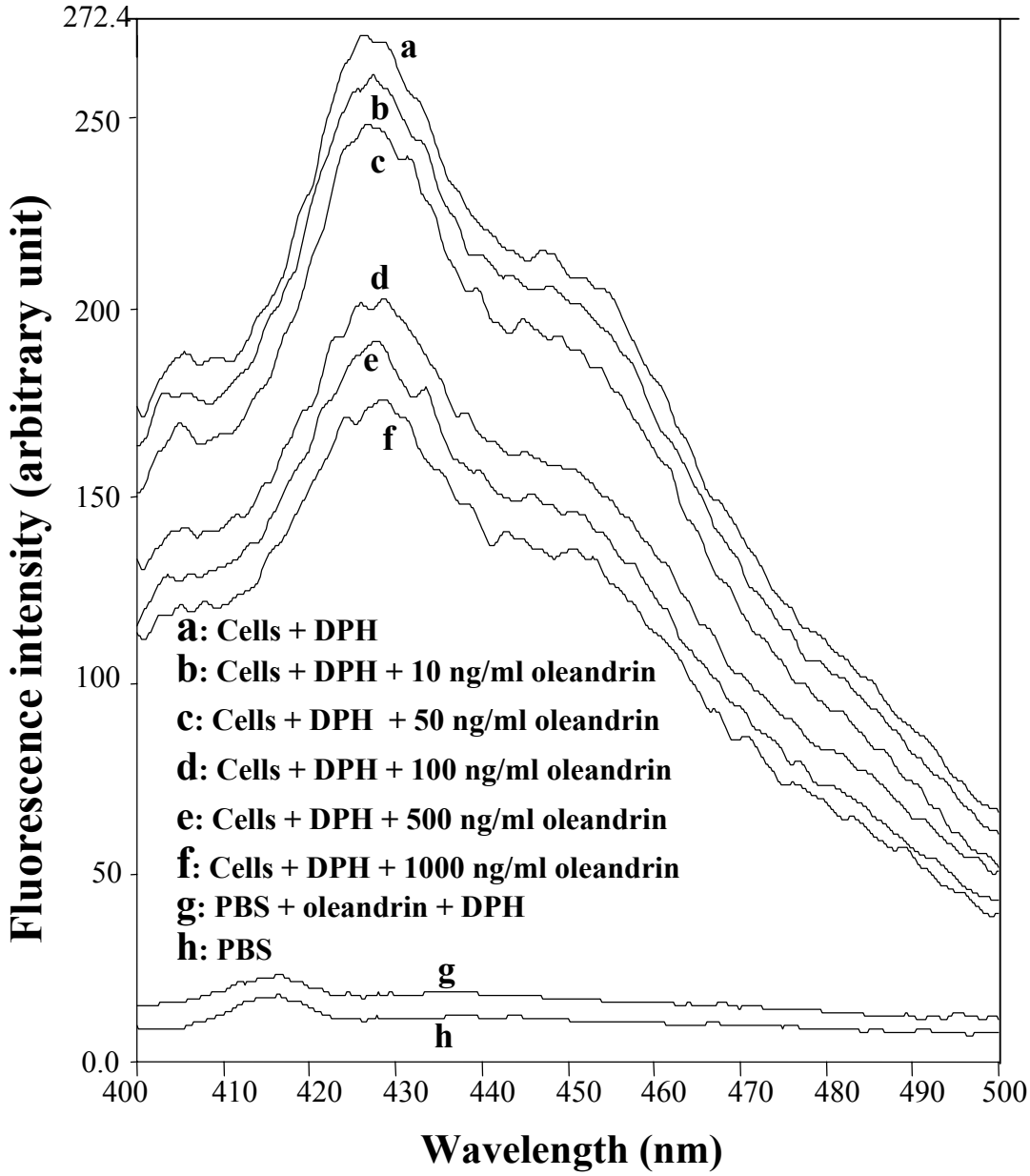


Fig 5.31 Effect of oleandrin on DPH fluorescence and microviscosity in neutrophils. Cells were treated with different concentrations of oleandrin for 3 h at 37°C. After washing, DPH (1 pM) was added to each well, and all tubes were kept at 37°C for 2 h with stirring. After washing, the cells were excited at 365 nm, and the emission spectrum was measured at 430 nm. The fluorescence spectrum obtained at 430 nm (expressed in arbitrary units) presented

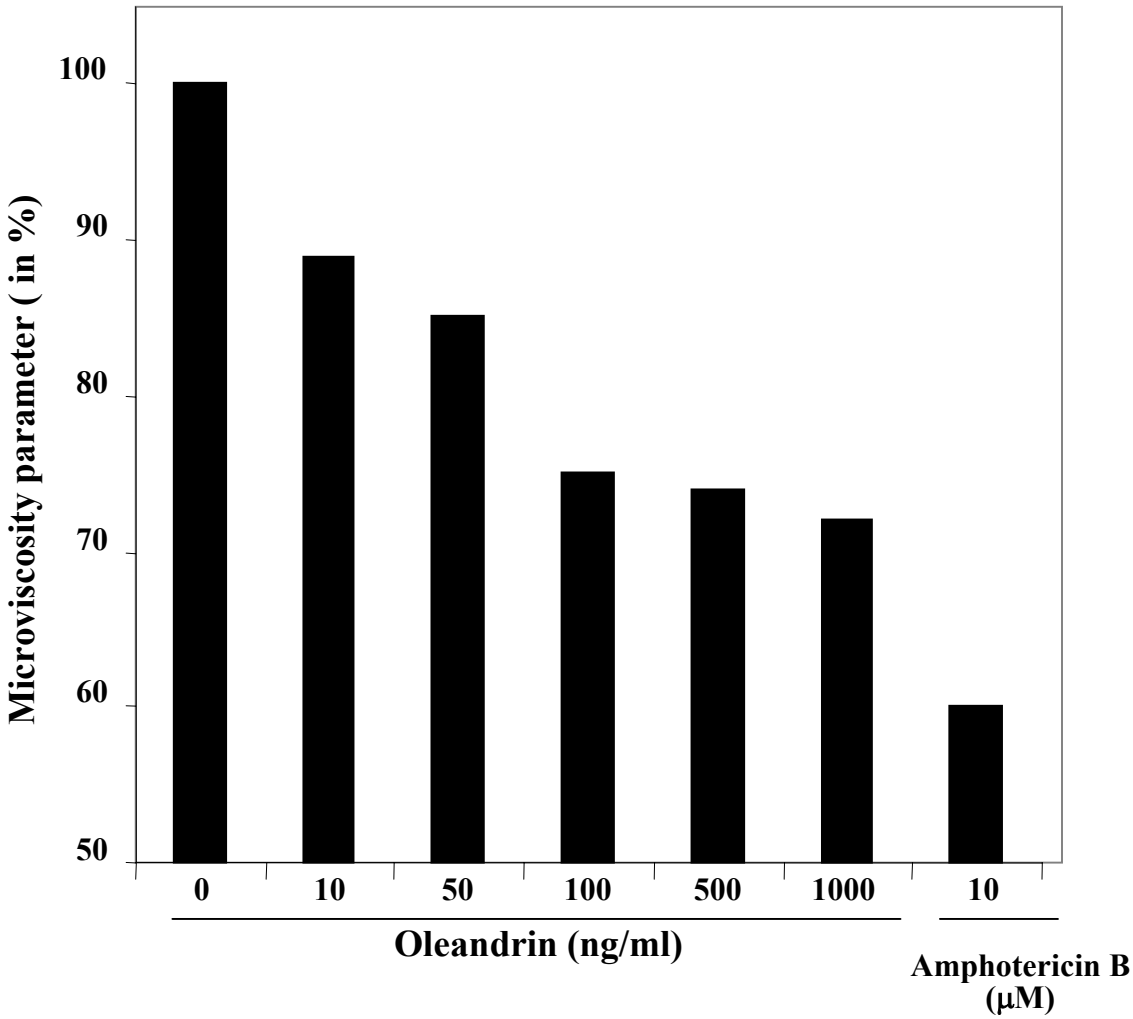


Fig 5.32 Effect of oleandrin on DPH fluorescence and microviscosity in neutrophils. Cells were treated with different concentrations of oleandrin for 3 h at 37°C. After washing, DPH (1 pM) was added to each well, and all tubes were kept at 37°C for 2 h with stirring. After washing, the cells were excited at 365 nm, and the fluorescent anisotrophy was measured. The results indicated are of values of the microviscosity parameter calculated using the formula mentioned in Materials and Methods.

mediated biological responses. In our experiments, we found that oleandrin blocked IL-8-, NGF-, EGF-, or FMLP-, but not IL-1- or TNF-induced NF-κB activation and oxidative burst response. Oleandrin's effect was shown in different cell types. Oleandrin mediated inhibition of several biological responses induced by IL-8, FMLP, NGF, or EGF is driven through its action on the cell surface resulting in altered microviscosity and fluidity of cell membrane. This inhibition by oleandrin is partially protected by different phospholipids.

TNF is a potent inducer of NF-κB and its dependent genes activation in several tumor cells. TNF induces IKKs, which mediates IκBα phosphorylation followed by degradation leading to p65-p50 (NF-κB) translocation to the nucleus and thereby activating several genes for cell survival and apoptosis. Oleandrin was unable to block TNF-induced NF-κB activation and its dependent genes activation in primary cells like macrophages and neutrophils. Although IL-8 induces NF-κB activation through IRAK-TRAF6 pathway (Manna and Ramesh, 2005), and oleandrin inhibited IL-8-induced IKKs activation and IκBα phosphorylation followed by degradation of IκBα in macrophages. However, oleandrin did not interfere with the activity of IKKs *in vitro*. These results suggest that oleandrin mediated action lies upstream of IL-8 signalling. Several of NF-κB-dependent gene products including cyclooxygenase and adhesion molecules are involved in tumorigenic and inflammatory responses. Oleandrin inhibited IL-8-, but not TNF-induced Cox2 and ICAM1 expressions suggesting inhibition of IL-8-induced NF-κB activation in macrophages.

IL-8 interacts with its cell surface receptors to activate several biological responses. These include degranulation of several proteolytic enzymes such as myeloperoxidase, alkaline phosphatase and β-D-glucuronidase and oxidative burst response for its first line of defence (Manna *et al.*, 1997). All these functions are downmodulated by oleandrin in macrophages. FMLP is a potent inducer of oxidative burst response (Chen *et al.*, 2003). Oleandrin inhibited not only IL-8-induced but also FMLP- and NGF-, but not TNF-induced oxidative burst response suggesting that oleandrin might act in a common pathway shared by IL-8, FMLP, and NGF. Oleandrin-mediated downregulation of IL-8-mediated NF-κB activation was observed in most cell types whereas TNF-induced cell signaling was restricted in tumor cells suggesting oleandrin's action in primay cells may be different to that in tumor cells.

Oleandrin decreased IL-8, NGF, or EGF, but not IL-1 or TNF binding (Fig.5.15) suggesting specific receptors downregulation by oleandrin. IL-8 and FMLP receptors belong to G-protein coupled receptors (GPCRs). Oleandrin downregulated IL-8- and FMLP-induced biological responses suggesting downregulation of GPCRs. Though NGF and EGF receptors are structurally different than GPCRs, oleandrin downregulates NGFR and EGFR. This provides scope for looking into the mode of action mediated by oleandrin in downmodulating NGFR and EGFR . Altered fluidity might affect specific receptor conformation for its ligand binding. NGF exerts its effect not only in neuronal cells but also in other cell types (Garaci *et al.*, 1999; Hikawa *et al.*, 2002; Ricci *et al.*, 2004). Expression of EGFR and NGFR in breast cancer (Decamps *et al.*, 2001; El-Rehim *et al.*, 2004; Lev *et al.*, 2004) gives us much attention to detect the levels in addition to other receptors. Suprisingly, oleandrin downmodulated NGF and EGF binding as well as NGF- and EGF-mediated responses. Oleandrin inhibited labeled IL-8 uptake at 37^0C (Fig.5.20) suggesting the downmodulation of viable IL-8 receptors. Receptors, after binding with its corresponding ligands are internalized and recycled back to cell surface to follow the same cycle repeatedly at 37^0C. Oleandrin inhibited this process, which may be by interfering with the binding of IL-8 with its receptors. ^{125}I-IL-8 binding was not inhibited in macrophages when incubated with oleandrin at 4^0C (data not shown), suggesting oleandrin has no role in the IL-8 binding domain of IL-8Rs. Oleandrin treated macrophages showed almost 80% IL-8Rs downregulation as observed by labeled IL-8 binding, detected by Scatchard analysis (Fig.5.21) and crosslinkined IL-8-IL-8Rs (Fig.5.20). Oleandrin downregulated both types of IL-8Rs as detected by radiolabeld IL-8 crosslinked IL-8Rs (Fig.5.22) and competitive binding with MGSA. MGSA specifically binds with IL-8R type 2 with high affinity, whereas IL-8 binds with both types of IL-8R (Type 1 and 2) with high affinity (Jones *et al.*, 1996). Oleandrin downregulated IL-8Rs in purified macrophage membrane, but not in affinity purified IL-8Rs. Ligand or anti-IL-8R antibodies did not protect this downregulation of IL-8Rs. Oleandrin-mediated downregulation of IL-8Rs was not due to production of IL-8 as detected by ELISA (Fig.5.26). Different protease inhibitors were unable to protect IL-8Rs from oleandrin-mediated downregulation indicating proteases are not involved in oleandrin's action.

DPH is a fluorophore that has been extensively used to monitor the hydrophobicity of the membrane microenvironment. DPH itself in aqueous solution shows very weak emission

because of its effective quenching of fluorescence. As soon as DPH interacts with the cell membrane, the intensity of fluorescence markedly enhances at 430 nm (Fig. 5.31). The possible reason for this increased intensity of fluorescence is that the fluorophore (DPH) bound to the nonpolar hydrophobic environment of the membrane. The intensity of DPH fluorescence was reduced in oleandrin-treated cells in a concentration-dependent manner (Fig.5.31), primarily due to disturbed microenvironment for DPH binding with membrane lipids. Oleandrin interferes with the fluidity and microviscosity of the membrane possibly by interacting with the phospholipids of the membrane. As a result, the non-polar environment required for enhanced DPH fluorescence intensity was impaired. The partial protection given by some of the phospholipids against the effect of oleandrin suggests a possible effect on membrane fluidity, which finally results in the downregulation of the receptors for IL-8, FMLP, NGF, and EGF.

Residential macrophages and migrated neutrophils release proteases not only to clear the microbes in case of infection but also to destroy the surrounding tissues in case of inflammation. Oleandrin interacts with cells membrane and specifically downregulates specific growth and G-protein-coupled receptors including IL-8Rs thus decreasing excessive migration and release of several proteases in several inflammatory diseases, which may help to ameliorate the distress in these patients. With its ability to downregulate IL-8-mediated biological responses in neutrophils, macrophages, and several tumor cells oleandrin may be considered as potent anti-inflammatory and anti-angiogenic therapeutic agent.

Chapter Six

Inhibition of Akt phosphorylation and induction of FasL expression by oleandrin

6.1 Introduction.

A plethora of transcription factors, kinases, phosphatases and other signaling molecules are involved in the regulation of apoptosis mediated by chemotherapeutic agents. The complex regulatory mechanisms, which govern these factors, play a vital role in the apoptosis inducing ability of chemotherapeutic agents. Some of the major protein molecules involved in the progression of tumors and apoptosis pertaining relevance to the study are elaborated in this prelude.

6.1.1 Akt.

Akt encodes a serine/theronine kinase that has an amino-terminal PH domain, a central catalytic domain and a short carboxy terminal regulatory domain. There are three members of the Akt family (Akt 1, Akt 2 and Akt 3), which, in general are broadly expressed. Growth factor mediated PI3K activation results in increases in 3'-phosphorylated phosphoinositides, which results in both the translocation of Akt to the plasma membrane and a conformational change that renders the regulatory phosphorylation sites accessible to PDKs (PI3K dependent kinases). Akt is rapidly phosphorylated by PDK1 at two positions, the activation loop (Thr 308) and the hydrophobic phosphorylation motif (Ser 473). In unstimulated cells, Akt is present in the cytosol in a conformation that blocks access of PDK-1 to the activation loop specifically, the PH domain masks Thr-308. Mitogen stimulation results in the membrane recruitment of Akt, unmasking of the activation loop, and allowing the phosphorylation of Thr-308 by PDK1. The phosphorylation of PDK1 renders Akt catalytically competent and causes the autophosphorylation of Ser-473 of the hydrophobic phosphorylation motif.

6.1.1.1 Significance of Akt activation.

The activation of Akt has profound biological consequences, which can be catalogued into three headings- survival, proliferation (increase in cell number) and growth (increase of cell size).

6.1.1.1.1 Cell survival.

As mentioned in earlier chapters, cancer cells have devised several mechanisms to inhibit apoptosis and prolong their survival. Akt functions in an apoptotic pathway, because dominant-negative alleles of Akt block survival that is mediated by insulin like growth factor

1(IGF 1) and constitutively active Akt rescues. PTEN mediated apoptosis (Dudek *et al*., 1997; Li *et al*., 1998). Akt directly phosphorylates several components of the cell death machinery. For example, BAD is a pro-apoptotic member of the BCl-X_L. Phosphorylation of BAD by Akt prevents this interaction restoring Bcl-X_L's anti-apoptotic function (Datta *et al*., 1997). Also, phosphorylation of FKHR a member of the forkhead family of transcription factors by Akt sequesters it in the cytosol thus inhibiting activation of FKHR gene targets which include BIM and Fas ligand (Brunet *et al*., 1999). Akt can also influence cell survival by means of indirect effects on two central regulators of cell death-NF-κB and p53 (Romashkova and Makarov, 1999). Akt can exert a positive effect on NF-κB function by phosphorylation and activation of IκB kinase (IKK). Akt influences the activity of the pro-apoptotic tumor suppressor p53, through phosphorylation of the p53 binding protein MDM2. MDM2 is a negative regulator of p53 for degradation by the proteasome through its E3 ubiquitin ligase activity.

6.1.1.1.2 Cell proliferation.

Cyclin D1 levels, which are important in the G1/S phase transition, are regulated at the transcriptional, post-transcriptional and post-translational level by distinct mechanisms. The cyclin D1 kinase, glycogen synthase kinase-3β (GSK3β) phosphorylates cyclinD1 marking it for degradation by the proteasome Akt directly phosphorylates GSK3β and blocks its kinase activity thereby allowing cyclin D1 to accumulate (Diehl *et al*., 1998).

6.1.1.1.3 Cell growth.

Growth refers to the synthesis of macromolecules which results in increased cell mass or size, a process that is enhanced in cancer cells to meet the biosynthetic requirements that are imposed by the augmented degree of proliferation. One of the central regulators of cell growth is mTOR, a serine/threonine kinase that serves as a molecular sensor that regulates protein synthesis on the basis of the availability of nutrients. mTOR is a direct target of Akt and its activity can be suppressed by the PI3K inhibitor wortmannin (Nave *et al*.,1999).

6.1.2 Forkhead transcription factors.

Recent studies have revealed an evolutionary conserved signaling module in higher eukaryotes in which Akt can directly phosphorylate and inactivate a family of Forkhead box

class O (FOXO) transcription factor. Members of the forkhead superfamily are characterized by the presence of a conserved 110 amino acid DNA binding domain, called the forkhead or winged domain (Kaufmann and Knochel, 1996). A new nomenclature for these factors has been adopted and they are now denoted Forkhead box (Fox) factors, within this larger family of Fox factors lays the subfamily the FOXO factors. Three mammalian proteins belong to this family namely, forkhead in rhabdomyosarcoma (FKHR), FKHR-like 1 (FKHRL1) and acute-lymphocytic leukemia-1 fused gene from chromosome X (AFX). FKHR is now known as FOXO1, FKHRL1 as FOXO3a and AFX is known as FOXO4. These FOXO proteins interact preferentially with a core consensus recognition motif 5'-TTGTTTAC-3', although the residues flanking this core contribute to the DNA binding specificity. The FOXO factors were initially identified at chromosomal break points in several human tumors. These genetic alterations of FOXOs in human cancers strongly suggested that they play a role in the regulation of proliferation, survival or differentiation in higher organisms. In the presence of survival factors, activated Akt phosphorylates forkhead members resulting in their sequestration in the cytoplasm. In the absence of Akt activity, forkhead family members translocate to the nucleus, where they initiate a program of gene expression including FasL gene. FasL, acting in an autocrine or paracrine manner, activates the cell surface Fas protein, which in turn, activates a caspase cascade, and causes cell death. Evidence forkhead family members can regulate apoptosis stems from the observation that the non-phosphorylatable mutant of FKHR or FKHRL1, which are potent transcriptional activation in the nucleus, triggering apoptosis in multiple cell types (Brunet *et al*., 1999; Tang *et al*., 1999). The finding that FKHRL1 binds to sites present in the promoter of the FasL gene, and induces expression of a reporter gene driven by the FasL promoter has led to the hypothesis that in the absence of survival factors, when Akt is inactive, Forkhead family members may induce endogenous FasL gene transcription (Datta *et al*., 1999).

6.1.3 Nuclear transcription factor of activated T-cells (NFAT).

The NFAT family of transcription factors encompasses five proteins evolutionarily related to the Rel/NF-κB family (Chytil and Verdine, 1996; Graef *et al*.,2001). The primodial family member is NFAT5, the only NFAT related protein represented in the drosophila genome and is

identical to Ton EBP (tonacity element binding protein), a transcription factor crucial for cellular responses to hypertonic stress (Lopez Rodriguez *et al*.,1999; Miyakawa *et al*.,1999).

The NFAT proteins have a conserved N-terminal transactivation domain and a central DNA binding domain with homology to the DNA binding domains of Rel/NF-κB factors. Between these regions lies a regulatory domain, which contains phosphorylation sites, nuclear import and export signals and a binding site for the cellular phosphatases calcineurin (Scott *et al.,* 2001).

The distinguishing feature of NFAT is its regulation by Ca^{2+} and the Ca^{2+}/calmodulin dependent serine phosphatases calcineurin. NFAT proteins are phosphorylated and reside in the cytoplasm in resting cells; upon stimulation, they are dephosphorylated by calcineurin, translocate to the nucleus and become transcriptionally active, thus provide a direct link between intracellular Ca^{2+} signaling and gene expression. NFAT activity is further modulated by additional inputs from diverse signaling pathways, which affect NFAT kinases and nuclear partner proteins. NFAT is capable of stimulating the transcription of target genes containing NFAT response elements in their upstream enhancer sequences. Such elements are widely distributed (Kel et.al.,1999) and are almost ubiquitous in cytokine promoters. NFAT can stimulate transcription alone (Kim *et al*., 2000; Macian *et al*., 2000) but shows comparative and synergistic binding with a number of other transcription factors with the most widely documented being activator protein-1 (AP-1) (Jain *et al*., 1993; Serfling *et al*., 2000). NFAT nuclear accumulation is rapid, occurring within 5 to 10 minutes of stimulation and reversible (Kehlenbach *et al*., 1998; Shibasaki *et al*., 1996). Export occurs following rephosphorylation of NFAT by kinases, most likely by remasking the NLS and allowing a constitutively active nuclear export signal to dominate (Beals *et al*., 1997; Zhu *et al*., 2000). Thus, a balance between cellular phosphatases and kinase activities determines NFAT localization, with the outcome being dependant on levels of intracellular Ca^{2+}.

Sequence analysis of the upstream region of fas ligand (FasL) gene has revealed potential binding sites for a number of transcription factors. These include NFAT (Latinis et.al.,1997a; Holtz-Heppelmann *et al*., 1998), AP-1 (Kasibhatla *et al*., 1998; Faris *et al*., 1998) and NF-κB (Kasibhatla *et al*., 1999; Matsui *et al*., 1998). The physiological role of NFAT in activation induced FasL gene expression has been demonstrated through both biochemical and genetic studies (Fleck *et al*., 1999; Forrester *et al*., 1992; Kel *et al*., 1999; Kim *et al*., 2000). Mutation

of the NFAT binding sites in FasL promoter completely blocks the inducible transcription from this promoter (Latinis *et al*., 1997a; Rivera *et al*., 1998). Also promoter transcription studies reveal the removal of the promoter distal region of FasL gene, which contains the binding sites for AP-1 and NF-κB, has no effect on the inducibility of the FasL promoter (Latinis *et al*., 1997a; 1997b).

Pre-clinical studies have demonstrated that the cardiac glycoside, oleandrin has excellent activity against a variety of human solid tumor cell lines. However, a thorough understanding of the anti-neoplastic activities of oleandrin has not been fully addressed. Interestingly, this study was undertaken to decipher the signal transduction pathway(s) involved in oleandrin-mediated apoptosis of tumor cell lines.

6.2 Results

6.2.1 Oleandrin induces cell death in human tumor cells.

This experiment was done to investigate as to whether oleandrin is able to mediate cell death in tumor cells derived from different tissue background. The viability of cells after 72 hours of incubation with or without oleandrin treatment was analyzed using the formazone based MTT dye. It was seen that oleandrin-mediated cell death in a variety of cells, which includes HL-60, U937, Jurkat, HuT-78, MCF-7, SKBr3 and Hela as shown is figure 6.1. This adds to the earlier shown results that oleandrin mediated cell death is not cell type or lineage dependent.

6.2.2 Downregulation of Akt phosphorylation in oleandrin treated cells.

In the previous chapter it was shown that oleandrin down regulated several growth receptor signaling and since Akt being the downstream kinase activated upon this signaling the influence on Akt activity under oleandrin pressure was investigated. The phosphorylation of Akt activates it and subsequently mediates other signaling cascades. Upon oleandrin treatment there was a marked decrease in Akt phosphorylation (Thr 308) and subsequent degradation of Akt protein at further time point as shown in Figure 6.2. Western blot analysis for the structural protein tubulin was done which serves as protein loading control. Hence oleandrin mediates down regulation of Akt phosphorylation.

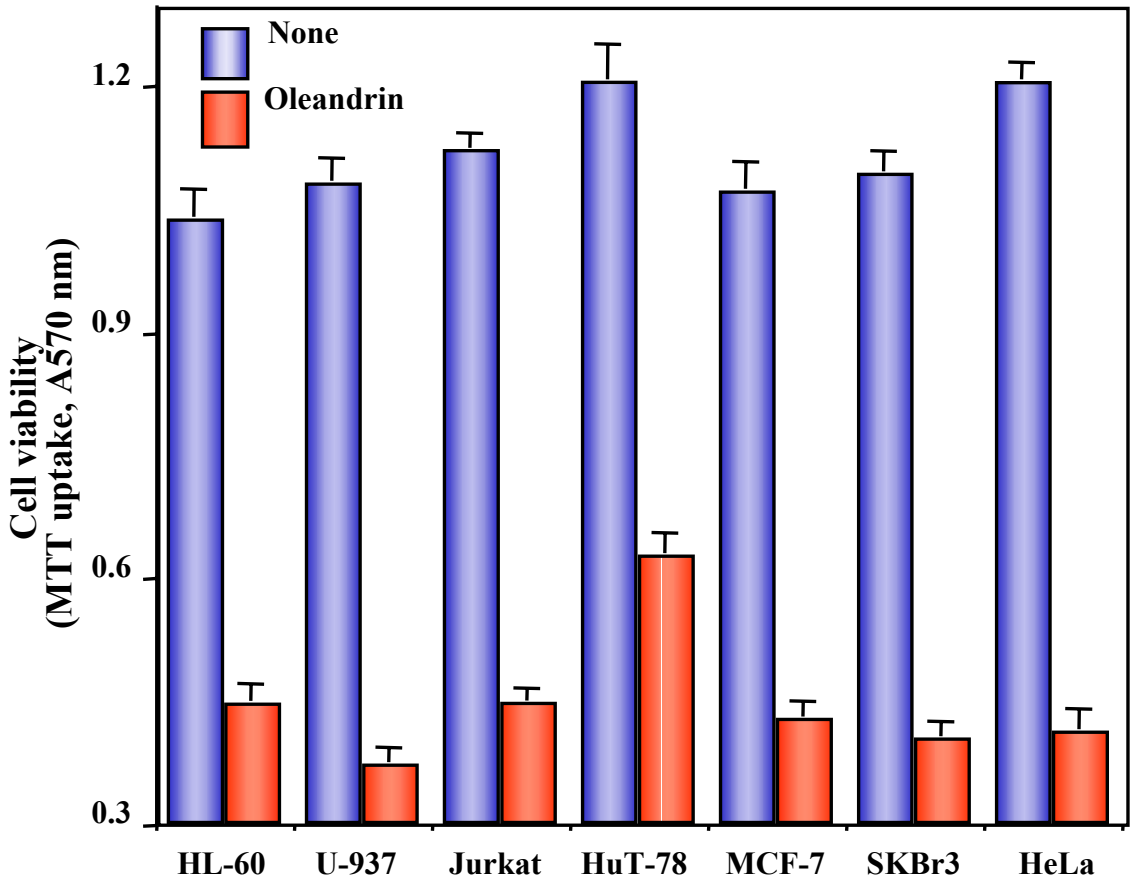


Fig 6.1 Oleandrin induced cytotoxicity in different human tumor cell lines. Different human tumor cell lines were cultured either in the presence or absence of oleandrin(0.1μg/ml) for 72 h. The MTT assay was done and absorbance taken at 570nm. The result indicated was mean OD of triplicate assay.

6.2.3 Oleandrin mediates nuclear localization of forkhead transcription factor.

One of the proteins that are phosphorylated by the kinase, Akt are the members of forkhead family of transcription factors. Non-activity of Akt renders the forkhead being not phosphorylated allowing it to traverse into the nucleus and drive transcription of dependent genes. The forkhead members are known to induce apoptosis primarily by inducing the expression of FasL. Experiments were carried out to check the status of forkhead factor upon oleandrin treatment. The cytoplasmic and nuclear extracts were partitioned from untreated or oleandrin treated U937 cells for various time intervals. Western blot analysis was carried out and probed for FKHR in the nuclear and cytoplasmic extracts. As shown in figure 6.3, upon six hours post treatment of oleandrin FKHR localizes to the nucleus and a subsequent decrease of the same in the cytoplasm was noticed (Fig.6.3). Hence it seems that upon oleandrin treatment forkhead factors traverse into the nucleus tracing the downstream signaling effects of deregulation of Akt phosphorylation.

6.2.4 Induction of FasL and CD95 in U937 cells treated with oleandrin.

Several scientific observations have documented that forkhead transcription factors can drive the expression of the death ligand FasL. According to the earlier observations of this study it was seen that upon oleandrin treatment forkhead factors translocates into the nucleus. Hence the expression pattern of FasL in U937 cells treated with oleandrin was investigated. It is seen that six hours post incubation with oleandrin there is an increase in the expression of FasL which was analyzed from the whole cell extracts prepared from untreated U937 cells or cells treated with oleandrin for various time intervals. Surprisingly, it was seen that there was also an increase in the expression levels of CD95 or the Fas death domain receptor. The increase in the expression of the Fas receptor level was at a later time point (Fig.6.4) from the inception of FasL expression in oleandrin treated U937 cells. Taken together the results show that there is an induction of FasL and CD95 expression upon treatment with oleandrin in U937 cells.

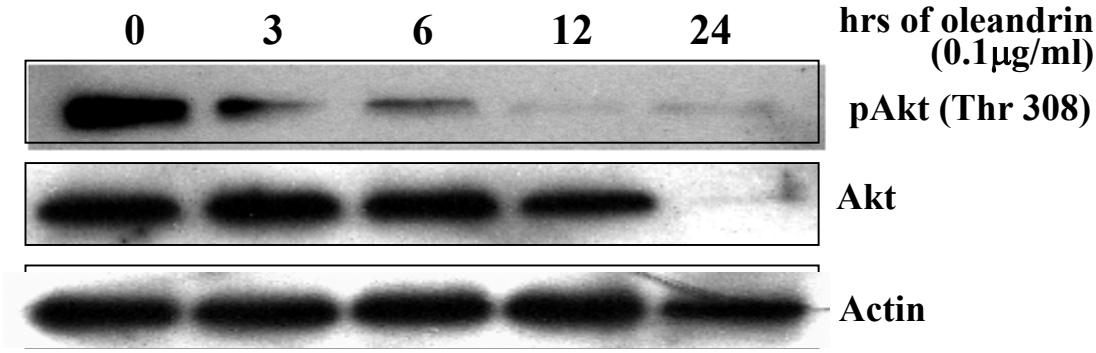


Fig 6.2 Effect of oleandrin on the phosphorylation and expression of Akt. U937 cells were incubated with 0.1 μg/ml of oleandrin for various time intervals and subsequently the whole cell lyaste was assayed for pAkt(Thr 308) and total Akt. The same blot was reprobed with actin.

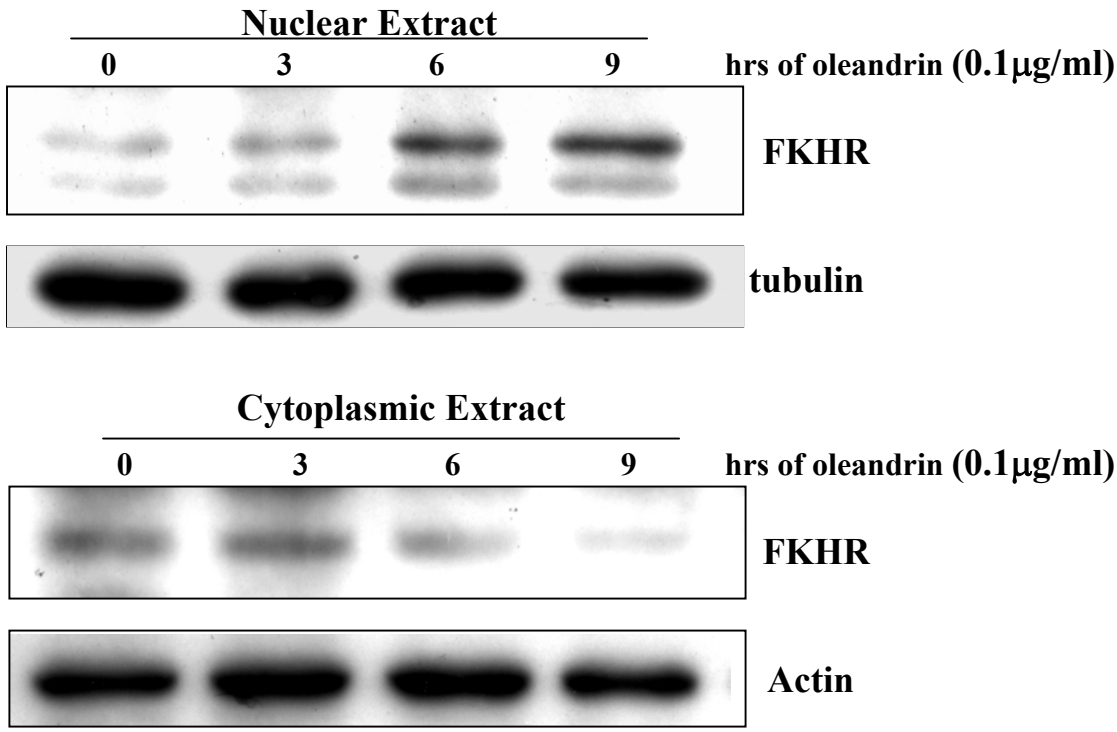


Fig. 6.3 Effect of oleandrin on the nuclear translocation of FKHR. Cells Treated with oleandrin for various time intervals were partitioned for cytoplasmic and nuclear extracts. These extracts were separately assayed for FKHR, actin or tubulin.

6.2.5 Oleandrin activates caspase 8 and caspase 3

The binding of FasL to its receptor CD95 activates the cytoplasmic death domain of the receptor, which then recruits the adaptor molecule FADD. FADD has an N-terminal death domain which interacts with the death domain of CD95 and a c-terminal death effector domain that recruits the initiator caspase 8 forming a complex called the death inducing signaling complex (DISC). Subsequent recruitment of various caspase 8 proteins to the DISC triggers an autocatalytic cleavage of the pro-caspase 8, forming the active caspase 8, which is explained by the induced proximity model of caspase activation. Tracing the same events the activation of caspase 8 was investigated in oleandrin treated U937 cells. Western blot analysis of whole cell extract from U937 cells treated with oleandrin for various time intervals showed that initially there was only the pro-caspase 8. However upon subsequent time intervals of oleandrin treatment there was appearance of activate caspase 8 (Fig.6.5). Oleandrin treated cells also showed the activation of effector caspase 3 (Fig.6.6). The active form of caspase 8 has the ability to process the effector caspase 3 into its active form. Hence in oleandrin treated U937 cells activation of both caspase 8 and caspase 3 were observed.

6.2.6 Oleandrin to U937 induces PARP cleavage and apoptosis.

Activation of caspase leads to the cleavage of a plethora of cellular proteins. Similarly, the poly ADP ribose polymerase (PARP) protein involved in the DNA repair mechanism is a direct proteolytic target of active caspase-3. PARP is a 118 kDa protein, which cleaves into 85 kDa fragment and is well documented as a molecular marker of apoptosis in general and activation of caspase 3 in particular. In this regard the total cellular extracts of untreated and oleandrin treated cells were subjected to western blot analysis and probed with anti-PARP antibody, which recognizes both the 118 kDa and 85 kDa proteins. Results as shown in figure 6a indicate that upon oleandrin treatment there is gradual disappearance of the 118 kDa protein and appearance of the cleaved 85 kDa fragment over a period of time. It is clear from the results that upon oleandrin treatment there is onset of the apoptotic programme in U937 cells. Further, FACS analysis of the annexin V phycoerythrin stained untreated and oleandrin treated (30 hours post) cells revealed that oleandrin treatment induces apoptosis in U937 cells (Fig.6.7).

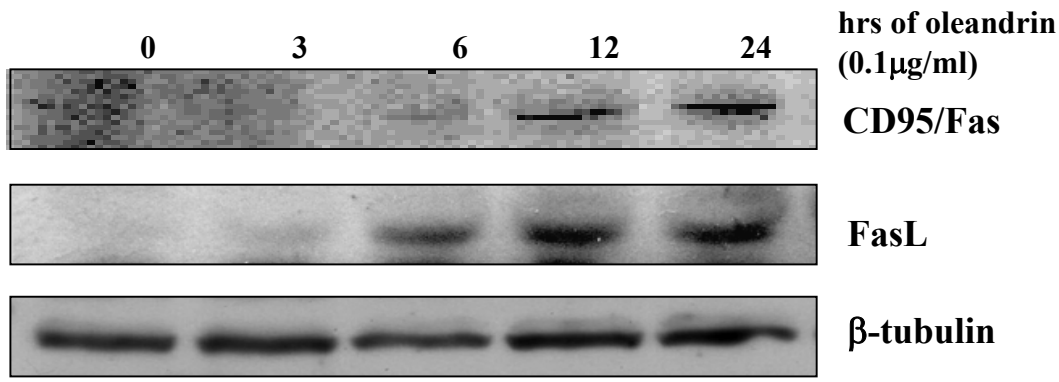


Fig 6.4 Effect of oleandrin on the induction of FasL and CD95 expression. U937 cells were incubated with 0.1 μg/ml of oleandrin for various time intervals and subsequently the whole cell lyaste was assayed for CD95 and FasL. The same blot was reprobed with anti tubulin antibodies.

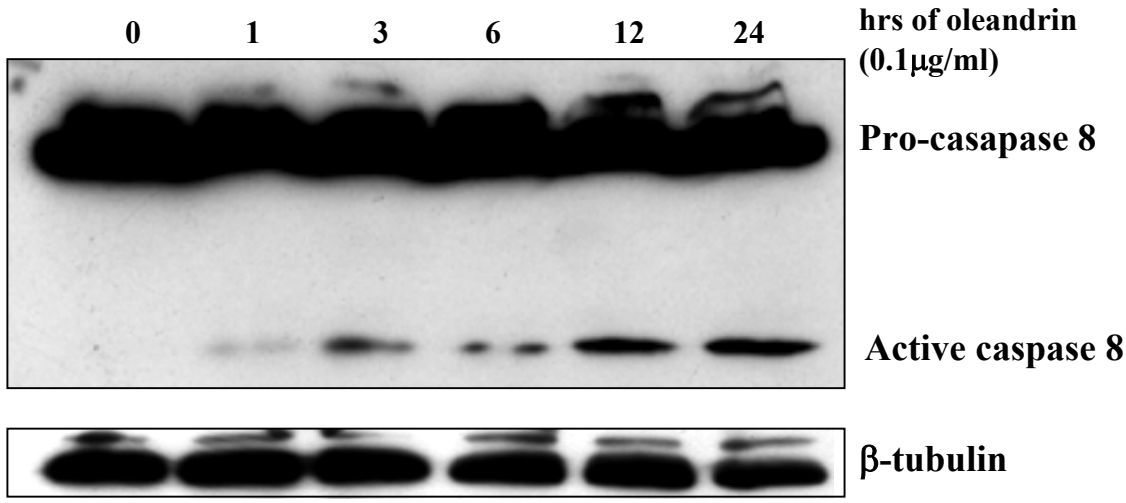


Fig 6.5 Activation of caspase-8 upon oleandrin treatment. Whole cell lysates were prepared from cells treated with oleandrin for various time intervals and subsequently assayed for caspase-8 using antibody that recognizes both the pro-caspase 8 and active form of caspase 8. The blot was reprobed with tubulin.

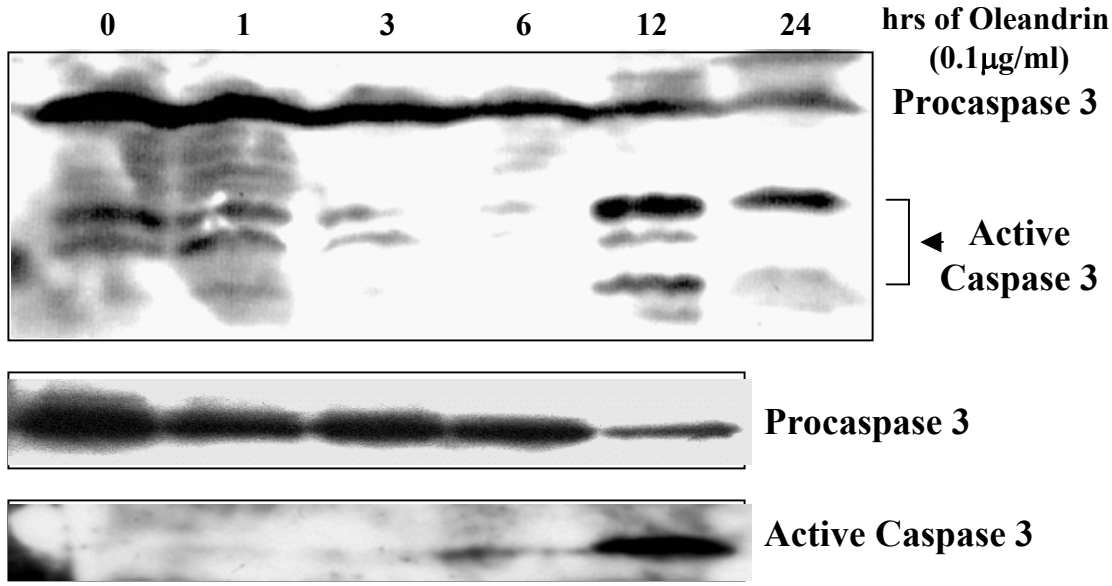


Fig 6.6 Activation of caspase-3 upon oleandrin treatment. Whole cell lysates were prepared from cells treated with oleandrin for various time intervals and subsequently assayed for caspase-3 using antibody that recognizes both the pro-caspase 3 and active form of caspase 3. The blot was reprobed with tubulin.

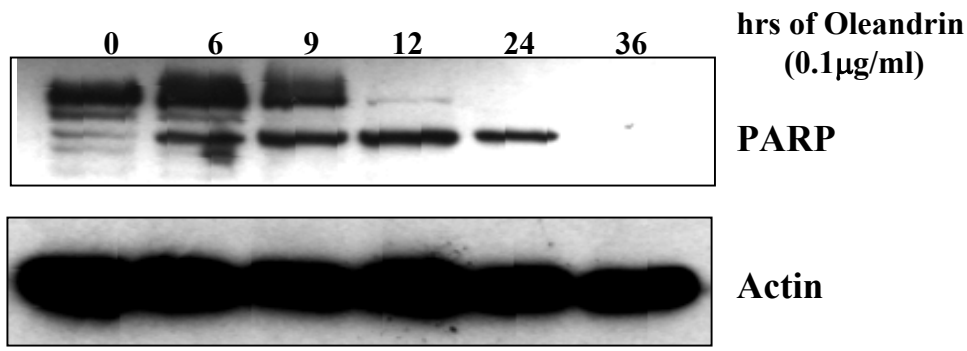


Fig 6.7 Effect of oleandrin on PARP cleavage. U937 cells, untreated or treated for various time intervals with 0.1 μg/ml oleandrin. Then cell extracts were prepared and 50 μg protein was analyzed by Western blot using anti-PARP mAb. The bands were located at 116 and 85 kDa. The blot was stripped and reprobed for actin.

6.2.7 Oleandrin treatment induces the nuclear localization of NFAT.

It was reported that oleandrin treatment to cells induces calcium efflux into the cytosol (McConkey *et al*., 2000). Presence of calcium in the cytosol activates calcineurin, which dephosphorylates the transcription factor, NFAT 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 NFAT. Hence it was appropriate to elucidate the role of NFAT in oleandrin treated cells. A gel retardation assay or EMSA (Fig.6.8) was done, wherein the nuclear extracts of U937 cells untreated or oleandrin treated for various time intervals was incubated with ^{32}P labeled NFAT binding oligo. According to the results as shown in figure oleandrin mediates the presence and DNA binding ability of NFAT in the nucleus from 6 hours post treatment onwards in U937 cells. Also immunolocalization experiments shows the presence of NFAT in the nucleus of U937 cells treated with oleandrin for 12 hours (Fig.6.8). Hence oleandrin treatment induces the nuclear localization of the transcription factor NFAT that could possibly mediate the expression of FasL.

6.2.8 Oleandrin treatment induces the activation of Erk and JNK in U937 cells.

It is reported that Erk induces the expression of Fas ligand via JNK mediated pathway (Suhara *et al*., 2002). The results from the kinase assay done (Fig.6.9) show that oleandrin increases the activity of both Erk and JNK at 6 hours post treatment onwards. Here phosphorylation domains of p17 (matrix protein of HIV1) and c-Jun were taken as substrates for Erk and JNK kinases respectively. Hence oleandrin treatment induces activity of Erk and JNK kinases in U937 cells.

6.3 Discussion.

Cancer arises from the stepwise accumulation of genetic changes that confer upon an incipient neoplastic cell the properties of unlimited, self sufficient growth and resistance to normal homeostatic regulatory mechanisms one among them being apoptosis. Although conventional chemotherapies have long been used to reduce the burden of disease in cancer patients, their side effects and the development of resistance by various forms of tumor has instigated the search for alternative agents that effectively kill cancer cells. Pre-clinical studies

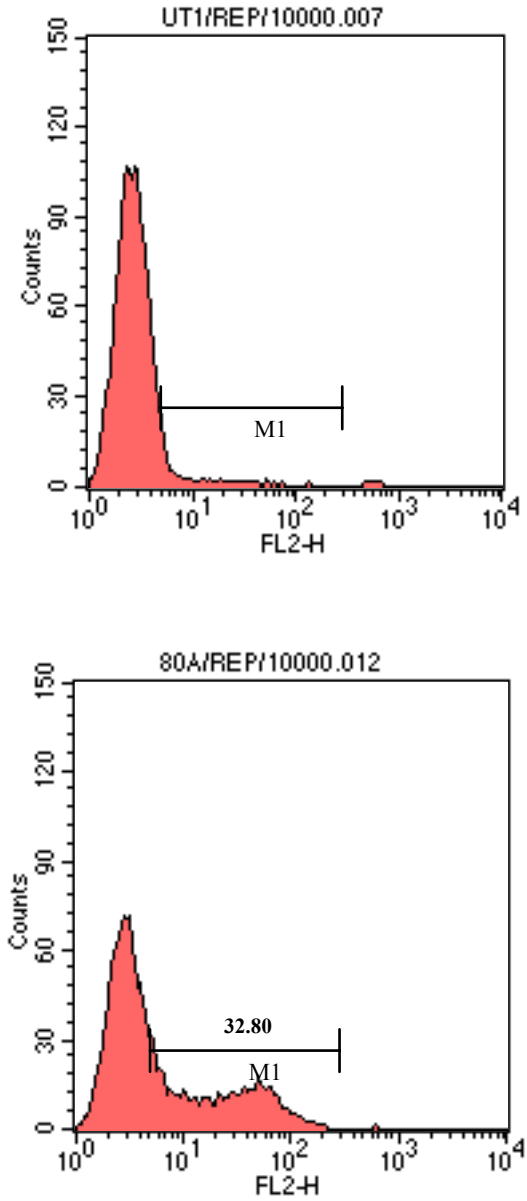


Fig 6.8 Oleandrin mediates apoptosis in U937 cells. Cells either untreated or treated with oleandrin for 30 h were pelleted and 10^6 cells were resuspended in Iml of annexin binding buffer. The cells were stained with Annexin V-PE and subsequently FACS anaylsis was done as described in Materials and Methods

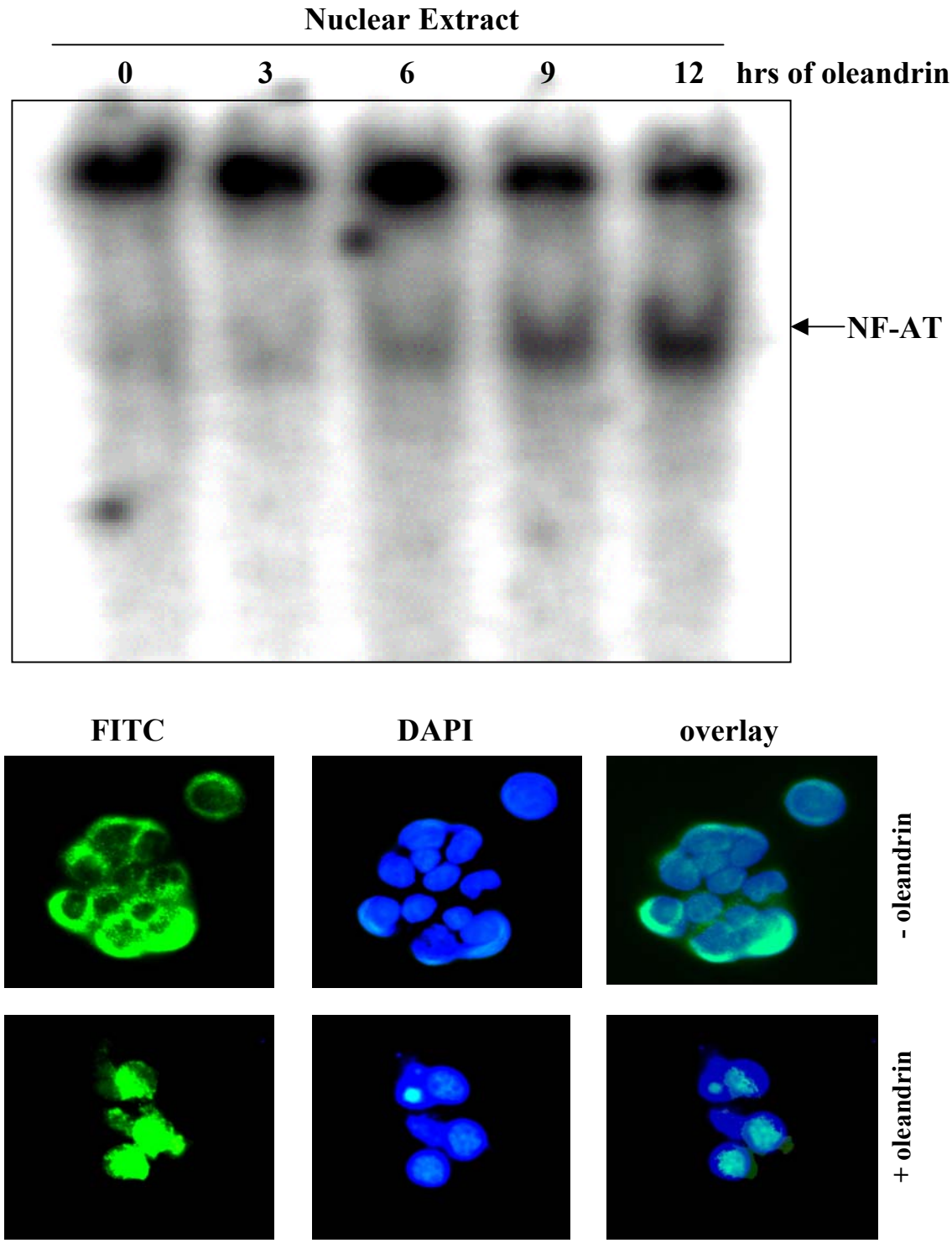


Fig 6.9 Effect of oleandrin on the nuclear localization of NF-AT. U937 cells were treated for various time intervals with oleandrin. After these treatments, nuclear extracts were prepared and then assayed for NF-AT by EMSA. Further U937 cells either treated with oleandrin for 12h or untreated were stained with FITC conjugated anti-NF-AT antibodies. These were further viewed under fluorescent microscope.

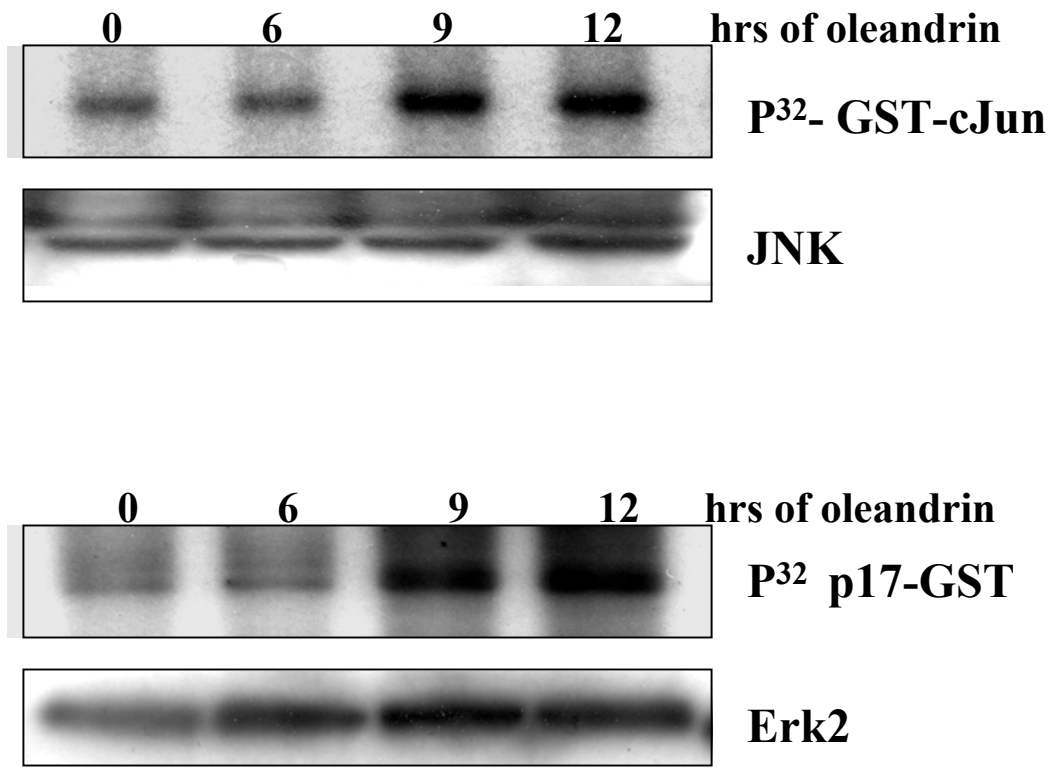


Fig 6.10 Effect of oleandrin on the activity of JNK and Erk2. Whole cell extracts were prepared from untreated or oleandrin treated cells for various time intervals. From these extracts JNK and Erk2 were immunoprecipated separately and kinase assay was done using GST-cJun and p17-GST as substrates as described in Materials and Methods.

have demonstrated that oleandrin has excellent activity against a variety of human solid tumor cell lines. But a thorough understanding of the apoptotic activities of oleandrin remains elusive. To begin with the ability of oleandrin to induce apoptosis in a variety of human tumor cells were checked upon. The results (Fig.6.1) showed that oleandrin was able to mediate apoptosis in cells which includes HL-60, U937, Jurkat, HuT-78, MCF-7, SKBr3 and HeLa proving that oleandrin mediated cell death cuts across wide array of tissue types. The serine/threonine kinase Akt is the major downstream target of growth factor receptor tyrosine kinases that signal via the phosphotidylinositol 3 kinase (PI_3K) (Testa and Bellacosa, 2001). Among its pleiotropic effects, activated Akt is a well-established survival factor, exerting anti-apoptotic activity by preventing release of cytochrome C from the mitochondria and inactivating forkhead transcription factor known to induce expression of pro-apoptotic factors such as Fas ligand. Akt phosphorylates and inactivates the pro-apoptotic factors BAD and pro-caspase 9. Moreover, Akt activates IκB kinase, a positive regulator of NF-κB, which results in transcription of anti-apoptotic genes (Testa and Bellacosa, 2001). Results obtained previously (chapters 3, 4 and 5) show that oleandrin downregulated NF-κB activation and also blocked the biological activities mediated by various growth factor receptors like IL-8, NGF and EGF receptor. Also results published earlier show that oleandrin inhibits FGF2 signaling (Smith *et al*., 2000). Treatment of oleandrin (6 hours post treatment onwards) down regulated Akt phosphorylation (Thr-308) in U937 cells whereas there was no decrease in the expression levels of Akt protein (Fig.6.2). The phosphorylation of Thr-308 of Akt by PDK1 renders Akt catalytically competent and causes the autophosphorylation of Ser-473 (Toker and Newton, 1999). Further downstream to Akt signaling is the regulation of the forkhead transcription factors. In a resting cell the forkhead transcription factors are phosphorylated by Akt, which renders them in the cytoplasm. In lines with the signaling cascade treatment of oleandrin to U937 cells activates the localization of the forkhead factor (FKHR) into the nucleus (fig 6.3). Dephosphorylation of the forkhead factors leads to the activation of genes like the FasL, which bears apoptotic potential. Upon treatment of oleandrin there was an increase in the expression of FasL and also its receptor CD95. However, the mechanism by which oleandrin induces the expression of CD95 remains elusive. There was induction of caspase 8 and caspase 3 (Fig.6.5 & 6.6) activation and subsequently PARP cleavage and apoptosis in oleandrin treated U937 cells (Fig.6.7 & 6.8). The results suggest that oleandrin activates the expression of FasL via the

forkhead factors, which leads to the activation of caspase 8, and subsequently triggering an apoptotic cascade in U937 cells leading to cell death. However the role of other transcription factors in mediating FasL expression was to be checked upon. The transcription factor NFAT has been reported to induce the expression of FasL. NFAT is regulated by the calcium induced Ca^{2+}/calmodulin dependent serine phosphatases calcineurin. In an earlier report published showed that treatment of cardiac glycosides induced the efflux of Ca^{2+} into the cytosol of cells (McConkey *et al*., 2000). Hence the role of NFAT in oleandrin treated cells was investigated. Oleandrin seemed to activate the nuclear translocation of NFAT upon treatment (6 hours post treatment onwards) (Fig.6.9). Also there was an increase in the activity of Erk and JNK kinases upon oleandrin treatment in U937 cells (Fig.6.10). The activation of Erk kinases is reported to induce the expression of FasL via JNK activation (Suhara *et al*., 2002).

Taken together the results from this study highlight the induction of FasL expression and subsequent apoptosis mediated via caspase 8 activation. The fact that oleandrin treatment inactivates Akt is very promising as Akt plays a central role in tumorigenesis. These data presented in this chapter suggest that oleandrin mediates more than one pathway that converges upon FasL expression. However the exact quantitative input of each of these pathways contributes towards the expression of FasL remains elusive. Whether these pathways compliment each other or one of them is thoroughly necessary is not clear. Also the possibility that other signaling pathways that bear the ability to drive FasL expression cannot be ruled out. The role of oleandrin as an agent that triggers multiple pathway(s) in the mediation of apoptosis bears immense potential for anticancer drug therapy especially in the regime of combination therapy to counter the chemo resistance phenomenon.

Summary

Cells are the structural and functional units of all living things and have a unique ability to reproduce them. The division of healthy cells goes in a regulated and systematic fashion, however some cells divide rapidly in a haphazard manner typically piling up into a non-structured mass or tumor. This mass or tumor becomes cancerous when it acquires the ability to spread to other parts of the body and start of new growth. Hence, cancer could be defined as a group of diseases characterized by uncontrolled growth and spread of normal cells. About 7 million reportedly died due to cancer in the year 2003 and 12.6% of all death each year is due to cancer. More than 10 million new cases are diagnosed each year and from a total of 10.8 million cases in 2002 it is estimated to reach to about 16.5 million cases by 2020.

Rapid advance since a quarter centaury, cancer research has generated a rich and complex body of knowledge, revealing cancer to be a disease involving dynamic changes in the genome. However, the search for the origin and treatment of this disease will continue over the next quarter centaury adding further layers of complexity to the existing scientific literature. On the contrary cancer research is developing into a logical science where the complexities of the disease, described in the laboratory and clinic will become understandable in terms of a small number of underlying principles that govern the transformation of normal cells into malignant cancer (The hallmarks of cancer according to Weinberg and Hanahan). The principles are self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. According to various scientific observations recorded, it is seen that NF-κB has a role in most of the above-mentioned principles.

Nuclear factor κB (NF-κB) is a transcription factor belonging to a family (REL family) of structurally related eukaryotic transcription factors that promote the expression of genes involved in a variety of cellular processes including cell growth and proliferation. The members of the family form homo and hetero dimmers, and the most common active form being the heterodimer of p50 and p65 (Rel A), which bind to a sequence motif known as the κB site found in the promoter region of various genes. It is reported that there is a constitutive expression of NF-κB in tumor cells as compared to the normal cells in the body. Constitutive

expression of NF-κB has been implicated as one of the causes for drug resistance in tumors. Chemotherapy has remained as the mainstay for tumor intervention till date. A chemotherapeutic agent interacts with a specific target, causing dysfunction and injury, which is interpreted by susceptible cancer cells as an instruction to undergo apoptosis. Apoptosis or programmed cell death is a well orchestered morphological phenomenon programmed in most of the cells characterized by chromatin condensation and nuclear fragmentation, plasma membrane blebbing and cell shrinkage, eventually breaking the cells into small apoptotic bodies, which are eaten up by phagocytes without inciting an inflammatory response in the vicinity. Of late, a major problem has been encountered that limits the effectiveness of chemotherapy called the chemo resistance phenomenon. Invariably a tumor can intrinsically be resistant to a chemotherapeutic agent prior to treatment or could be acquired while in treatment with the agent. 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. Also there is a requirement of new drugs in the market. Of the therapeutic drugs in use today, 70% of them are plant derived.

Oleandrin is a polyphenolic cardiac glycoside derived from the leaves of a tropical flowering weed *Nerium oleander*. According to reports, oleandrin was used in treating cardiac abnormalities in Russia and China, produced beneficial side effects in patients with Ewings sarcoma, prostrate and breast cancer. Pre-clinical studies on oleandrin show excellent activity against human tumor cell lines. In this scenario the objectives of the study were to decipher- the signal transduction pathway(s) of apoptosis mediated by the cardiac glycoside, oleandrin and also the ability of oleandrin in inhibiting any signaling event(s), which mediate as the lifeline or hallmark of a tumor cell.

Given the fact that NF-κB mediates a major influence in tumorigenesis, the effect of oleandrin on NF-κB activation was investigated. Ceramide, a well-studied second messenger was used as an inducer to activate NF-κB. The activity of the transcription factor NF-κB was assayed by the use of a radiolabelled oligo bearing the κB consensus sequence and

subsequently doing an EMSA (electrophoretic mobility shift assay) with the same. It was seen that oleandrin was able to inhibit ceramide induced NF-κB activation in a dose dependent manner. Pre-treatment of oleandrin (100ng/ml) for a minimum period of four hours was able to shut down ceramide induce NF-κB activation. Also oleandrin was shown to block ceramide mediated IkBα degradation and p65 translocation into the nucleus. Oleandrin inhibited ceramide induced NF-κB dependent reporter gene expression and the inhibitory effect of oleandrin was not shown for NF-κB oligo and protein binding *in vitro*, indicating oleandrin-mediated inhibitory effects are result of signaling events. There was inhibition to the binding of NF-κB to the radiolabelled oligo showing that oleandrin mediated inhibitory effects are result of signaling events. However oleandrin seemed to potentiate ceramide induced reactive oxygen intermediates generation and lipid peroxidation. Since ceramide was able to mediate apoptosis in cells the role of oleandrin on the ceramide mediated cell death was studied. Oleandrin potentiated ceramide induced cell death as shown by cell viability experiments like radiolabelled thymidine incorporation assay and dye based cytotoxicity assay (MTT assay). Also oleandrin potentiated ceramide induced PARP cleavage, DNA and nuclear fragmentation. The cleavage of PARP and DNA fragmentation is a well-established hallmark of caspase-3 activation. Hence oleandrin inhibited ceramide induced NF-κB activation but potentiates apoptosis.

It is reported that classical inducers of apoptosis like TNFα and ceramide fail to trigger apoptosis in NF-κB expressing cells. The question asked was whether oleandrin is able to induce apoptosis in NF-κB expressing cells? The model system used was HuT-78 cells, which constitutively activate NF-κB and serum activated LPS treated Jurkat cells (SA-LPS/Jkt), which induces NF-κB activation for over 48 hours. Oleandrin down regulated NF-κB activation in NF-κB expressing cells (HuT-78 and SA-LPS/Jkt) as shown by EMSA experiments and also down regulates NF-κB dependent reporter gene (SEAP) expression. Since p65 is the subunit involved in the transactivation of NF-κB heterodimer. Phosphorylation on the p65 subunit, signals for the localization of co-activators like p300 and HDACs onto the promoter and subsequently the polymerase, thereby transactivating the transcription factor. Hence the levels of p65 under oleandrin pressure were examined. Oleandrin down regulated

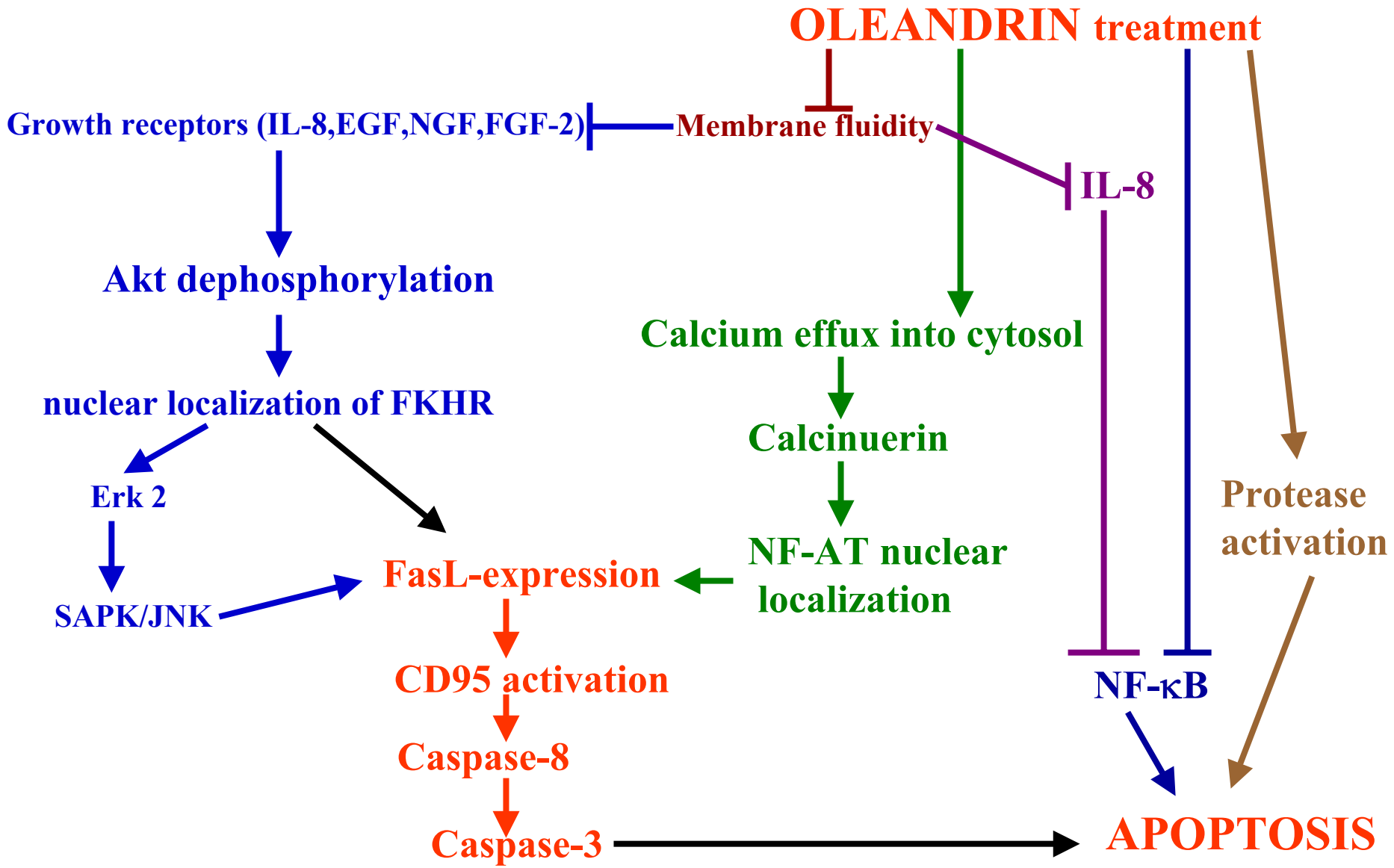
p65 levels in a time dependent manner (minimum of 6 hrs post treatment) but the levels of other transcription factors like SP-1 and p53 remained unaltered. Also oleandrin treatment did not change the expression levels of other REL members like p50 and Rel B. However c-Rel expression was down regulated upon oleandrin treatment but the levels of IkBα remained unaltered. The cysteine proteases, caspases are known to be the executioners of apoptosis and the effector caspases like caspase 3 mediate degradation of various cellular proteins. The activity of caspases 3,8 and 9 were assayed and oleandrin seems to activate all the above-mentioned caspases in a time dependent manner. However when ^{32}P-labelled p65 was incubated with caspases 3,8 and 9 immuno-precipitated from oleandrin treated HuT-78 cells for various time intervals, there was no down regulation of p65. Further z-VAD-fmk, a broad-spectrum caspase inhibitor was unable to block oleandrin mediated p65 degradation. Lactacystin, broad-spectrum proteosome inhibitor blocked the p65 degradation in oleandrin treated HuT-78 cells, also LMP-7, the inducible subunit of the proteosome was shown to be associated with p65. In most of the cases proteosomal degradation is preceded by ubiquitination of the target protein, but p65 did not shown any ubiquitination prior to degradation by the proteosome. The possibility of oleandrin directly degrading p65 was also ruled out by incubating oleandrin with bacterial expressed GST-p65 fusion protein, subsequently checking for p65 degradation. Oleandrin induced apoptosis in NF-κB expressing cells, but lactacystin alone was unable to fully prevent oleandrin mediated cell killing. However, lactacystin in combination with z-VAD-fmk was able to block most of the apoptosis in NF-κB expressing cells induced by oleandrin. Hence it was shown that oleandrin is able to mediate inhibition of NF-κB activation, degradation of p65 via the activation of proteosome and also induction of apoptosis in NF-κB expressing cells.

Interleukin (IL)-8 was first purified and cloned as a neutrophill chemotactic factor from LPS-stimulated mononuclear cell supernatants. It belongs to the CXC family of cytokines and functions by interactions with IL-8 receptors (IL-8Rs), which are G protein, coupled receptors with seven transmembrane domains. After ligand binding, IL-8Rs are internalized and subsequently recycled to reappear on the cell surface rapidly within 60 minutes. IL-8 elicits multiple effects on tumor growth, angiogenesis and metastasis. Hypoxia, has been shown to up-regulate IL-8 expression. IL-8 induces shedding of EGF ligands, which interact with the

EGF receptor promoting proliferative signals also IL-8 activates macrophages to produce growth factors near the tumor environment aiding in the proliferation of tumors. IL-8 is shown to mediate activation of metalloproteases and migration of endothelial cells for neo-vascularization. Oncogenic Ras, PI3K and MAPK activities in tumor cells promote the expression of IL-8. Also reactive oxygen species induces IL-8 expression via NF-κB activation. Hence the role of oleandrin in IL-8 mediated signaling was investigated. Our laboratory was the first to report that IL-8 mediates NF-κB activation via a TRAF-6 pathway. It was seen that oleandrin blocked IL-8, EGF or NGF, but not TNF or IL-21 mediated NF-κB activation. Further oleandrin inhibited IL-8, but not TNF induced reporter genes (SEAP driven by NF-κB promoter and luciferase driven by COX-2 promoter). Oleandrin mediated blocking of IL-8, EGF or NGF but not TNF or IL-1 ligand binding as shown by experiments using idonated (^{125}I) ligands. The effect of oleandrin was not extended to affinity purified IL-8Rs however when intact cell membranes of macrophages were isolated and incubated with oleandrin there was blocking of ^{125}I IL-8 binding. There are reports that some of the proteases pocess the ability to cleave the ligands or its receptors, hence a wide array of protease inhibitors was used in an effort to block oleandrin mediated down regulation of ligand binding. However, use of protease inhibitors did not revert the action of oleandrin on IL-8 but usage of lipid molecules like lecithin and sphingosine rescued the inhibition of IL-8 binding to its receptor. The results indicated that oleandrin interacts with lipid constituents of the cell membrane. Later it was seen that oleandrin induces reduction in membrane fluidity, as assayed by using DPH and checking for total fluorescence and microviscocity parameter for untreated and oleandrin treated macrophages for various time intervals. Hence oleandrin blocks IL-8 mediated signaling via influencing the membrane fluidity.

The inhibition of growth receptor signaling (EGF, NGF, IL-8, FGF-2) inhibits Akt phosphorylation. Akt is a serine/threonine kinase, which is activated upon phosphorylation by upstream kinases like PI3K, is reported to be over expressed in tumor cells and is implicated in giving the tumor cells a strong proliferative drive. Of the various downstream substrates of Akt is the forkhead transcription factor (FKHR). FKHR is phosphorylated by Akt thereby sequestering the former in the cytoplasm, upon dephosphorylation of FKHR due to non-activity of Akt; FKHR translocates to the nucleus and mediates expression of FasL via a JNK dependent pathway. According to the results it is seen that oleandrin mediates inhibition of Akt

Hypothetical model for oleandrin mediated apoptosis in human tumor cells



phosphorylation and at a later time down regulates Akt. Also oleandrin treatment induces activation of JNK and translocation of FKHR to the nucleus. There was induction of expression of FasL and CD95 in oleandrin treated U937 cells and subsequently apoptosis. According to one of the earlier reports cardiac glycosides have the ability to induce calcium efflux into the cytosol. Hence the possibility of induction of NF-AT which is one of the transcription factors implicated in the expression of FasL, in oleandrin treated U937 cells was investigated. It was seen that oleandrin mediates activation of NF-AT as shown by EMSA and immunolocalization experiments. Oleandrin like other cardiac glycosides mediate calcium efflux into the cytosol thereby activating calcineurin, a calcium dependent protein phosphatase and subsequently nuclear translocation of NF-AT. Here it is seen that oleandrin mediates induction of FasL expression via the activation of transcription factors like FKHR or NF-AT and subsequently triggering apoptosis.

Hence this study for the first time reports the role of various signaling pathways mediated by the cardiac glycoside, oleandrin which has the ability to cut the life lines of a cancer cell. To be more specific oleandrin cuts the proliferative signals by inhibiting NF-κB activation, Akt phosphorylation or inhibition of IL-8, EGF and NGF signaling, is able to trigger apoptosis by inducing the expression of FasL/CD95 and also by activating caspase and the proteosome. Additionally, oleandrin may also have an inhibitory effect on angiogenesis (as oleandrin inhibits IL-8 and FGF-2 signaling) and metastasis (inhibition of IL-8 and NF-κB signaling by oleandrin). Finally the cardiac glycoside, oleandrin is able to counter most of the earlier mentioned acquired hallmarks of a tumor cell making it a hypothetical drug model, implications of which could be used in the course of discovery for effective therapeutic intervention of tumors.

Appendix

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Mechanism of cytosine arabinoside-mediated apoptosis: role of Rel A (p65) dephosphorylation

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Nuclear transcription factor kappa B (NF-κB) has been shown both to block apoptosis and to promote cell proliferation, and hence has been considered an important target for anticancer drug development. The pyrimidine analogue cytosine arabinoside (araC) is among the most effective agents used in the treatment of acute leukemia, and we demonstrate in this study that its chemotherapeutic activity may be mediated by its inhibition of NF-κB. We found that in Jurkat cells, although tumor necrosis factor (TNF), araC, or ceramide induced NF-κB, the induction was only transient in the case of araC. In both HuT-78 and serum-activated LPS-stimulated Jurkat (SA-LPS/Jkt) cells that expressed NF-κB, TNF or ceramide treatments did not affect the NF-κB expression whereas araC downregulated it. AraC, but not TNF or ceramide was able to induce apoptosis in these cells as detected by assays for lipid peroxidation, reactive oxygen intermediates generation, caspase activation, cytotoxicity, Bcl-2 degradation, and DNA fragmentation. AraC also potentiated apoptosis mediated by cis-platin, etoposide, or taxol in these cells. AraC was able to induce protein phosphatases (PP) 2A and 2B-A, and phosphorylation of p65 subunit of NF-κB in the HuT-78 and SA-LPS/Jkt cells was downregulated by araC treatment. Furthermore, calyculin A, a specific phospho-serine/phospho-threonine phosphatase inhibitor, protected HuT-78 and SA-LPS/Jkt cells from araC-mediated NF-κB downregulation and apoptosis. These observations collectively suggest that araC induces apoptosis in NF-κB-expressing cells by dephosphorylating the p65 subunit of NF-κB.
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Keywords: NF-κB; araC; apoptosis; phosphatase; p65

Introduction

The major problem in cancer therapy by cytokines and chemotherapeutic agents is the development of resistance to apoptosis (Baldini, 1997), the mechanisms for which are not fully understood. Inactivation of p53 and related proteins, or expression of p-glycoprotein, Bcl-2, glutathione *S*-transferase, protein kinase C, transglutaminase, and heat shock proteins (e.g. hsp 27) has each been suggested to play an important role in resistance to anticancer agents (Bellamy and Dalton, 1994; Harrison, 1995; Reed, 1995). The activated form of NF-κB has also recently been implicated in the development of resistance to TNF (Beg and Baltimore, 1996; Van Antwerp *et al*., 1996; Wang *et al*., 1996; Giri and Aggarwal, 1998; Manna and Aggarwal, 1999).

NF-κB, a nuclear transcription factor, was first identified by Sen and Baltimore (1986). It regulates the expression of various gene products such as various adhesion molecules (ICAM-1, VCAM-1, ELAM-1), cyclooxygenase-2, and matrix metalloprotease-9, which play critical roles in inflammation, viral replication, tumor initiation, tumor promotion, metastasis, and apoptosis (Collins *et al*., 1995; Roshak *et al*., 1996; Baeuerle and Baichwal, 1997; Baichwal and Baeuerle, 1997; Lee and Burckart, 1998; Wang *et al*., 1999b; Waddick and Uckun, 1999). As part of the stress response, NF-κB is activated in response to various inflammatory stimuli including cytokines, mitogens, bacterial products, viral proteins, and apoptosis-inducing agents; consequently, this factor is currently a target of pharmaceutical interest (Lee and Burckart, 1998). NF-κB is also an ideal target for anticancer drug development, as it has been shown to block apoptosis and to promote proliferation (Van Antwerp, 1996; Wang *et al*., 1996, 1998). NF-κB activation induces resistance to chemotherapeutic agents (Wang *et al*., 1999a, b) and constitutive expression of NF-κB induces proliferation of tumor cells (Nakshatri *et al*., 1997; Giri and Aggarwal, 1998).

NF-κB is a heterodimer of two subunits p50 (NF-κB1) and p65 (RelA), that 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 (Egan *et al*., 1999). In this study, time course-dependent increase or decrease in NF-κB levels in nuclear extracts has been referred to as upregulation or downregulation of NF-κB, respectively. Interestingly, several chemotherapeutic agents such as doxorubicin and

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daunorubicin (Boland *et al*., 1997; Das and White, 1997), taxol, vinblastine and vincristine (Das and White, 1997), camptothecin (Piret and Piette, 1996), and etoposide (Perez, 1997) also cause NF-κB upregulation.

Constitutive upregulation of NF-κB has recently been correlated with progression of breast cancer, melanoma, and juvenile myelomonocytic leukemia (Kochetkova *et al*., 1997; Nakshatri *et al*., 1997; Raziuddin *et al*., 1997; Izban *et al*., 2000). How NF-κB is constitutively up-regulated in some tumor cells and what role it plays in acquiring resistance to apoptosis is not clear. It has been shown that HuT-78, a cutaneous lymphoid T-cell line that constitutively expresses NF-κB, is resistant to the apoptotic effects induced by TNF (Manna and Aggarwal, 1999). In T cells, NF-κB also activates the expression of Bcl-2, an antiapoptotic factor (Heckman *et al*., 2002). On the other hand, in endothelial cells, Bcl-2 and Bcl-XL have been shown to inhibit NF-κB-mediated gene transcription, by inhibiting IκBα degradation (Badrichani *et al*., 1999).

Resistance to chemotherapy is a major problem in the treatment of cancers. Combination therapy may be a useful tool in this regard, and it has been shown that a combination of thalidomide plus dexamethasone is effective in myeloma (Rajkumar *et al*., 2002), while the combination of dacarbazine, nimustine hydrochloride, vincristine sulfate, and interferon-β is useful in anorectal melanoma (Terada *et al*., 2002).

Cytosine arabinoside (araC) has been shown to induce apoptosis through various mechanisms. AraC and its different metabolites contribute to its cytotoxicity including incorporation of AraCTP into DNA and AraUMP into RNA, inhibition of polymerase α and β, and impairment of repair mechanisms (Braess *et al*., 1999). Besides this DNA synthesis impairment, araC engages an array of signaling events, including activation of PKC and MAPK (Kharbanda *et al*., 1994) and upregulation of AP-1 and NF-κB (Brach *et al*., 1992a,b). Simultaneous activation of both pathways for apoptosis by impairing DNA repair and for survival by inducing antiapoptotic influence of MAPK mediated by araC are interesting. In this study, we have examined the effect of the anticancer agent araC on apoptosis in cells expressing NF-κB.

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. In this study, we demonstrate, for the first time, that araC mediates apoptosis in NF-κB-expressing cells, HuT-78 and SA-LPS/Jkt cells. In these cells, araC downregulates NF-κB by activation of phosphatases that dephosphorylate its p65 subunit. We also demonstrate the sensitivity of araC in NF-κB expressed cells treated with different anticancer agents.

Results

In the present report, we investigated the role of NF-κB in apoptosis induced by TNF, araC, or ceramide in two human T-cell lines, Jurkat and HuT-78. HuT-78 cells constitutively express the nuclear transcription factor NF-κB. In Jurkat cells, NF-κB expression was induced with serum-activated lipopolysaccharide, as described in Materials and methods.

AraC downregulates NF-κB activation in Jurkat cells

To investigate the effects of TNF, araC, or ceramide on the levels of NF-κB, Jurkat cells (1×10^6/ml) were incubated with 1 nM TNF, 50 μM araC, or 10 μM ceramide for different times at 37°C. NF-κB activity in nuclear extracts was determined by electrophoretic mobility shift assay (EMSA) as indicated in Materials and methods using ^{32}P-labeled oligonucleotide carrying NF-κB binding sequence. As shown in Figure 1a, TNF and ceramide treatments led to persistent induction of NF-κB activity, whereas araC treatment was associated with an initial increase in NF-κB activity in 2 h followed by complete loss of NF-κB activity at and beyond 4 h. In control experiments, the levels of Oct1, a house-keeping transcription factor, were assayed and shown to be roughly the same in all the samples (Figure 1b).

Of the various possible oligomeric combinations of p50 and p65 (i.e., p50–p50 or p65–p65 homodimer, or p50–p65 heterodimer) NF-κB is able to bind to specific sequences in DNA. To show that the retarded band visualized by EMSA in araC-treated cells was indeed NF-κB, we incubated the nuclear extracts from ceramide-activated cells with antibodies (Abs) to p50 and p65 alone or in combination, and then conducted EMSA. Abs to either subunit of NF-κB shifted the band to a higher m.w. (Figure 1c), thus suggesting that the araC-activated complex consisted of both p50 and p65 subunits. Neither preimmune serum nor irrelevant Abs such as anti-c-Rel or anticyclin D1 had any effect on the mobility of NF-κB. The complex completely disappeared in the presence of a 50-fold molar excess of cold NF-κB indicating its specificity as that of NF-κB.

AraC downregulates IκBα level in Jurkat cells

The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα. To test the roles of TNF, araC, or ceramide on IκBα levels, the cytoplasmic extracts from the experiment described in the previous section were examined by Western blot analysis. Upon treatment with TNF or ceramide, the levels of IκBα showed an initial decline followed by slow recovery up to 24 h; in the case of TNF, the levels again declined from 12 h. With araC treatment, the IκBα level showed a sustained decrease up to 24 h (Figure 1d). We interpret the changes in IκBα levels as being the consequence (rather than the cause) of changes in NF-κB. Upon re-probing, all these gels with anti-p50 antibody, we found that the band intensities in all the lanes were uniform, indicating equal loading of extracted protein in the lanes. These results indicate that in TNF- or ceramide-treated cells, first there is degradation of IκBα leading to upregulation of NF-κB, and then resynthesis of IκBα since the latter is itself an NF-κB-dependent gene product. TNF-induced

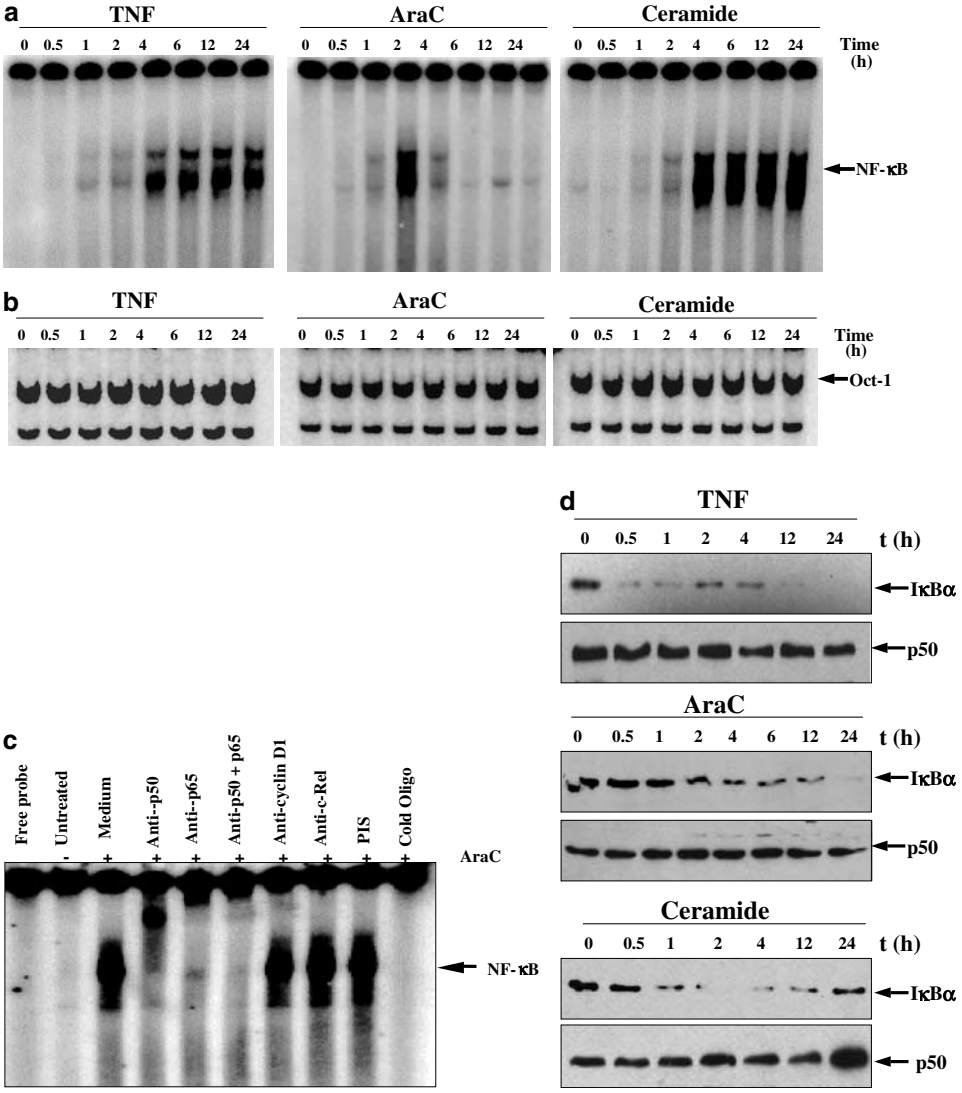

Figure 1 (**a**, **b**) Effect of TNF, araC, or ceramide on NF-κB activation in Jurkat cells. Jurkat cells (2×10^6/ml) were incubated with TNF (1 nM), araC (50 μM), or ceramide (10 μM) for different times as indicated in the figure. After these treatments, cytoplasmic and nuclear extracts were prepared. Nuclear extracts were assayed for NF-κB (**a**) and Oct1 (**b**), as described in Materials and methods. (**c**) Nuclear extracts were prepared from untreated or araC-treated Jurkat cells, incubated for 15 min with different Abs and cold NF-κB oligo, and then assayed for NF-κB, as described in Materials and methods. (**d**) Effect of TNF, araC, or ceramide on IκBα degradation in Jurkat cells. Cytoplasmic extracts were assayed for IκBα and p50 by Western blot analysis

IκBα degradation at later time points may be because of the availability of TNF in the medium as we stimulated cells with 1 nM TNF and it is very stable in aqueous phase. AraC-mediated downregulation of NF-κB correlates with the degradation of IκBα without resynthesis.

AraC inhibits NF-κB activation in SA-LPS/Jkt and HuT-78 cells

Jurkat, SA-LPS-stimulated Jurkat, or HuT-78 cells (2×10^6/ml each) were incubated with different concentrations of TNF, araC, or ceramide for 4 h at 37°C. Nuclear extracts were prepared and assayed for NF-κB by EMSA. As shown in Figure 2, all these agents induced NF-κB activation in Jurkat cells. SA-LPS/Jkt cells showed sustain levels of upregulated NF-κB and HuT-78 cells showed basal levels of NF-κB upregulation, which were marginally activated by TNF or ceramide. On the other hand, araC suppressed NF-κB in SA-LPS/Jkt and HuT-78 cells, as evidenced from the disappearance of the shifted band in the corresponding nuclear extracts. All the lanes exhibited equivalent intensities of Oct1, assayed as the loading control.

AraC, but not TNF or ceramide, inhibits NF-κB reporter gene expression in SA-LPS/Jkt and HuT-78 cells

Jurkat, SA-LPS/Jkt, and HuT-78 cells were each transfected with a construct expressing NF-κB-SEAP (secretory alkaline phosphatase) reporter gene fusion and dominant-negative IκBα. The cells were aliquoted, cultured for 24 h, and stimulated with different

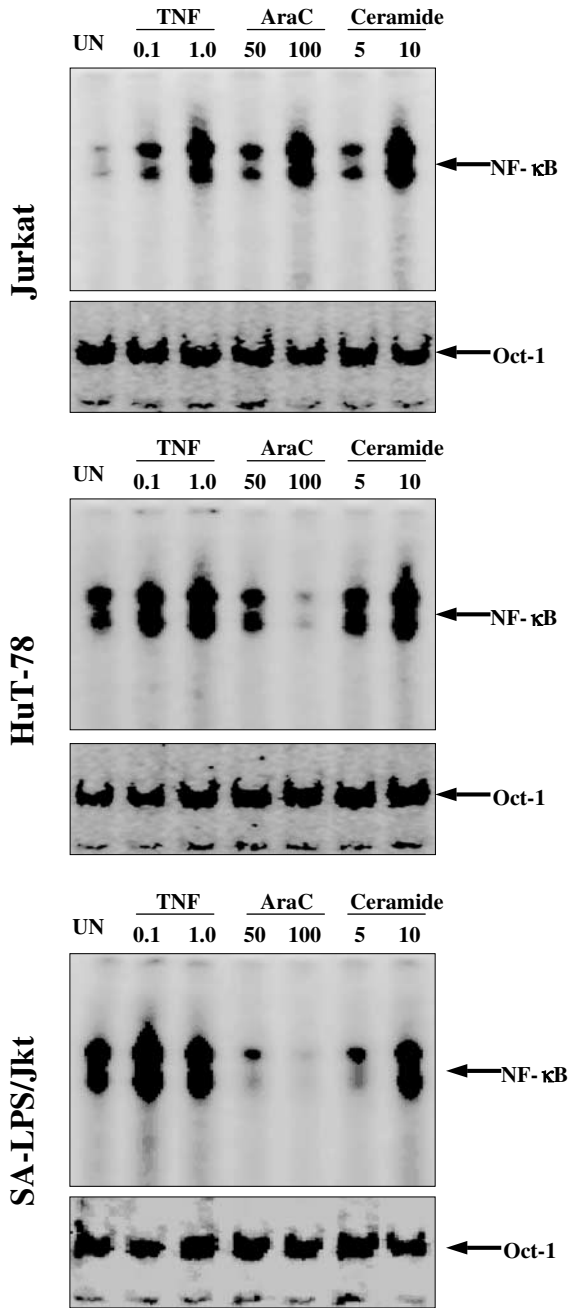

Figure 2 Effect of TNF, araC, or ceramide on NF-κB activation in Jurkat, SA-LPS/Jkt, and HuT-78 cells. LPS (1 μg) was taken in 200 μl of human serum and incubated for 1 h at 37°C. A volume of 20 μl of this mixture (SA-LPS) was used to make LPS (100 ng/ml) for activation of Jurkat cells for all experiments. Jurkat, HuT-78, and SA-LPS/Jkt cells (2×10^6/ml) were incubated with different concentrations of TNF, araC, or ceramide for 4 h. After these treatments, nuclear extracts were prepared and then assayed for NF-κB and Oct1 as described in Materials and methods

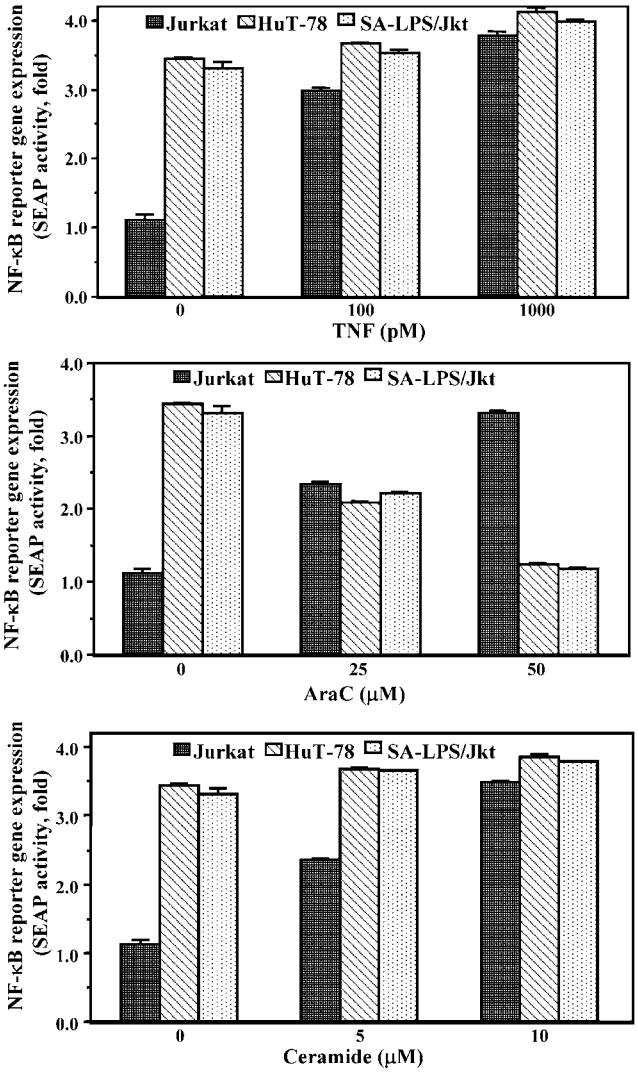

Figure 3 Effect of TNF, araC, or ceramide on NF-κB reporter gene expression in Jurkat, SA-LPS/Jkt, and HuT-78 cells. Jurkat, HuT-78, and SA-LPS/Jkt cells (1×10^6/ml) were transiently transfected with pcDNA vector (2.5 μg) and NF-κB-SEAP (0.5 μg) for 12 h, cultured for 24 h and then treated with different concentrations of TNF, araC, or ceramide indicated in the figure for 6 h. Cells culture supernatant was assayed for secreted alkaline phosphatase (SEAP) activity as described in Materials and methods and mean SEAP activity was indicated as fold of activation above unstimulated cells

concentrations of TNF, araC, or ceramide for 6 h. The culture supernatants were then assayed for secretory alkaline phosphatase (SEAP) activity. Dose-dependent induction of SEAP activity by TNF or ceramide was observed in Jurkat cells. HuT-78 and SA-LPS/Jkt cells showed a high basal activity of SEAP that was not further induced with TNF (Figure 3, upper panel) or ceramide (Figure 3, lower panel). With araC, SEAP activity was induced in a dose-dependent manner in Jurkat cells, but the high basal activity of SEAP in HuT-78 and SA-LPS/Jkt cells was progressively reduced with increasing concentrations of araC (Figure 3, middle panel). Jurkat cells transfected with dominant-negative IκBα showed no induction of levels of SEAP activity upon treatments of TNF, araC, or ceramide. The high basal levels of SEAP activity were downregulated in HuT-78 and SA-LPS/Jkt cells, when transfected with dominant-negative IκBα (data not shown). These results

correlate with the upregulation of NF-κB as detected by EMSA in the previous experiment. Although araC downregulates NF-κB activity in Jurkat cells, it shows upregulation of SEAP activity. As araC induces Jurkat cells initially, it induces NF-κB-dependent SEAP which is secreted into the medium.

AraC induces apoptosis in HuT-78 and SA-LPS/Jkt cells

To investigate the effects of TNF, araC, or ceramide on induction of apoptosis in Jurkat and NF-κB expressed cells (HuT-78 and SA-LPS/Jkt cells) different markers of apoptosis (lipid peroxidation, reactive oxygen intermediates (ROIs) generation, PARP and Bcl-2 cleavage, DNA fragmentation, and nuclear fragmentation) were assayed.

AraC induces lipid peroxidation and ROI generation in HuT-78 and SA-LPS/Jkt cells

As lipid peroxidation is a marker of apoptosis, we examined the effect of TNF, araC, or ceramide on lipid peroxidation in Jurkat, SA-LPS/Jkt, and HuT-78 cells through the detection of levels of malondialdehyde (MDA) production. In Jurkat, but not in HuT-78 or SA-LPS/Jkt cells, TNF or ceramide induced lipid peroxidation in a dose-dependent manner (Figure 4a, left and right panels). AraC induced lipid peroxidation

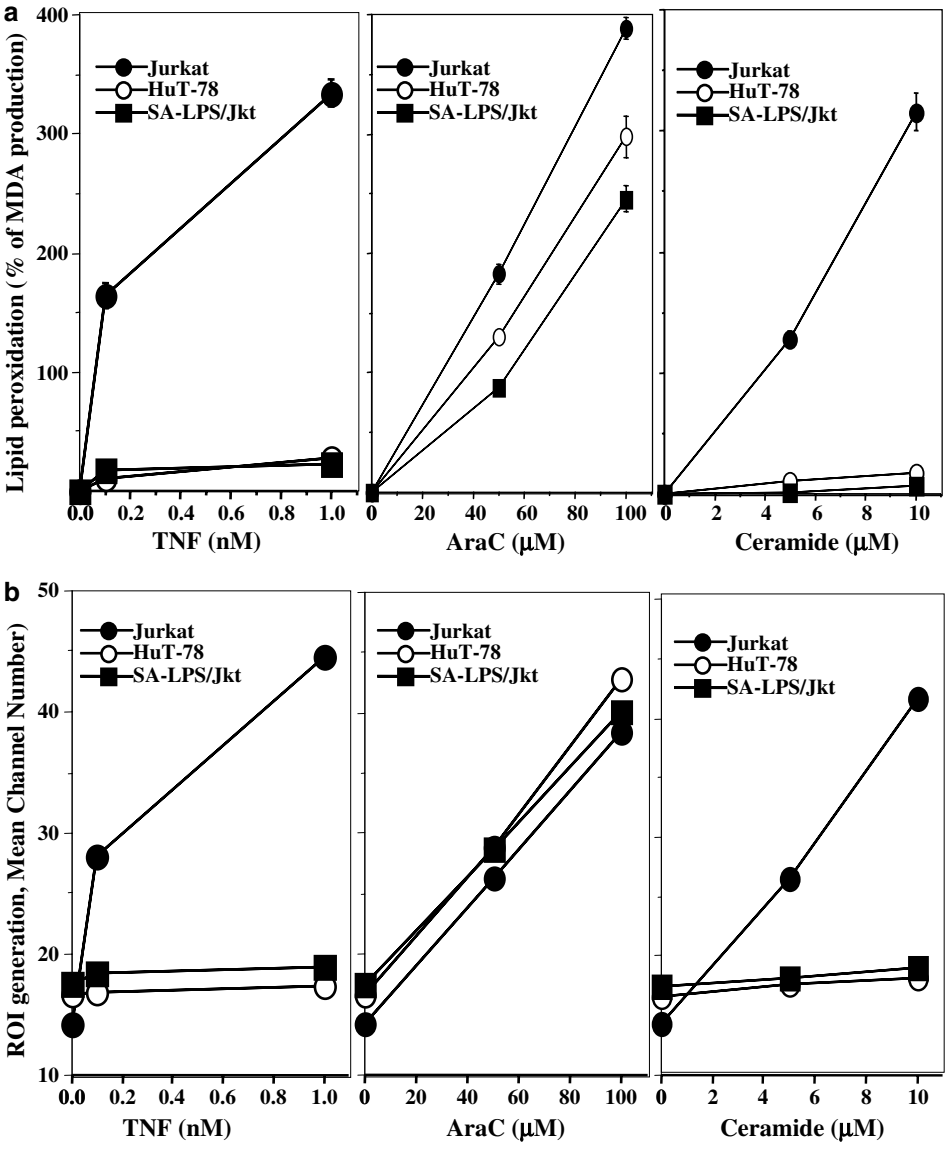

Figure 4 (**a**) Effect of TNF, araC, or ceramide on lipid peroxidation in Jurkat, HuT-78, and SA-LPS/Jkt cells. Jurkat, HuT-78, and SA-LPS/Jkt cells (5×10^6) were incubated with different concentrations of TNF, araC, or ceramide for 6 h. Cell extracts were prepared by the freeze–thaw method and 500 μg proteins were used for TBA-SDS reactionable MDA assay as described in Materials and methods. The results are indicated as MDA production in percentage above control (0.562 ± 0.122 nmol MDA/mg protein). (**b**) Effect of TNF, araC, or ceramide on ROI generation in Jurkat, HuT-78 and SA-LPS/Jkt cells. Jurkat, HuT-78, and SA-LPS/Jkt cells (1×10^6) were incubated with dihydrorhodamine for 2 h and then treated with different concentrations of TNF, araC, or ceramide for 6 h. The results shown are representative of two independent experiments

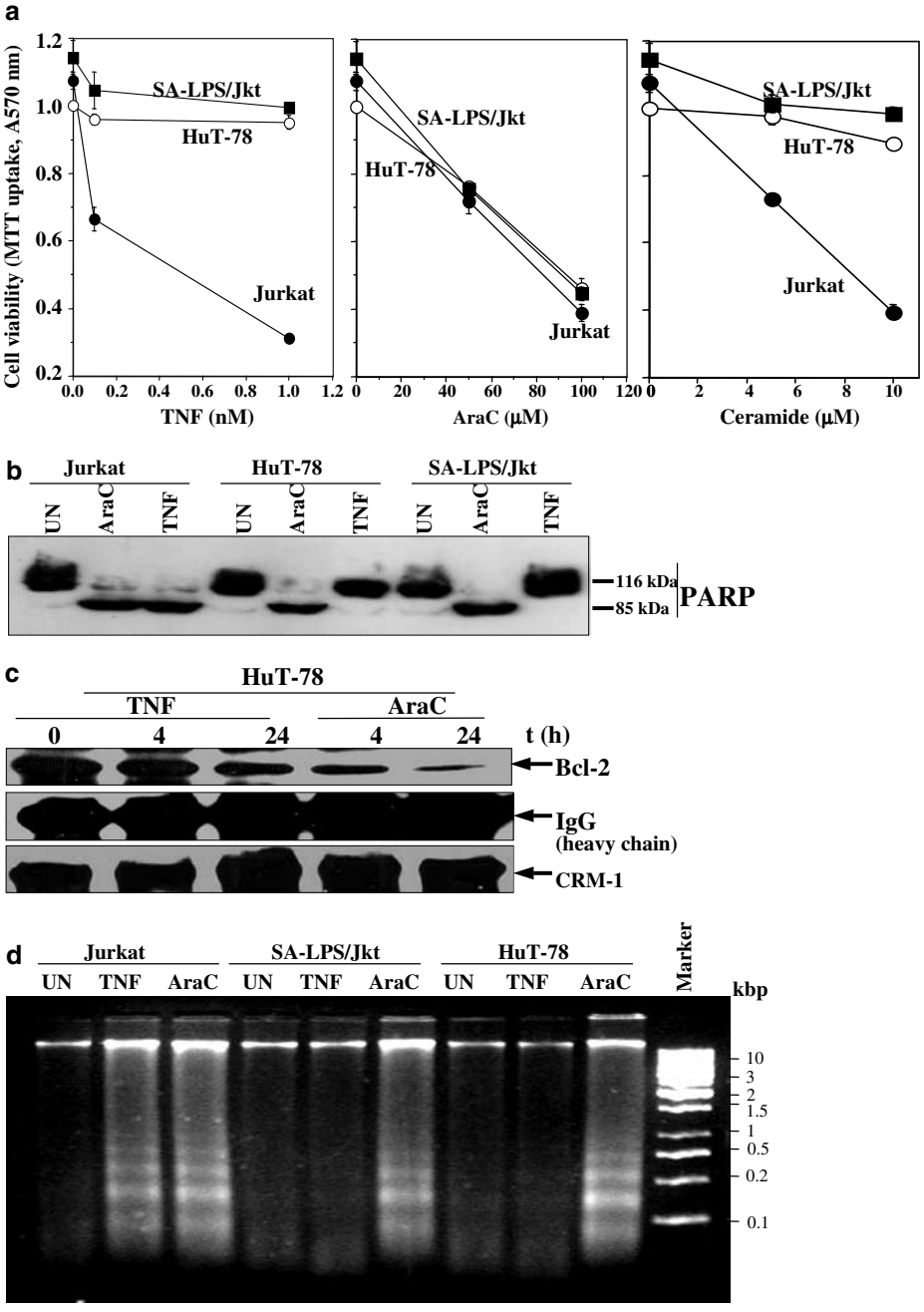

Figure 5 Effect of TNF, araC, or ceramide on cytotoxicity, PARP and Bcl-2 cleavage, and DNA fragmentation in Jurkat, HuT-78 and SA-LPS/Jkt cells. (**a**) Jurkat, HuT-78, and SA-LPS/Jkt cells (10^4/0.1 ml) were incubated with different concentrations of TNF, araC, or ceramide for 72 h. Cell viability was assayed by MTT dye uptake. The results are indicated as mean OD of triplicate assays. (**b**) Jurkat, HuT-78, and SA-LPS/Jkt cells (2×10^6) cells were treated with araC (50 μM) or TNF (1 nM) for 24 h at 37°C in a CO_2 incubator. Then cell extracts were prepared and 50 μg protein was analysed by 7.5% SDS–PAGE to detect PARP by Western blot using anti-PARP mAb. (**c**) HuT-78 (2×10^6) cells were treated with araC (50 μM) and TNF (1 nM) for different times at 37°C in a CO_2 incubator. Then cell extracts were prepared and 400 μg protein was immunoprecipitated against anti-Bcl-2 and –CRM1 Ab and analysed by 10% SDS–PAGE to detect Bcl2 by Western blot using anti-Bcl2 Ab. The level of IgG heavy chain was visualized from the same X-ray film. The same blot was reprobed with anti-CRM1 antibody and the level of CRM1 was detected by Western blot analysis. (**d**) Jurkat, HuT-78, and SA-LPS/Jkt cells (5×10^6) were treated with araC (50 μM) or TNF (1 nM) for 36 h at 37°C in a CO_2 incubator. Then cells were washed, DNA was prepared, and 2.0 μg DNA was analysed by 2% agarose gel. (**e**) Jurkat, HuT-78, and SA-LPS/Jkt cells (2×10^6) were treated with araC (50 μM) or TNF (1 nM) for 36 h at 37°C in a CO_2 incubator. Then cells were scraped, washed, fixed with methanol and stained with PI. Cells were then taken in a slide and visualized through a fluorescence microscope. The picture showed the Jurkat, HuT-78, and SA-LPS/Jkt cells of untreated, TNF-treated, or araC-treated in phase contrast as well as in fluorescent microscope view

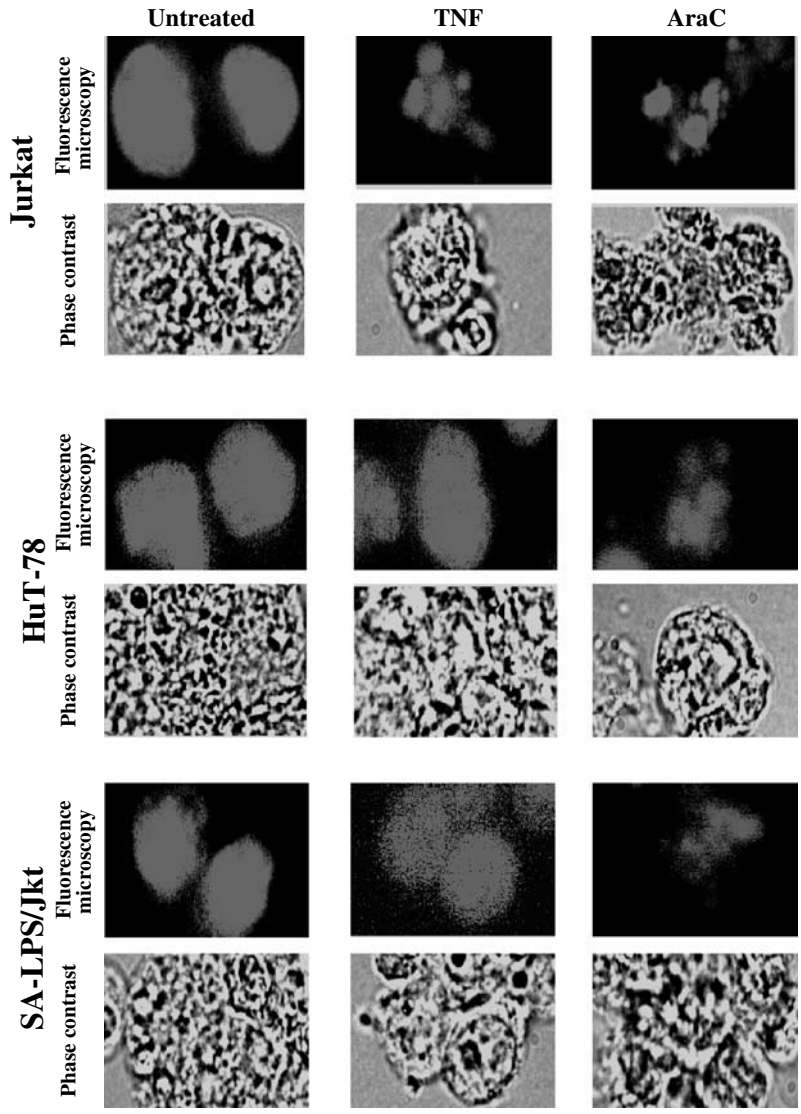

Figure 5 *Continued*

in Jurkat, HuT-78, or SA-LPS/Jkt cells in a dose-dependent manner (Figure 4a, middle panel), indicating that araC induces apoptosis in NF-κB expressed cells.

ROI generation is an intermediate step in apoptosis induced by different agents. TNF-, araC-, or ceramide-induced ROI generation in Jurkat, SA-LPS/Jkt, and HuT-78 cells was examined with dihydrorhodamine dye conversion to rhodamine as described in Materials and methods. As shown in Figure 4b left and right panels, TNF or ceramide induced ROI generation in a dose-dependent manner in Jurkat cells, but not in HuT-78 or SA-LPS stimulated Jurkat cells. AraC induced ROI generation in Jurkat, HuT-78, or SA-LPS stimulated Jurkat cells in a dose-dependent manner (Figure 4b, middle panel). These data indicate that araC induces ROI generation in NF-κB expressed cells.

AraC induces cytotoxicity, PARP and Bcl-2 cleavage, and DNA fragmentation in HuT-78 and SA-LPS/Jkt cells

To detect TNF-, araC-, or ceramide-mediated apoptosis, Jurkat, SA-LPS/Jkt, and HuT-78 cells (10^4 cells/0.1 ml) were incubated with different concentrations of TNF, araC, or ceramide for 72 h and then cytotoxicity was assayed by MTT assay. As shown in Figure 5a left and right panels, TNF or ceramide induced cytotoxicity in a dose-dependent manner in Jurkat cells but not in HuT-78 or SA-LPS/Jkt cells. AraC induced cytotoxicity in Jurkat, HuT-78, or SA-LPS/Jkt cells in a dose-dependent manner (Figure 5a, middle panel).

Cell viability is reflected in caspase activation, which cleaves a lot of proteins including PARP, Bcl-2, etc., induces DNA fragmentation and nuclear fragmentation. TNF or ceramide induced PARP cleavage in Jurkat, but not in HuT-78 or SA-LPS/Jkt cells. AraC induced

PARP cleavage in Jurkat, HuT-78, or SA-LPS/Jkt cells (Figure 5b). To detect the level of Bcl-2, HuT-78 cells (2 x10^6) were treated with araC (50 μM) or TNF (1 nM) for different times at 37°C, extracted proteins were immunoprecipitated with anti-Bcl-2 and -CRM1 Ab and then analysed for Bcl-2 by Western blot. The levels of Bcl-2 almost did not alter in TNF-treated cells but were downregulated in araC-treated cells (Figure 5c) indicating araC-mediated apoptosis in NF-κB expressed cells. In the same blot, the levels of IgG heavy chain were equal in all these lanes, indicating equal addition of the antibody. The same blot was probed with anti-CRM1 Ab and the levels of CRM1 were equal in each lane indicating loading control. TNF or ceramide enhanced DNA fragmentation in Jurkat, but not in HuT-78 or SA-LPS/Jkt cells, whereas araC induced DNA fragmentation in Jurkat, HuT-78, or SA-LPS/Jkt cells as shown by DNA laddering (Figure 5d). Propidium iodide (PI)-stained cells also showed nuclear fragmentation (Figure 5e).

AraC dephosphorylates p65 in SA-LPS/Jkt and HuT-78 cells

As phosphorylation of the p65 subunit of the NF-κB is required for NF-κB-mediated gene transcription and araC downregulates NF-κB in HuT-78 or SA-LPS/Jkt cells, we tested the role of araC in p65 phosphorylation. Jurkat, HuT-78, or SA-LPS/Jkt cells (5×10^6) were incubated with [^{32}P]orthophosphate and treated with TNF, araC, or ceramide for 4 h. Extracts were prepared, immunoprecipitated with anti-p65 antibody and analysed for p65 as described in Materials and methods. In Jurkat cells all these agents induced p65 phosphorylation. The high levels of p65 phosphorylation were shown in HuT-78 or SA-LPS/Jkt cells that were not further induced by TNF or ceramide, whereas araC treatment led to the loss of the p65-phosphorylated band in these cells (Figure 6a, upper panel). As control for loading artefacts, aliquots of 50 μg proteins from the same extracts were analysed by 9% SDS–PAGE, and bands were shown by chemiluminescence to have equivalent levels of p65 (Figure 6a, lower panel). The results indicate that araC suppresses the phospho-p65 band of HuT-78 or SA-LPS/Jkt cells.

Calyculin A protects araC-induced downregulation of p65 phosphorylation, NF-κB activation, and cytotoxicity

To understand the mechanism of downregulation of phosphorylated p65, we used calyculin A, a specific serine/threonine phosphatase inhibitor (Suganuma *et al*., 1990), and $ZnCl_2$, a protein-tyrosine phosphatase inhibitor (Tonks *et al*., 1991), to investigate the mechanism of araC-induced signaling (cytotoxicity, p65 phosphorylation, and NF-κB activation) in NF-κB expressed cells. HuT-78 or SA-LPS/Jkt cells were pretreated with calyculin A or $ZnCl_2$ and then treated with different concentrations of araC for 72 h, following which cytotoxicity was assayed by MTT. The results indicate that calyculin A, but not $ZnCl_2$ protects HuT-78 or SA-LPS/Jkt cells from araC-induced cell killing (Figure 6b).

HuT-78 or SA-LPS/Jkt cells were incubated with [^{32}P]orthophosphate and calyculin A (10 nM) for 4 h and then stimulated with araC (50 μM) for 4 h. The cell extracts were then immunoprecipitated with anti-p65 antibody and analysed for the labeled p65 band. The results shown in Figure 6c indicate that araC mediates the de-phosphorylation of p65 in HuT-78 and SA-LPS/Jkt cells which in turn is blocked by calyculin A. When calyculin-A-pretreated HuT-78 or SA-LPS/Jkt cells were exposed to araC for 4 h and the nuclear extracts were then assayed for NF-κB, we observed complete protection conferred by calyculin A on araC-mediated downregulation of NF-κB activation in these cells (Figure 6D).

AraC induces protein phosphatases 2A and 2B-A, which dephosphorylate p65 in vitro

To detect the phosphatase(s) involved in araC-mediated dephosphorylation of p65, Jurkat cells were stimulated with araC (50 μM) for 2 and 6 h. The cell extracts were immunoprecipitated with antibodies against PP1, PP2A, and PP2B-A and the complexes were incubated with ^{32}P-p65 for 2 h at 37°C as described in Materials and methods. The intensity of the labeled p65 band was decreased in PP2A and PP2B-A with time of incubation but not in the case of PP1 indicating the activation of PP2A and PP2B-A by araC (Figure 6e). The 80 μg of the extracted protein from the same extracts was analysed in 9% SDS–PAGE and Western blot performed against anti-PP1, -PP2A, and -PP2B-A. The bands showed equal intensity in each case indicating that the level of expression of those phosphatases is not changed with the increased time of araC treatment (Figure 6f).

To detect the role of araC in p65 (RelA) and PP2A interaction *in vitro*, equal amounts of ^{32}P-p65 proteins were incubated with araC and PP2A (immunoprecipitated from 500 μg unstimulated Jurkat cells extract) alone or in combination for 2 h at 37°C in microfuge tubes. Then the levels of labeled p65 proteins were analysed. The levels of the labeled p65 band showed equal intensities (Figure 6g), indicating that araC has no role in the interaction of p65 and PP2A *in vitro*.

AraC potentiates cis-platin-, etoposide-, or taxol-mediated cytotoxicity in SA-LPS/Jkt and HuT-78 cells

NF-κB is known as an antiapoptotic factor (Kochetkova *et al*., 1997; Nakshatri *et al*., 1997; Raziuddin *et al*., 1997; Manna and Aggarwal, 1999; Izban *et al*., 2000). In order to detect the role of NF-κB on cell viability, HuT-78 or SA-LPS/Jkt cells were incubated with 10 μM each of cis-platin, vincristine, doxorubicin, etoposide, adriamycin, or taxol and 50 μM of araC for 72 h and MTT-assayed. As shown in Figure 7a, all those agents decreased cell viability in Jurkat cells. Cis-platin, etoposide, taxol, or araC partially blocked cell viability in HuT-78 or SA-LPS/Jkt cells. We observed that araC downregulated NF-κB in NF-κB expressed cells.

To understand the effect of other inducers of apoptosis in NF-κB downmodulated cells, araC-pretreated HuT-78 and SA-LPS/Jkt cells were incubated with different inducers of apoptosis. Then cell viability was assayed by MTT dye uptake. AraC-pretreated cells showed potentiation of cytotoxicity induced by cisplatin, etoposide, or taxol in HuT-78 (Figure 7b1) and SA-LPS/Jkt (Figure 7b2) cells.

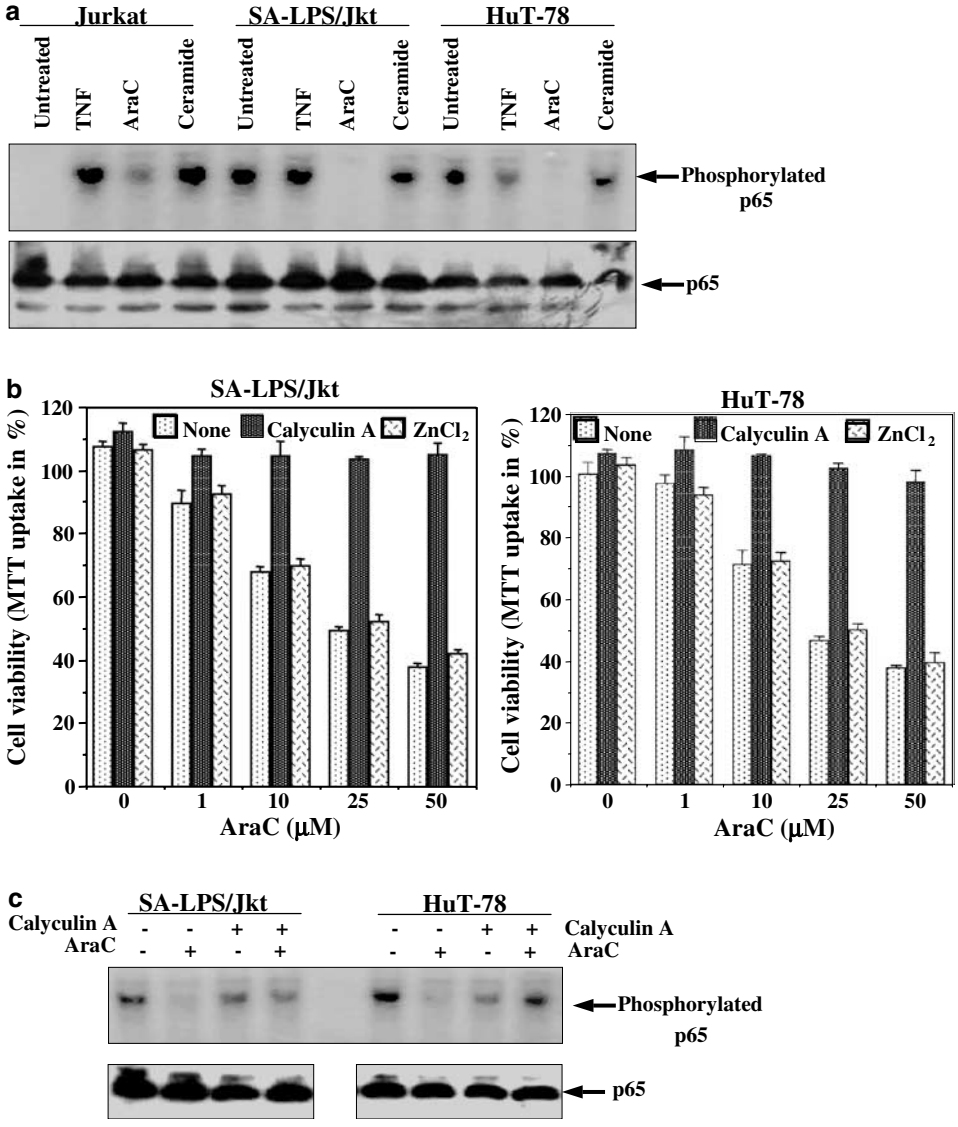

Figure 6 (**a**) Effect of TNF, araC, or ceramide on p65 phosphorylation in SA-LPS/Jkt and HuT-78 cells. Jurkat, HuT-78, and SA-LPS/Jkt cells (5×10^6) were incubated with 2 mCi [^{32}P]orthophosphate for 6 h in phosphate-free medium. Cells were treated with TNF (1 nM), araC (50 μM), or ceramide (10 μM) for 4 h and extract was prepared, 500 μg of the extract protein was immunoprecipitated using anti-p65 antibody and analysed in 9% SDS–PAGE for p65 radioactive band. Aliquots of 50 μg proteins from the same extracts were analysed for p65 in 9% SDS–PAGE and bands were detected by Western blot. (**b**) Effect of calyculin A and $ZnCl_2$ on araC-induced cytotoxicity. SA-LPS/Jkt and HuT-78 (10^4cells/0.1 ml) cells were pretreated with calyculin A (10 nM) and $ZnCl_2$ (1 mM) for 4 h and then cells were washed and incubated with different concentrations of araC for 72 h. Cell viability was assayed by MTT assay. (**c**) Effect of calyculin A on araC-induced downregulation of p65 phosphorylation. HuT-78 and SA-LPS/Jkt (2×10^6) cells were pretreated with calyculin A (10 nM) for 4 h and then stimulated with araC (50 μM) for 4 h. Cell extracts were prepared, immunoprecipitated (500 μg protein) and analysed in 9% SDS–PAGE for p65 radioactive band. Aliquots of 50 μg proteins from the same extracts were analysed for p65 in 9% SDS–PAGE and bands were detected by Western blot. (**d**) Effect of calyculin A on araC-induced downregulation of NF-κB activation. HuT-78 and SA-LPS/Jkt (2×10^6) cells were pretreated with calyculin A (10 nM) for 4 h and then stimulated with araC (50 μM) for 4 h. Nuclear extracts were prepared and analysed for NF-κB. (**e**) Effect of araC on activation of PP1, PP2A, and PP2B-A. Jurkat cells were treated with araC (50 μM) for different times and cell extracts were prepared. Cell extract proteins were immunoprecipitated with antibodies against PP1, PP2A, and PP2B-A and then complexes were incubated with ^{32}P-p65 for 2 h at 37°C. Then the samples were analysed in 9% SDS–PAGE and gel was exposed and scanned in a PhosphorImager. (**f**) Effect of araC on expression of PP1, PP2A, and PP2B-A. Jurkat cells extract proteins (80 μg) obtained from previous experiments were analysed in 9% SDS–PAGE and Western blot was performed against anti-PP1, -PP2A, and -PP2B-A. (**g**) Effect of araC on p65 and PP2A interaction *in vitro*. Unstimulated Jurkat cell extract immunoprecipitated with anti-PP2A and incubated ^{32}P-p65 proteins in the presence or absence of araC for 2 h at 37°C in microfuge tubes. Then labeled p65 proteins were detected by 9% SDS–PAGE, gel was exposed and scanned in a PhosphorImager

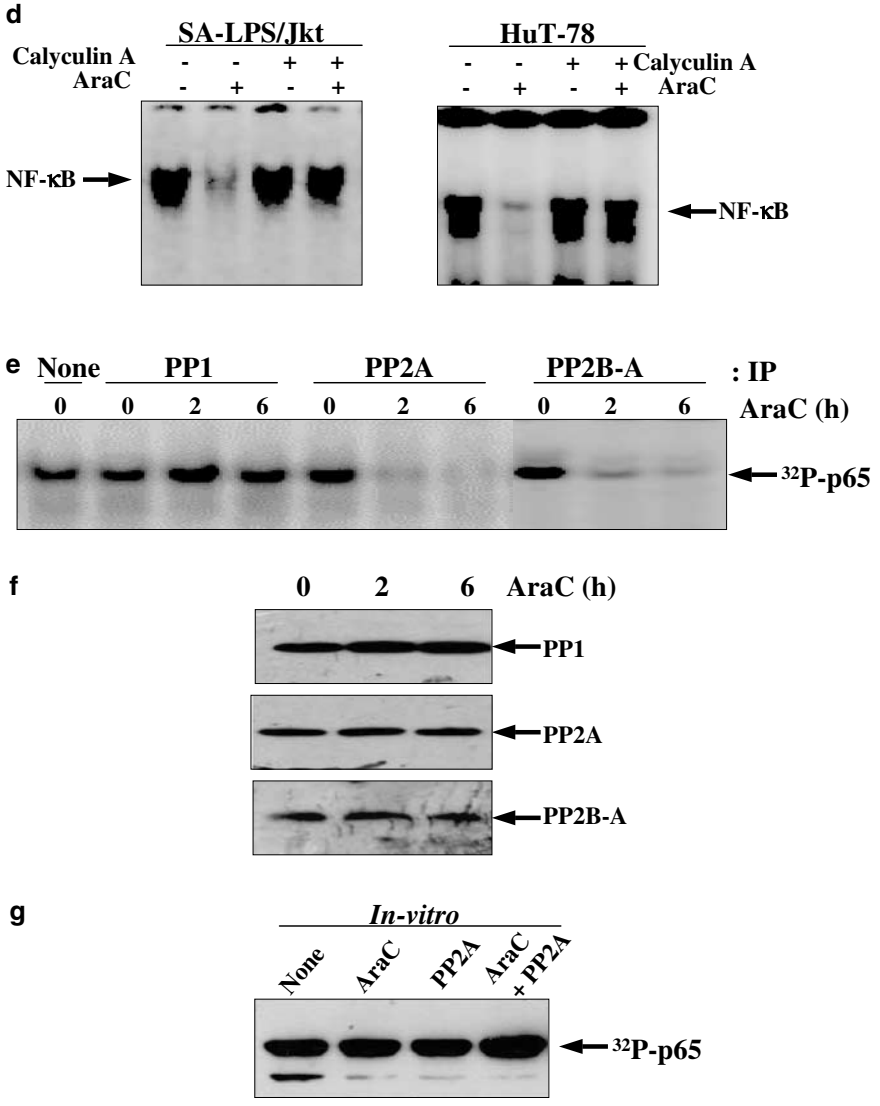

Figure 6 *Continued*

Discussion

The mechanism coupling araC-induced DNA damage to the initiation of cell death have not yet been fully established. Myeloid leukemia cells have been shown to be treated with araC. The mechanism of araC-mediated cell death has been shown by inhibition of mitogen-activated protein kinase (MAPK) and interruption of protein kinase C (Jarvis *et al*., 1998). TNF and ceramide have been shown to induce apoptosis in different pathways (Manna and Aggarwal, 2000; Manna *et al*., 2000a). To dissect the signaling mechanism induced by different apoptotic inducers, we used TNF, araC, and ceramide as these inducers act through different mechanisms and to understand the role of NF-κB on the mechanism of drug resistance. TNF is a well-known inducer of apoptosis through death domain activation and caspase recruitment (Baker and Reddy, 1998). Ceramide, an intermediate messenger molecule induced by a variety of apoptotic inducers including TNF though the mechanism of cell signaling mediated by ceramide differs from TNF (Manna and Aggarwal, 2000; Manna *et al*., 2000b). All these inducers follow NF-κB activation as a downstream signal. However surprisingly, when we incubated Jurkat cells with TNF, araC, or ceramide for different time periods it has been shown that NF-κB activation was maintained till 24 h of incubation by TNF and ceramide but not by araC (Figure 1). To understand the mechanism of araC-mediated downregulation of NF-κB, we used HuT-78, constitutively expressed NF-κB cells and endotoxin-stimulated Jurkat cells. Serum-activated LPS stimulates Jurkat T cells for induction of NF-κB potently and maintains the level of expression for a long time (Manna and Aggarwal, 1999). LPS interacts with lipopolysaccharide binding protein (LBP) of serum and forms

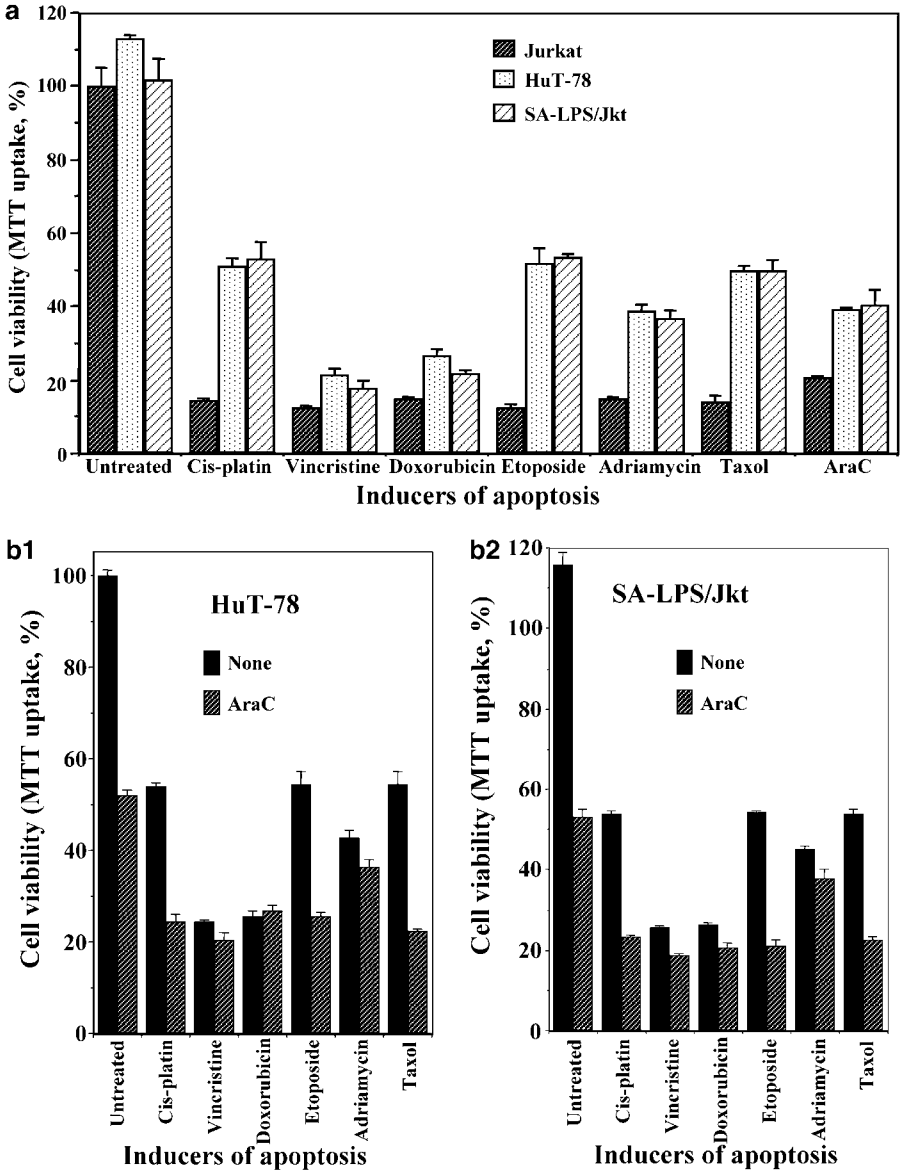

Figure 7 (**a**) Effect of cis-platin, vincristine, doxorubicin, etoposide, adriamycin, taxol, or araC on cytotoxicity in Jurkat, SA-LPS/Jkt, and HuT-78 cells. Cells were treated with 10 μM of cis-platin, vincristine, doxorubicin, etoposide, adriamycin, or taxol and 50 μM of araC for 72 h. Then cytotoxicity was assayed by MTT assay. (**b**) Effect of cis-platin, vincristine, doxorubicin, etoposide, adriamycin, and taxol on araC-induced HuT-78 and SA-LPS/Jkt cells. Cells, pre-treated with 50 μM of araC for 4 h, were treated with 10 μM of cis-platin, vincristine, doxorubicin, etoposide, adriamycin, or taxol for 72 h. Then cytotoxicity was assayed by MTT assay. The results are presented as mean percentage of viable cells $\pm$ s.d. of triplicate samples

LPS-LBP complex (also known as endotoxin) and it acts through CD14 cell surface receptors for cell stimulation. The LPS used for activation in the form of SA-LPS was 100 ng/ml, which is not toxic to cells for a long time as detected by cell viability.

Our results indicate that TNF, araC, or ceramide induced NF-κB in Jurkat cells, but araC, downregulates NF-κB activation after 4 h (Figure 1a). Ceramide- and TNF-induced NF-κB activation is correlated with IκBα degradation (Figure 1d). IκBα is an NF-κB-dependent gene product. As TNF or ceramide induces NF-κB, IκBα degradation was observed initially but later it is resynthesized. The TNF-mediated IκBα resynthesis occurred at 2 h but again it degraded at 12 and 24 h time points. Possibly the effect of TNF is long-lasting and presence in the medium exerts its effect further. AraC has been shown to induce IκBα degradation until 24 h. As araC downregulates NF-κB, IκBα, an NF-κB-dependent gene, is not resynthesized further. TNF-mediated apoptosis was not observed in HuT-78 and serum-activated Jurkat cells, which is consistent with our earlier report (Manna and Aggarwal, 1999). Ceramide-induced apoptosis was also not observed in those NF-κB expressed cells indicating the common

pathway followed by TNF and ceramide for apoptosis. AraC has been shown to downregulate NF-κB, as indicated by gel retardation assay (Figure 2) and NF-κB-dependent reporter gene expression in NF-κB expressed cells (Figure 3) indicating a novel mechanism followed by araC.

TNF- and ceramide-mediated cell apoptosis was not observed in NF-κB expressed cells, either HuT-78 (constitutively) or SA-LPS/Jkt (induced) cells, as detected by MTT assay (Figure 5a), PARP cleavage (Figure 5b), Bcl2 degradation (Figure 5c), DNA fragmentation (Figure 5d), and visualized by nuclear fragmentation (Figure 5e). NF-κB expressed cells showed antiapoptotic activity by inhibiting apoptosis induced by TNF or ceramide. AraC-induced apoptosis was shown in NF-κB expressed cells, indicating a different mechanism of action from that of TNF or ceramide. In NF-κB expressed cells, TNF or ceramide was unable to induce lipid peroxidation as detected by MDA production and ROIs generation as detected by the conversion of dihydrorhodamine to rhodamine and analysed in FACScan, further indicating that the cells are not following the apoptosis pathway. On the other hand, araC-induced lipid peroxidation and ROI generation were observed in NF-κB expressed cells (Figure 4). Bcl-2 has been shown to exert an anti-apoptotic activity in cells and it is an NF-κB-dependable gene product (Heckman *et al*., 2002). Overexpressed Bcl-2 again downregulates NF-κB and dependent gene products (Badrichani *et al*., 1999). In this report we provide the evidence that araC downregulates Bcl-2 level in NF-κB expressed cells. As araC downregulates NF-κB, it may correlate with the downregulation of NF-κB-mediated synthesis of Bcl-2 (Heckman *et al*., 2002) or araC-mediated apoptotic pathways that may degrade Bcl-2.

As reported previously, araC hypophosphorylated retinoblastoma protein, phosphorylated by cyclin-dependent kinases and stimulates cell cycle in HL-60 cells (Dou *et al*., 1995) and later it was proved that araC induced tyrosine phosphatase, which dephosphorylates retinoblastoma protein causing apoptosis (Dou and Lui, 1995; Wang *et al*., 2001). As NF-κB is an ideal target for anticancer drug development, it is important to detect its role on araC-mediated cell signaling. An active NF-κB dependent gene expression requires the p65 subunit of NF-κB to be phosphorylated (Egan *et al*., 1999; Manna *et al*., 2000a). We observed that TNF, araC, or ceramide induced p65 phosphorylation (Figure 6a), which was correlated with NF-κB activation and dependent gene expression. Surprisingly, in NF-κB expressed cells, TNF or ceramide did not interfere with the high level of p65 phosphorylation but araC downregulated the phospho-p65 protein level in both NF-κB expressed cells (induced and constitutive) (Figure 6c). Zinc is believed to be an inhibitor of protein-tyrosine phosphatase inhibitor. AraC-mediated inhibition of NF-κB or apoptosis was not modulated by $ZnCl_2$ whereas addition of calyculin A completely protected araC-induced inhibition of cytotoxicity (Figure 6b) and downregulation of NF-κB (Figure 6d). AraC activates phospho-serine/phospho-threonine phosphatase, which dephosphorylates the p65 subunit of NF-κB and makes cells more apoptotic. AraC activates PP2A and PP2B-A but not PP1. Activated PP2A and PP2B-A dephosphorylate basal phospho-p65 in NF-κB expressed cells (Figure 6e) without changing the level of those phosphatases expression (Figure 6f). AraC does not interfere with the interaction between p65 and PP2A *in vitro* (Figure 6g). So, araC has a potential role to make cells sensitive to cell death which attain resistance to apoptosis because of NF-κB. This is the first report that the p65 subunit of NF-κB undergoes dephosphorylation by phosphatases activity induced by araC and cells become sensitive to apoptosis.

Pretreatment of araC and then stimulation of different anticancer agents also shows the potentiation of apoptosis (Figure 7). Combinatorial therapy is an important aspect in regulating cancer progression and metastasis. As chemotherapeutic agent-induced resistance is the main problem for cancer, the understanding of the basic mechanism will be helpful in addressing this problem. NF-κB expressed cells showed 20–40% cell survival in cis-platin-, etoposide-, adriamycin-, taxol-, or araC-mediated cytotoxicity (Figure 7a). These agents showed potentiation of cell killing in combination with AraC in NF-κB expressed cells (Figure 7b).

Overall, our results suggest that dephosphorylation of a key protein, p65, by protein phosphatases may be crucial for the initiation of apoptosis in NF-κB expressed cells, and further support the concept of protein phosphatase serving as a potential target for anticancer therapy. Our studies suggest that the dephosphorylation of the p65 subunit of NF-κB might be an important strategy for treating drug-resistant cancers.

Materials and methods

Materials

AraC, C2 ceramide (*N*-acetyl-D-sphingosine), MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)], glycine, calyculin A, $ZnCl_2$, doxorubicin, vincristine, taxol, cis-platin, etoposide, adriamycin, and LPS (*Escherichia coli*, 055:B5) were obtained from Sigma (St Louis, MO, USA). Penicillin, streptomycin, RPMI-1640 medium, and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY, USA). TNF was obtained from PeproTech Inc., NJ, USA. Dihydrorhodamine was purchased from Molecular Probe, The Netherlands. Oct1 double-stranded oligonucleotide and antibodies (Abs) against PP1, PP2A, PP2B-A, IκBα, p50, p65, cyclin D1, c-Rel, CRM1, and Bcl2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Poly ADP-ribose polymerase (PARP) antibody (Ab) was purchased from PharMingen (San Diego, CA, USA).

Cell lines

Jurkat and HuT-78 (T cells) cells, obtained from American Type Culture Collection (Manassas, VA, USA), were cultured in RPMI-1640 medium supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) and were mycoplasma free, as tested by Gen-probe mycoplasma rapid detection kit (Fisher Scientific, Pittsburgh, PA, USA) as

described (Giri and Aggarwal, 1998; Manna and Aggarwal, 1999).

HuT-78 cells constitutively express the nuclear transcription factor NF-κB. In Jurkat cells, NF-κB expression was induced with serum-activated lipopolysaccharide (100 ng LPS was incubated with 20 μl of rabbit serum for 1 h at 37°C to make SA-LPS and this was used for treatment) for 24 h at 37°C to upregulate NF-κB. The concentration used for LPS activation did not show any cytotoxicity, as detected by MTT assay, up to 72 h (cell viability was 98.02 ±3.24, 96.34±3.36, 94.78±3.24, 91.42±4.48, and 92.24±5.52 at 0, 24, 36, 48, and 72 h of incubation, respectively).

NF-κB activation assay

NF-κB activation was assayed following the method described (Manna *et al*., 1998). Nuclear extract (8 μg protein) was incubated with 4 ng of ^{32}P end-labeled 45-mer double-stranded NF-κB oligonucleotide from the HIV-LTR, 5′-TTG TTA CAA **GGG ACT TT**C CGC TG**G GGA CTT TC**C AGG GAG GCG TGG-3′ (bold indicates NF-κB binding site) and assayed for NF-κB using 6.6% native PAGE by EMSA. Similarly, 8 μg nuclear extract protein was incubated with ^{32}P-labeled double-stranded Oct1 oligonucleotide and assayed in 6% native PAGE by gel retardation assay.

Cytotoxicity assay

The cytotoxicity was measured by MTT assay (Manna *et al*., 1998). Briefly, cells (1×10^4 cells/well of 96-well plate) were treated with inducers for 72 h and thereafter, 25 μl of MTT solution (5 mg/ml in phosphate buffer saline (PBS)) was added to each well. After a 2-h incubation at 37°C, 0.1 ml of the extraction buffer (20% SDS, 50% dimethylformamide) was added. After an overnight incubation at 37°C, the absorbance was read at 570 nm.

Western blot of PARP and Bcl-2

Apoptosis was examined by the proteolytic cleavage of PARP (Manna *et al*., 1998). Briefly, cells were treated with different inducers and whole-cell extracts were prepared. Cell extract protein (50 μg) was resolved on 7.5% SDS–PAGE, electrotransferred onto a nitrocellulose membrane, blotted with mouse anti-PARP Ab, and then detected by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Apoptosis was represented by the cleavage of 116-kDa PARP into an 85-kDa product (Manna *et al*., 1998; Manna and Aggarwal, 1999). TNF- and araC-treated HuT-78 cell extracts were immunoprecipitated with anti-Bcl-2 and -CRM1 (nuclear export receptor protein) Ab. Then the blot was used to detect Bcl-2 and CRM1 levels by Western blot analysis.

NF-κB-dependent reporter gene transcription

The effect of different inducers of apoptosis on NF-κB-dependent reporter gene transcription was measured as previously described (Darnay *et al*., 1999). Briefly, cells were transiently transfected by the calcium phosphate method with 1 ml medium containing 0.5 μg NF-κB promoter DNA linked to the heat-stable SEAP gene. After 24 h of culture, cells were stimulated with TNF, araC, and ceramide for 6 h and then the culture-conditioned medium was harvested, and 25 μl was analysed for alkaline phosphatase activity using 4-methylumbelliferyl phosphate as substrate essentially as per the CLONTECH protocol (Palo Alto, CA, USA). This reporter system was specific, because ceramide-induced NF-κB SEAP activity was inhibited by overexpression of IκBα mutants lacking either Ser^{32} or Ser^{36}.

Determination of lipid peroxidation and measurement of ROIs

Lipid peroxidation was determined by detection of thiobarbituric acid-reactive MDA, an end product of the peroxidation of polyunsaturated fatty acids and related esters as described (Bowie *et al*., 1997; Manna *et al*., 2000a). The production of ROIs was determined by flow cytometry as described (Manna *et al*., 1999). Treated or untreated cells were exposed to dihydrorhodamine 123 and then washed with PBS. Rhodamine 123 fluorescence intensity resulting from dihydrorhodamine 123 oxidation was measured by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) with excitation at 488 nm and was detected between 515 and 550 nm. Data analysis was performed using LYSYSII software (Becton Dickinson).

Determination of DNA and nuclear fragmentation

DNA was extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v/v) and precipitated by 100% ethanol at −20°C overnight (Ito *et al*., 1999) and ran on 1% agarose gel. DNA fragments were visualized as ladder with ethidium bromide under UV light.

After treatment, cells were fixed in 80% methanol overnight at 4°C and pellets were suspended in 100 μl of PI mixture for 30 min. Cells were then viewed under a fluorescence microscope to determine nuclear fragmentation (Nutku *et al*., 2001).

Immunoprecipitation of p65 from orthophosphate-labeled cells

To determine the phosphorylation of the p65 subunit of NF-κB (Manna *et al*., 2000a), Jurkat, HuT-78, and SA-LPS stimulated Jurkat cells (5×10^6) were labeled with [^{32}P]orthophosphate (Amersham) in phosphate-free medium for 4 h at 37°C, and then the cells were treated with TNF (1 nM), araC (50 μM), and ceramide (10 μM) for 4 h at 37°C. Then the cell-extracted proteins were immunoprecipitated with anti-p65 polyclonal Ab (Santa Cruz Biotechnology) followed by protein A/G sepharose beads. Washed beads were then boiled with SDS sample buffer for 5 min, and subjected to SDS–PAGE (9%). The gel was dried, exposed to PhosphorImager screen, and analysed by a PhosphorImager (Fuji, Japan). To confirm equal loading, 50 μg protein was resolved on 10% SDS–PAGE, electrotransferred to nitrocellulose filters, and probed with the anti-p65 Ab, and the bands were detected by chemiluminescence (ECL, Amersham).

In-vitro phosphatases activity assay

Jurkat cells (5×10^6), labeled with [^{32}P]orthophosphate (Amersham) in phosphate-free medium for 4 h at 37°C, were stimulated with TNF (1 nM) for 1 h. Cell extract proteins were incubated with anti-p65 antibody followed by immunoprecipitation with protein A/G sepharose beads. To assay the activities of different phosphatases these beads were used. Jurkat cells were stimulated with araC for different times and cell extract was prepared. Cells extracts were incubated with different antiphosphatase antibodies followed by immunoprecipitation by protein A/G sepharose. Then these beads were incubated with ^{32}P-labeled p65 in 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 1 μM DTT for 2 h at 37°C. Reactions were stopped with the addition of 15 μl of 2 × SDS sample buffer, boiled for 5 min, and subjected to SDS–PAGE (9%). The gel was dried and ^{32}P-p65 was analysed by a PhosphorImager (Fuji, Japan).

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References

Badrichani AZ, Stroka DM, Bilbao G, Curiel DT, Bach FH and Ferran C. (1999). *J. Clin. Invest*., **103,** 543–553.
Baeuerle PA and Baichwal VR. (1997). *Adv. Immunol.*, **65,** 111–137.
Baichwal VR and Baeuerle PA. (1997). *Curr. Biol.*, **7,** R94–R96.
Baker SJ and Reddy EP. (1998). *Oncogene*, **17,** 3261–3270.
Baldini N. (1997). *Nat. Med.*, **3,** 378–380.
Beg AA and Baltimore D. (1996). *Science*, **274,** 782–784.
Bellamy WT and Dalton WS. (1994). *Adv. Clin. Chem.*, **31,** 1–61.
Boland MP, Foster SJ and O'Neill LAJ. (1997). *J. Biol. Chem.*, **272,** 12952–12960.
Bowie AG, Moynagh PN and O'Neill LAJ. (1997). *J. Biol. Chem.*, **272,** 25941–25950.
Brach MA, Herrmann F, and Kufe DW. (1992a). *Blood*, **79,** 728–734.
Brach MA, Kharbanda SM, Herrmann F, and Kufe DW. (1992b). *Mol. Pharmacol*., **41,** 60–63.
Braess J, Wegendt C, Feuring-Buske M, Riggert J, Kern W, Hiddemann W and Schleyer E. (1999). *Br. J. Haematol.*, **105,** 388–393.
Collins T, Read MA, Neish AS, Whitley MZ, Thanos D and Maniatis T. (1995). *FASEB J.*, **9,** 899–909.
Darnay B, Ni J, Moore PA and Aggarwal BB. (1999). *J. Biol. Chem.*, **274,** 7724–7731.
Das KC and White CW. (1997). *J. Biol. Chem.*, **272,** 14914–14920.
Dou QP, An B and Will PL. (1995). *Proc. Natl. Acad. Sci. USA*, **92,** 9019–9023.
Dou QP and Lui VW. (1995). *Cancer Res.*, **55,** 5222–5225.
Egan LJ, Mays DC, Huntoon CJ, Bell MP, Pike MG, Sandborn WJ, Lipsky JJ and McKean DJ. (1999). *J. Biol. Chem*., **274,** 26448–26453.
Giri DK and Aggarwal BB. (1998). *J. Biol. Chem.*, **273,** 14008–14014.
Harrison DJ. (1995). *J. Pathol.*, **175,** 7–12.
Heckman CA, Mehew JW and Boxer LM. (2002). *Oncogene*, **21,** 3898–3908.
Ito T, Yang M and May WS. (1999). *J. Biol. Chem.*, **274,** 15427–15432.
Izban KF, Ergin M, Qin JZ, Martinez RL, Pooley Jr RJ, Saeed S and Alkan S. (2000). *Hum. Pathol.*, **31,** 1482–1490.
Jarvis DW, Fornari Jr FA, Tombes RM, Erukulla RK, Bittman R, Schwartz GK, Dent P and Grant S. (1998). *Mol. Pharmacol.*, **54,** 844–856.
Kharbanda S, Emoto Y, Kisaki H, Saleem A and Kufe D. (1994). *Mol. Pharmacol.*, **46,** 67–72.
Kochetkova M, Iversen PO, Lopez AF and Shannon MF. (1997). *J. Clin. Invest.*, **99,** 3000–3008.
Lee JI and Burckart GJ. (1998). *J. Clin. Pharmacol.*, **38,** 981–993.
Manna SK and Aggarwal BB. (1999). *J. Immunol.*, **162,** 1510–1518.
Manna SK and Aggarwal BB. (2000). *J. Immunol.*, **164,** 5156–5166.
Manna SK, Mukhopadhyay A and Aggarwal BB. (2000a). *J. Immunol.*, **164,** 6509–6519.
Manna SK, Mukhopadhyay A, Van NT and Aggarwal BB. (1999). *J. Immunol.*, **162,** 6800–6809.
Manna SK, Sah NK and Aggarwal BB. (2000b). *J. Biol. Chem.*, **275,** 13297–13306.
Manna SK, Zhang HJ, Yan Y, Oberley LW and Aggarwal BB. (1998). *J. Biol. Chem.*, **273,** 13245–13254.
Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet Jr RJ and Sledge Jr GW. (1997). *Mol. Cell. Biol.*, **17,** 3629–3639.
Nutku E, Zhuang Q, Soussi-Gounni A, Aris F, Mazer BD and Hamid Q. (2001). *J. Immunol*. **167,** 1039–1046.
Perez C, Vilaboa NE, Garcia-Bermejo L, de Blas E, Creighton AM and Aller P. (1997). *J. Cell Sci.*, **110**(pt 3)**,** 337–343.
Piret B and Piette J. (1996). *Nucleic Acids Res.*, **24,** 4242–4248.
Rajkumar SV, Hayman S, Gertz MA, Dispenzieri A, Lacy MQ, Greipp PR, Geyer S, Iturria N, Fonseca R, Lust JA, Kyle RA and Witzig TE (2002). *J. Clin. Oncol.*, **20,** 4319–4323.
Raziuddin A, Court D, Sarkar FH, Liu Y-L, Kung HF and Raziuddin R. (1997). *J. Biol. Chem.*, **272,** 15715–15720.
Reed JC. (1995). *Curr. Opin. Oncol.* **7,** 541–546.
Roshak AK, Jackson JR, McGough K, Chabot-Fletcher M, Mochan E and Marshal LA. (1996). *J. Biol. Chem.*, **271,** 31496–31501.
Sen R and Baltimore D.(1986). *Cell*, **47,** 921–928.
Suganuma M, Fujiki H, Furuya-Suguri H, Yoshizawa S, Yasumoto S, Kato Y, Fusetani N and Sugimura T. (1990). *Cancer Res*., **50,** 3521–3525.
Terada R, Ito S, Kobayashi M, Akama F, Tsujimura M and Ooe H. (2002). *Hepatogastroenterology*, **49,** 1545–1548.
Tonks NK, Diltz CD and Fischer EH. (1991). *Methods Enzymol.*, **201,** 442–451.
Van Antwerp DJ, Martin SJ, Kafri T, Green DR and Verma IM. (1996). *Science* (Washington DC), **274,** 787–789.
Waddick KG. (1999). *Biochem. Pharmacol.*, **57,** 9–17.
Wang CY, Cusack Jr JC, Liu R and Baldwin Jr AS. (1999a). *Nat. Med*, **5,** 412–417.
Wang CY, Guttridge DC, Mayo MW and Baldwin Jr AS. (1999b). *Mol. Cell. Biol.*, **19,** 5923–5929.
Wang CY, Mayo MW and Baldwin Jr AS. (1996). *Science* **274,** 784–787.
Wang CY, Mayo MW, Korneluk RG, Goeddel DV and Baldwin Jr AS. (1998). *Science*, **281,** 1680–1683.
Wang RH, Liu CW, Avramis VI and Berndt N. (2001). *Oncogene*, **20,** 6111–6122.

Bibiliography

Abd El-Rehim, D. M., Pinder S. E., Paish, C. E., Bell, J. A., Rampaul, R. S., Blamey, R. W., Robertson, J. F., Nicholson, R. I., and Ellis, I. O. 2004. Expression and co-expression of the members of the epidermal growth factor receptor (EGFR) family in invasive breast carcinoma Br J Cancer 91:1532-1542.

Abe, F., Yamaguchi, T. 1978. Digitoxingenin oleandroside and 5 alpha-adynerin in the leaves of Nerium odorum. Chem Pharm Bull 26:3023-3027.

Abe, F., Yamaguchi, T. 1979. Oleasides-novel cardenolides with an unusual framework on Nerium. Chem Pharm Bull 27:1604-1610.

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., Yuan, J. 1996. Human ICE/CED-3 protease nomenclature. Cell. 87:171-180.

Ambrosini, G., Adida, C. & Altieri, D. C. 1997. A novel antiapoptosis gene, survivin, expressed in cancer and lymphoma. Nature Med 3:917-921.

Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I., Gottesman M.M. 1999 Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 39:361-398.

Aplin, A.E., Howe, A., Alahari, S.K., and Juliano, R.L. 1998. Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. Pharmacol Rev 50:197-263.

Ashkenazi, A, Dixit, V.M. 1998. Death receptors: signaling and modulation. Science 281(5381):1305-8.

Baeuerle, P. A., Baichwal, V. R. 1997. NF-kappa B as a frequent target for immunosuppressive and anti-inflammatory molecules Adv Immunol 65:111-137.

Baggiolini, M., Dewald, B., and Moaer, B. 1994. Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. Adv lmmunol 55:97-179.

Baichwal, V. R., and Baeuerle, P. A. 1997. Activate NF-kappa B or die? Curr. Biol. 7, R94-R96.

Baldwin, A.S. 2001. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. J Clin Invest 107:241-246.

Belperio, J. A., Keane, M. P., Arenberg, D. A., Addison, C. L., Ehlert, J. E., Burdick, M. D., and Strieter, R. M. 2000. CXC chemokines in angiogenesis J Leukoc Biol 68:1-8.

Beltinger, C., Fulda, S., Kammertoens, T., Meyer, E., Uckert, W., Debatin, K.M. 1999. Herpes simplex virus thymidine kinase/ganciclovir-induced apoptosis involves ligand-independent death receptor aggregation and activation of caspases. Proc Natl Acad Sci U S A 96(15):8699-704.

Bender, K., Gottlicher, M., Whiteside, S., Rahmsdorf, H.J., Herrlich, P. (1998) Sequential DNA damage-independent and dependent activation of NF-kappaB by UV. EMBO J 17:5170-5181.

Bertino, J.R. 1993. Karnofsky memorial lecture. Ode to methotrexate. J Clin Oncol 11:5-14.

Blume-Jensen, P., Hunter, T. 2001. Oncogenic kinase signalling. Nature 411:355-365.

Boise, L. H. 1993. BCL-X, a BCL-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597-608.

Bonizzi, G., Piette, J., Merville, M. P., and Bours, V. 1997. Distinct signal transduction pathways mediate nuclear factor-kappaB induction by IL-1beta in epithelial and lymphoid cells J Immunol 159:5264-5274.

Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z., Kolesnick, R. 1995. Ceramide synthase mediates daunorubicin-induced apoptosis: An alternative mechanism for generating death signals. Cell 82:405-410.

Bouck, N., Stellmach, V., Hsu, S.C. 1996. How tumors become angiogenic. Adv Cancer Res 69:135-174.

Boyer, J., McLean, E.G., Aroori, S., Wilson, P., McCulla, A., Carey, P.D. 2004. Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin and irinotecan. Clin Cancer Res 10:2158-2167.

Brasier, A. R., Jamaluddin, M., Casola, A., Duan, W., Shen, Q., Garofalo, R. P. 1998. A promoter recruitment mechanism for tumor necrosis factor-alpha-induced interleukin-8 transcription in type II pulmonary epithelial cells. Dependence on nuclear abundance of Rel A, NF-kappaB1, and c-Rel transcription factors J Biol Chem 273:3551-3661.

Brivanlou, A. H., Darnell, J. E., Jr. 2002. Signal transduction and the control of gene expression. Science 295:813-818.

Bryan, T.M., and Cech, T.R. 1999. Telomerase and the maintenance of chromosome ends. Curr Opin Cell Biol 11:318-324.

Bui, N.T., Livolsi, A., Peyron, J.F., Prehn, J.H. 2001. Activation of nuclear factor kappaB and Bcl-x survival gene expression by nerve growth factor requires tyrosine phosphorylation of IkappaB alpha. J Cell Biol 152:753-764

Bull, H.A., Brickell, P.M., and Dowd, P.M. 1994. Src-related protein tyrosine kinases are physically associated with the surface antigen CD36 in human dermal microvascular endothelial cells. FEBS Lett 351:41-44.

Burkhart, C.A., Kavallaris, M., Band Horwitz. S. 2001. The role of betatubulin isotypes in resistance to antimitotic drugs. Biochim Biophys Acta 1471: O1-O9.

Chaney, S.G., Sancar, A. 1996. DNA repair: enzymatic mechanisms and relevance to drug response. J Natl Cancer Inst 88:1346-1360.

Cheng, J. 1994. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science 263:1759-1762.

Chen, J., Flannery, J.G., LaVail, M.M., Steinberg, R.H., Xu, J., Simon, M.I. (1996) bcl-2 overexpression reduces apoptotic photoreceptor cell death in three different retinal degenerations. Proc Natl Acad Sci USA 93:7042-7047.

Chen, Q., Powell, D. W., Rane, M. J., Singh, S., Butt, W., Klein, J. B., and McLeish, K. R. (2003) Akt phosphorylates p47phox and mediates respiratory burst activity in human neutrophils J Immunol 170:5302-5308.

Chen, X., Yeung, T.K., Wang, Z. 2000. Enhanced drug resistance in cells coexpressing ErbB2 with EGF receptor or ErbB3. Biochem Biophys Res Commun 277:757-763.

Chen, Z.J., Parent, L., Maniatis, T. 1996. Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. Cell 84:853-862

Chu, E., Voeller, D.M., Jones, K.L., Takechi, T., Maley, G.F., Maley, F. 1994. Identification of a thymidylate synthase ribonucleoprotein complex in human colon cancer cells. Mol Cell Biol 14:207-213.

Chung, J.Y., Huang, C., Meng, X., Dong, Z., Yang, C.S. 1999. Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in H-ras-transformed cells: structure-activity relationship and mechanisms involved. Cancer Res 59:4610-7.

Cogswell, P. C., Guttridge, D. C., Funkhouser, W. K. & Baldwin, A. S. Jr. 2000. Selective activation of NF-ÎºB subunits in human breast cancer: potential roles for NF-κB2/p52 and for BCL3. Oncogene 19:1123-1131.

Collins, T., Read, M.A., Neish, A.S., Whitley, M.Z., Thanos, D., Maniatis, T. 1995. Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. FASEB J 9:899-909.

Cordon-Cardo, C., and Prives, C. 1999. At the crossroads of inflammation and tumorigenesis. J Exp Med 190:1367-1370.

Counter, C.M., Avilion, A.A., LeFeuvre, C.E., Stewart, N.G., Greider,C.W., Harley, C.B., and Bacchetti, S. 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 11:1921-1929.

Coussens, L.M., Raymond, W.W., Bergers, G., Laig-Webster, M.,Behrendtsen, O., Werb, Z., Caughey, G.H., and Hanahan, D. 1999. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. Genes Dev 13:1382-1397.

Cryns, V., Yuan, Y. Proteases to die for. 1999. Genes Dev 13:371-382.

Dallegri, F., and Ottonello, L. 1997. Tissue injury in neutrophilic inflammation. Inflamm Res 46:382-391.

Datto, M.B., Hu, P.P., Kowalik, T.F., Yingling, J., and Wang, X.F. 1997. The viral oncoprotein E1A blocks transforming growth factor b-mediated induction of p21/WAF1/Cip1 and p15/INK4B Mol Cell Biol 17:2030-2037.

Deffie, A.M., McPherson, J.P., Gupta, R.S., Hedley, D.W., Goldenberg, G.J. 1992. Multifactorial resistance to antineoplastic agents in drugresistant P388 murine leukemia, Chinese hamster ovary, and human HeLa cells, with emphasis on the role of DNA topoisomerase II. Biochem Cell Biol 70:354-364.

De Larco, J.E., Wuertz, B.R., Yee, D., Ricket, B.L., and Furcht, L.T. 2003. Atypical methylation of the interleukin-8 gene correlates strongly with the metastatic potential of breast carcinoma cells. Proc Natl Acad Sci USA 100(24):13988-13993.

Denhardt, D. T. 1996. Oncogene-initiated aberrant signaling engenders the metastatic phenotype: synergistic transcription factor interactions are targets for cancer therapy. Crit Rev Oncog 7:261-291.

Descamps, S., Toillon, R-A., Adriaenssens, E., Pawlowski, V., Cool, S. A., Nurcombe, V., Bourhis, X. L., Boilly, B., Peyrati, J. P., and Hondermarck, H. 2001. Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways J Biol Chem 276:17864-17870.

Diasio RB, Harris BE. 1989. Clinical pharmacology of 5-fluorouracil. Clin Pharmacokinet 16: 215-237.

DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E., Karin, M. 1997. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature 388:548-554.

Digicaylioglu, M., Lipton, S.A. 2001. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NfkappaB signalling cascades. Nature 412:641-647.

Dmitrieva, R.I., Doris, P.A. 2000. Cardiotonic steroids: potential endogenous sodium pump ligands with diverse function. Exp Biol Med (Maywood). 227(8):561-9.

Dole, M. G. 1995. BCL-XL is expressed in neuroblastoma cells and modulates chemotherapy-induced apoptosis. Cancer Res 55:2576-2582.

Dong, G., Chen, Z., Kato, T., Van Waes, C. 1999. The host environment promotes the constitutive activation of nuclear factor-kappa B and proinflammatory cytokine expression during metastatic tumor progression of murine squamous cell carcinoma. Cancer Res 59:3495-504.

Dumontet, C., Sikic, B.I. 1999. Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. J Clin Oncol 17:1061-1070.

Durand, R. E., Olive, P. L. 2001. Resistance of tumor cells to chemo- and radiotherapy modulated by the threedimensional architecture of solid tumors and spheroids. Methods Cell Biol. 64:211-233.

Dyson, N., Howley, P.M., Munger, K., and Harlow, E. 1989. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 243:934-937.

Egan, L.J., Mays, D.C., Huntoon, C.J., Bell, M.P., Pike, M.G., Sandborn, W.J., Lipsky, J.J., McKean, D.J. 1999. Inhibition of interleukin-1-stimulated NF-κB RelA/p65 phosphorylation by mesalamine is accompanied by decreased transcriptional activity. J Biol Chem 274:26448-53.

Eichhorst, S.T, Muller, M., Li-Weber, M., Schulze-Bergkamen, H., Angel, P., Krammer, P.H. 2000. A novel AP-1 element in the CD95 ligand promoter is required for induction of apoptosis in hepatocellular carcinoma cells upon treatment with anticancer drugs. Mol Cell Biol 20(20):7826-37.

Epperly, M. Bray, J., Kraeger, S., Zwacka, R., Engelhardt, J., Travis, E. Greenberger, J. 1998. Prevention of late effects of irradiation lung damage by manganese superoxide dismutase gene therapy. Gene Ther 5:196-208.

Evrard, A., Cuq, P., Ciccolini, J., Vian, L., Cano, J.P. 1999. Increased cytotoxicity and bystander effect of 5-fluorouracil and 5-deoxy-5-fluorouridine in human colorectal cancer cells transfected with thymidine phosphorylase. Br J Cancer 80:1726-1733.

Fedi, P., Tronick, S.R., and Aaronson, S.A. 1997. Growth factors.In Cancer Medicine, J.F. Holland, R.C. Bast, D.L. Morton, E. Frei, D.W. Kufe, and R.R. Weichselbaum, eds. (Baltimore, MD: Williams and Wilkins), pp. 41-64.

Feng, D., Nagy, J. A., Pyne, K., Dvorak, H. F., and Dvorak, A. M. 1998. Vesiculo-vacuolar organelles and the regulation of venule permeability to macromolecules by vascular permeability factor, histamine, and serotonin. J Exp Med 187:903-915.

Fichtner, I., Slisow, W., Gill, J., Becker, M., Elbe, B., Hillebrand, T. 2004. Anticancer drug response and expression of molecular markers in early-passage xenotransplanted colon carcinomas. Eur J Cancer 40:298-307.

Finco, T.S.A., Beg, A.A., Baldwin, A.S. 1994. Inducible phosphorylation of IκBα is not sufficient for its dissociation from NF- κB and is inhibited by protease inhibitors. Proc Natl Acad Sci USA 91:11884-8.

Findley, H. W., Gu, L., Yeager, A. M. & Zhou, M. 1997. Expression and regulation of BCL-2, BCL-XL, and BAX correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukemia. Blood 89:2986-2993.

Fink, D., Aebi, S., Howell, S.B. 1998. The role of DNA mismatch repair in drug resistance. Clin Cancer Res 4:1-6.

Fisher, M. J. 2001. Nucleotide substitution in the ectodomain of trail receptor DR4 is associated with lung cancer and head and neck cancer. Clin. Cancer Res. 7:1688-1697.

Fogar, P., Basso, D., Pasquali, C., De Paoli, C., Sperti, C., Roveroni,G., Pedrazzoli, G., and Plebani, M. 1997. Neural cell adhesion molecule (N-CAM) in gastrointestinal neoplasias. Anticancer Res 17:1227-1230.

Foley, K.P., and Eisenman, R.N. 1999. Two MAD tails: what the recent knockouts of Mad1 and Mx1 tell us about the MYC/MAX/MAD network. Biochim Biophys Acta 1423:M37-47.

Folkman, J. 1997. Tumor angiogenesis. In Cancer Medicine, J.F.Holland, R.C. Bast, D.L. Morton, E. Frei, D.W. Kufe, and R.R. Weichselbaum, eds. (Baltimore, MD: Williams and Wilkins), pp. 181-204.

Foulds, L. 1954. The Experimental Study of Tumor Progression.Volumes I-III (London: Academic Press).

Friche, E., Danks, M.K., Schmidt, C.A., Beck, W.T. 1991. Decreased DNA topoisomerase II in daunorubicin-resistant Ehrlich ascites tumor cells. Cancer Res 51: 4213-4218.

Friesen, C., Herr, I., Krammer, P.H., Debatin, K.M. 1996. Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat Med 2(5):574-7.

Fulda, S. 1997. Betulinic acid triggers CD95 (APO-1/Fas) and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. Cancer Res 57:4956-4964.

Fulda, S., Sieverts, H., Friesen, C., Herr, I., Debatin, K.M. 1997. The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. Cancer Res 57(17):3823-9.

Fulda, S., Strauss, G., Meyer, E., Debatin, K.M. 2000. Functional CD95 ligand and CD95 death-inducing signaling complex in activation-induced cell death and doxorubicin-induced apoptosis in leukemic T cells. Blood 95(1):301-8.

Fulda, S., Susin, S.A., Kroemer, G., Debatin, K.M. 1998. Molecular ordering of apoptosis induced by anticancer drugs in neuroblastoma cells. Cancer Res 58(19):4453-60.

Furuta, T., Ueda, T., Aune, G., Sarasin, A., Kraemer, K.H., Pommier, Y. 2002. Transcription-coupled nucleotide excision repair as a determinant of cisplatin sensitivity of human cells. Cancer Res 62:4899-4902.

Fynan, T.M., Reiss, M. 1993. Resistance to inhibition of cell growth by transforming growth factor-b and its role in oncogenesis. Crit. Rev Oncog 4:493-540.

Gangur, V., Birmingham, N.P., Thanesvorakul, S. 2002. Chemokines in health and disease.Vet. Immunol. Immunopathol. 86, 127-36

Garaci, E., Caroleo, M. C., Aloe, L., Aquaro, S., Piacentini, M., Costa, N., Amendola, A., Micera, A, Calio, R., Perno, C. F., Levi-Montalcini, R. 1999. Nerve growth factor is an autocrine factor essential for the survival of macrophages infected with HIV Proc. Natl. Acad. Sci. U S A. 96:14013-14018.

Geisler, S., Lonning, P.E, Aas, T., Johnsen, H., Fluge, O., Haugen, D.F. 2001. Influence of TP53 gene alterations and c-erbB-2 expression on the response to treatment with doxorubicin in locally advanced breast cancer. Cancer Res 61:2505-2512.

Gerharz, C. D. 1999. Resistance to CD95 (APO-1/Fas)-mediated apoptosis in human renal cell carcinomas: an important factor for evasion from negative growth control. Lab Invest 79:1521-1534.

Ghosh, S., May, M.J., Kopp, E.B. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 16:225-260.

Giancotti, F.G., and Ruoslahti, E. 1999. Integrin signaling. Science 285:1028-1032.

Gibbs, J. B. 2000. Mechanism-based target identification and drug discovery in cancer. Science 287:1969-1973.

Gilmore, T. D. 1999. Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel. Oncogene 18:6925-6937.

Goldman, B. 2003. Multidrug resistance: can new drugs help chemotherapy score against cancer? J Natl Cancer Inst 95:255-257.

Gorlick, R., Bertino, J.R. Clinical pharmacology and resistance to dihydrofolate reductase inhibitors. In Antifolate Drugs in Cancer Therapy, Jackman AL (ed). Humana Press: Totowa, NJ, 1999.

Gottesman, M.M., Fojo, T., Bates, S.E. 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. Nature Rev Cancer 2:48-58.

Green, D.R., Ferguson, T.A. 2001. The role of Fas ligand in immune privilege. Nat Rev Mol Cell Biol 2(12):917-24.

Green, S. K., Frankel, A. and Kerbel, R. S. (1999) Adhesiondependent multicellular drug resistance. Anticancer Drug Des 14:153-168.

Guo, W., Healey, J.H., Meyers, P.A., Ladanyi, M., Huvos, A.G, Bertino, J.R. (1999) Mechanisms of methotrexate resistance in osteosarcoma. Clin Cancer Res 5:621-627.

Gupta, R. S., and Chopra, A. 1985. Cross-resistance and biochemical studies with two classes of HeLa cell mutants resistant to cardiac glycosides. The unusual behavior of cardenolide SC4453 J Biol Chem 260:6843-6850.

Gupta, R. S., and Chopra, A. 1987. Human cell mutants affected in the interaction of the 12 beta-OH group of cardiac glycosides with the digitalis receptor Biochem Pharmacol 36:3829-3833.

Gupta, R. S., Chopra, A., and Stetsko, D. K. 1986. Cellular basis for the species differences in sensitivity to cardiac glycosides (digitalis). J Cell Physiol 127:197-206.

Guttridge, D. C., Albanese, C., Reuther, J. Y., Pestell, R. G. & Baldwin, A. S., Jr. 1999. NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol 19:5785-5799.

Hanahan, D., Folkman, J. 1996. Patterns and emerging mechahypoxianisms of the angiogenic switch during tumorigenesis. Cell 86:353-364.

Hanahan, D, Weinberg, RA. 2000. The hallmarks of cancer. Cell 100(1):57-70.

Hannon, G.J., and Beach, D. 1994. P15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature 371:257-261.

Hannun, Y.A. 1996. Functions of ceramide in coordinating cellular responses to stress. Science 274:1855-9.

Harris, C.C. 1996. p53 tumor suppressor gene: from the basic research laboratory to the clinic: an abridged historical perspectyrosinetive. Carcinogenesis 17:1187-1198.

Hayakawa, J., Ohmichi, M., Kurachi, H., Kanda, Y., Hisamoto, K.,Nishio, Y. 2000. Inhibition of BAD phosphorylation either at serine 112 via extracellular signal-regulated protein kinase cascade or at serine 136 via Akt cascade sensitizes human ovarian cancer cells to cisplatin. Cancer Res 60:5988-5994.

Hayflick, L. 1997. Mortality and immortality at the cellular level. A review. Biochemistry 62:1180-1190.

Herfarth, K.K., Brent, T.P., Danam, R.P., Remack, J.S., Kodner, I.J., Wells, S.A, Jr. 1999. A specific CpG methylation pattern of the MGMT promoter region associated with reduced MGMT expression in primary colorectal cancers. Mol Carcinog 24:90-98.

Herr, I., Wilhelm, D., Bohler, T., Angel, P., Debatin, K.M. 1997. Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. EMBO J 16(20):6200-8.

Hikawa, S., Kobayashi, H., Hikawa, N., Kusakabe, T., Hiruma, H., Takenaka, T., Tomita, T., and Kawakami, T. 2002. Expression of neutrophins and their receptors in peripheral lung cells of mice. Histochem. Cell Biol 118:51-58.

Hirano, M., Osada, S., Aoki, T., Hirai, S., Hosaka, M., Inoue, J., Ohno, S. 1996. MEK kinase is involved in tumor necrosis factor alpha induced NF-κB activation and degradation of IκBα. J Biol Chem 271:13234-8.

Holmes, W. E., Lee, J., Kuang, W. J., Rice, G. C., and W. I. Wood, W. I. 1991. Structure and functional expression of a human interleukin-8 receptor. Science. 253:1278-1280.

Houghton, J.A., Harwood, F.G., Tillman, D.M. 1997. Thymineless death in colon carcinoma cells is mediated via fas signaling. Proc Natl Acad Sci U S A 94(15):8144-9.

Houghton, J.A., Houghton, P.J. 1983. Elucidation of pathways of 5-fluorouracil metabolism in xenografts of human colorectal adenocarcinoma. Eur J Cancer Clin Oncol 19:807-815.

Huang, S., Mills, L., Mian, B., Tellez, C., McCarty, M., Yang, X-D., Gudas, J. M., and Bar-Eli, M. 2002. Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL8) inhibit angiogenesis, tumor growth, and metastasis of human melanoma. Am. J. Pathol. 161:125-134.

Hudson, J.D., Shoaibi, M.A., Maestro, R., Carnero, A., Hannon, G.J.,and Beach, D.H. 1999. A proinflammatory cytokine inhibits p53 tumor suppressor activity. J Exp Med 190:1375-1382.

Hwang, D., Jang, B.C., Yu, G., Boudreau, M. 1997. Expression of mitogen-inducible cyclooxygenase induced by lipopolysaccharide: mediation through both mitogen-activated protein kinase and NF-κB signaling pathways in macrophages. Biochem Pharmacol 54:87-96.

Iademarco, M.F., McQuillan, J.J., Rosen, G.D., Dean, D.C. 1992. Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1). J Biol Chem 267:16323-9.

Igney, F.H. and Krammer, P.H. 2002. Death and antideath: Tumour resistance to apoptosis. Nat Rev Can 2:277-288.

Imbert, V., Rupec, R.A., Livols,i A., Pahl, H.L., Traenckner, E.B.,Mueller-Dieckmann, C., Farahifar, D., Rossi, B., Auberger, P., Baeuerle, P.A., Peyron, J.F. 1996. Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. Cell 86:787-798.

Inada, A., Nakanishi, T., Konoshima, T., Kozuka, M., Tokuda, H., Nishino, H., Iwashima, A. 1993. Anti-tumor promoting activities of natural products. II. Inhibitory effects of digitoxin on two-stage carcinogenesis of mouse skin tumors and mouse pulmonary tumors. Biol Pharm Bull 16(9):930-1.

Inohara, N., Gourley, T.S., Carrio, R., Muniz, M., Merino, J., Garcia, I., Koseki, T., Hu, Y., Chen, S., Nunez, G. 1998. Diva, a Bcl-2 homologue that binds directly to Apaf-1 and induces BH3-independent cell death. J Biol Chem 273:32479-32486.

Inoue, K., Slaton, J. W., Eve, B. Y., Kim, S. J., Perrotte, P., Derya Balbay, D., Yano, S., Bar-Eli, M., Radinsky, R., Pettaway, C., and Dinney, C. P. N. 2000. Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer Clin Cancer Res 6, 2104-2119.

Inoue, K., Slaton, J. W., Kim, S. J., Perrotte, P., Eve, B. Y., Bea-Eli, M., Radinsky, R., and Dinney, C. P. N. 2000. Interleukin 8 expression regulates tumorigenicity and metastasis in androgen-independent prostrate cancer. Cancer Res 60:2290-2299.

Irmler, M. 1997. Inhibition of death receptor signals by cellular FLIP. Nature 388, 190-195

Israel, A. 1997. IκB kinase all zipped up. Nature 388:519-521.

Issa, J.P. 2000. The epigenetics of colorectal cancer. Ann N Y Acad Sci 910:140-153; discussion 153-155.

Itoh, Y., Joh, T., Tanida, S., Sasaki, M., Kataoka, H., Itoh, K., Oshima, T., Ogasawara, N., Togawa, S., Wada, T., Kubota, H., Mori, Y., Ohara, H., Nomura, T., Higashiyama, S., and Itoh, M. 2005. IL-8 promotes cell proliferation and migration through metalloproteinase-cleavage of proHB-EGF in human colon carcinoma cells. Cytokine 29(6):275-282.

Jackman, A.L., Taylor, G.A., Gibson, W., Kimbell, R., Brown, M.,Calvert, A.H. 1991. ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth in vitro and in vivo: a new agent for clinical study. Cancer Res 51:5579-5586.

JaffreÂ´zou, J.P., Levade, T., Bettaieb. A., Andrieu, N., Bezombes, C., Maestre, N., Vermeersch, S., Rousse, A., Laurent, G. 1996. Daunorubucin induced apoptosis: Triggering of ceramide generation through sphingomyelin hydrolysis. EMBO J 15:2417-2423.

Jain, R. K. (2001) Delivery of molecular and cellular medicine to solid tumors. Adv. Drug Deliv Rev 46:149-168.

Jansen, W.J., Kolfschoten, G.M., Erkelens, C.A., Van Ark-Otte, J., Pinedo, H.M., Boven, E. 1997. Anti-tumor activity of CPT-11 in experimental human ovarian cancer and human soft-tissue sarcoma. Int J Cancer 73:891-896.

Jobin, C., Bradham, C.A., Russo, M.P., Juma, B., Narula, A.S., Brenner, D.A., Sartor, R.B. 1999. Curcumin blocks cytokine-mediated NF-κB activation and proinflammatory gene expression by inhibiting inhibitory factor I-κB kinase activity. J Immunol 163:3474-83.

Johnson, J.P. 1991. Cell adhesion molecules of the immunoglobulin supergene family and their role in malignant transformation and progression to metastatic disease. Cancer Metastasis Rev 10:11-22.

Jones, S. A., Wolf, M., Qin, S., Mackay, C. R., and Baggiolini, M. 1996. Different functions for the interleukin 8 receptors (IL-8R) of human neutrophil leukocytes : NADPH oxidase and phospholipase D are activated through IL-8R1 but not IL-8R2. Proc Natl Acad Sci U S A 93:6682-6686.

Jordan, M.A., Toso, R.J., Throwe, D., Wilson, L. 1993. Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. Proc Natl Acad Sci U S A 90:9552-9556.

Jordan, M.A., Wendell, K., Gardiner, S., Derry, W.B., Copp, H., Wilson, L. 1996. Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. Cancer Res 56:816-825.

Kaiser, U., Auerbach, B., and Oldenburg, M. 1996. The neural cell adhesion molecule NCAM in multiple myeloma. Leuk. Lymphoma 20:389-395.

Karin, M. 1999. The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. J Biol Chem 274:27339-27342.

Karin, M., Ben-Neriah, Y. 2002. Regulatory functions of ubiquitination in the immune system. Nat Immunol 3:20-26

Karin, M., Cao, Y., Greten, F.R., and Zhi-Wei Li 2002. NF-κB in Cancer: From innocent bystander to major culprit. Nat Rev Can 2:301-310.

Karin, M., Delhase, M. 2000. The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signaling. Semin Immunol 12:85-98.

Karin, M., Delhase, M. 1998. JNK or IKK, AP-1 or NF-KB, which are the targets for MEK kinase1 action? Proc Natl Acad Sci USA 95:9067-9.

Karin, M., Lin, A. 2002. NF-kappaB at the crossroads of life and death. Nat Immunol 3:221-227.

Karin, M., Liu, Z.G., Zandi, E. 1997. AP-1 function and regulation. Curr Opin Cell Biol 9:240-6.

Kasibhatla, S., Brunner, T., Genestier, L., Echeverri, F., Mahboubi, A., Green, D.R. 1998. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. Mol Cell. 1(4):543-51.

Kato Jr T, Delhase M, Hoffmann A, Karin M. 2003. CK2 Is a C-Terminal IkappaB Kinase Responsible for NF-kappaB activation during the UV Response. Mol Cell 12:829-839

Kavallaris, M., Tait, A.S., Walsh, B.J., He, L., Horwitz, S.B., Norris, M.D. 2001. Multiple microtubule alterations are associated with vinca alkaloid resistance in human leukemia cells. Cancer Res 61: 5803-5809.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239-257.

Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Philipps, H.S., and Ferrara, N. 1993. Inhibition of vascular endothelial growth factor induced angiogenesis suppresses tumour growth in vivo. Nature 362:841-844.

King, B.L., Carcangiu, M.L., Carter, D., Kiechle, M., Pfisterer, J., Pfleiderer, A. 1995. Microsatellite instability in ovarian neoplasms. Br J Cancer 72: 376-382.

Klagsbrun, M., and Moses, M. A. 1999. Molecular angiogenesis Chem Biol 6:R217-224.

Kogan, S. C. 2001. BCL-2 cooperates with promyelocytic leukemia retinoic acid receptor alpha chimeric protein (PMLRAR) to block neutrophil differentiation and initiate acute leukemia. J Exp Med 193:531-543.

Kojima, A., Hackett, N.R., Crystal, R.G. 1998. Reversal of CPT-11 resistance of lung cancer cells by adenovirus-mediated gene transfer of the human carboxylesterase cDNA. Cancer Res 58:4368-4374.

Korsmeyer, S.J. 1992. Chromosomal translocations in lymphoid malignancies reveal novel proto-oncogenes. Annu Rev Immunol 10:785-807.

Krajewski, S. 1995. Reduced expression of proapoptotic gene BAX is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. Cancer Res 55:4471-4478.

Krammer, P.H. 2000. CD95's deadly mission in the immune system. Nature. 407(6805):789-95.

Krishna, R., Mayer, L.D. 2000. Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur J Pharm Sci 11:265-283.

Krueger, A., Baumann, S., Krammer, P. H. & Kirchhoff, S. 2001. FLICE-inhibitory proteins: regulators of death receptormediated apoptosis. Mol Cell Biol 21:8247-8254.

Kumar, A., Aggarwal, B.B. 1999. Assay for redox sensitive kinases. Methods Enzymol 300:339-45.

Kumar, A., Boriek, A.M. 2003. Mechanical stress activates the nuclear factor-kappaB pathway in skeletal muscle fibers: a possible role in Duchenne muscular dystrophy. FASEB J 17:386-396.

Kumar. A., Lnu, S., Malya, R., Barron, D., Moore, J., Corry, D.B., Boriek, A.M. 2003. Mechanical stretch activates nuclear factor-kappaB, activator protein-1, and mitogen-activated protein kinases in lung parenchyma: implications in asthma. FASEB J 17:1800-1811.

Kumar, A., Takada, Y., Boriek, A.M., Aggarwal, B.B. 2004. Nuclear factor-kappa B : its role in health and disease. J Mol Med. 82:434-448.

Lam, V., McPherson, J.P., Salmena, L., Lees, J., Chu, W., Sexsmith. E. 1999. p53 gene status and chemosensitivity of childhood acute lymphoblastic leukemia cells to adriamycin. Leuk Res 23:87-880.

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G., Earnshaw, W.C. 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 371:346-349.

Lee, F.S., Peters, R.T., Dang, L.C., Maniatis, T. 1998. MEKK1 activates both IkappaB kinase alpha and IkappaB kinase beta.Proc Natl Acad Sci USA 95:9319-9324

Lee, J., Horuk, R., Rice, G. C., Bennet, G. L., Camerato, T., and Wood, W. I. 1992. Characterization of two high affinity human interleukin-8 receptors.) J Biol Chem 267: 16283-16287.

Lee, J. I., and Burckart, G. J. 1998. Nuclear factor kappa B: important transcription factor and therapeutic target J. Clin Pharmacol 38:981-993.

Lee, S. H. 1999. Alterations of the DR5/TRAIL receptor 2 gene in non-small cell lung cancers. Cancer Res 59:5683-5686.

Leithauser, F. 1993. Constitutive and induced expression of APO-1, a new member of the nerve growth factor/tumor necrosis factor receptor superfamily, in normal and neoplastic cells. Lab Invest 69:415-429.

Lev, D. C., Kim, L. S., Melnikova, V., Ruiz, M., Ananthaswamy, H. N., and Price, J. E. 2004. Br J Cancer 91:795-802.

Leverkus, M., Yaar, M., Gilchrest, B.A. 1997. Fas/Fas ligand interaction contributes to UV-induced apoptosis in human keratinocytes. Exp Cell Res 232(2):255-62.

Lin, A., Karin, M. 2003. NF-kappaB in cancer: a marked target. Semin Cancer Biol 13: 107-114.

Ling, L., Cao, Z., Goeddel, D.V. 1998. NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176. Proc Natl Acad Sci USA 95:3792-3797.

Li, N., Karin, M. 1998. Ionizing radiation and short wavelength UV activate NF-kappaB through two distinct mechanisms. Proc Natl Acad Sci USA 95:13012-13017.

Li, N., Karin, M. 1999. Is NF-κB the sensor of oxidative stress? FASEB J 13:1137-43.

Lin, X., Cunningham, E.T. Jr, Mu, Y. 1999 Geleziunas R, Greene WC. The proto-oncogene Cot kinase participates in CD3/CD28 induction of NF-κB, acting through the NF-κB-inducing kinase and IκB kinases. Immunity. 10:271-80.

Li, Q., Verma, I.M. (2002) NF-kappaB regulation in the immune system. Nat Rev Immunol 2:725-734.

Liston, P. 2001. Identification of XAF1 as an antagonist of XIAP anti-caspase activity. Nature Cell Biol 3, 128-133.

Lukashev, M.E., and Werb, Z. 1998. ECM signaling: orchestrating cell behaviour and misbehaviour. Trends Cell Biol. 8, 437-441.

Mahon, T.M., O'Neill, L.A. 1995. Studies into the effect of the tyrosine kinase inhibitor herbimycin A on NF-κB activation in T lymphocytes: evidence for covalent modification of the p50 subunit. J Biol Chem 270:28557-64.

Manna, S. K., Samanta, S., and Samanta, A. K. 1997. Hamycin inhibits IL-8-induced biologic response by modulating its receptor in human polymorphonuclear neutrophils. J Immunol 159:5042-5052.

Manna, S. K., and Aggarwal, B. B. 1998a. Interleukin-4 down-regulates both forms of tumor necrosis factor receptor and receptor-mediated apoptosis, NF-kappaB, AP-1, and c-Jun N-terminal kinase. Comparison with interleukin-13. J Biol Chem 273:33333-33341.

Manna, S.K., Zhang, H.J., Yan, T., Oberley. L.W., Aggarwal, B.B. 1998b. Overexpression of Mn-superoxide dismutase suppresses TNF induced apoptosis and activation of nuclear transcription factor-B and activated protein-1. J Biol Chem 273:13245-54.

Manna, S.K., Mukhopadhyay, A., Van, N.T. Aggarwal, B.B. 1999a. Silymarin suppresses TNF-induced activation of nuclear transcription factor- [{kappa}] B, c-Jun N terminal kinase, and apoptosis J Immunol 162:6800-9.

Manna, S.K., Kuo, M.T., Aggarwal, B.B. 1999a. Overexpression of glutamylcysteine synthetase abolishes tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-B and activator protein-1. Oncogene 18:4371-82.

Manna, S.K., Mukhopadhyay, A, Aggarwal, B.B. 2000a. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. J Immunol 164:6509-19.

Manna, S.K., Sah, N.K., Aggarwal, B.B. 2000b. Protein tyrosine kinase p56lck is required for ceramide-induced but not tumor necrosis factor-induced activation of NF-kappa B, AP-1, JNK, and apoptosis. J Biol Chem 275:13297-306.

Manna, S.K., Sah, N.K., Newman, A., Cisneros, A., Aggarwal, B.B. 2000c. Oleandrin Suppresses Activation of Nuclear Transcription Factor- [{kappa}] B, Activator Protein-1, and c-Jun NH2-Terminal Kinase. Cancer Res 60:3838-47.

Manna, S. K., and Ramesh, G. T. 2005. Interleukin-8 induces nuclear transcription factor-kappaB through a TRAF6-dependent pathway J Biol Chem 280:7010-7021.

Markowitz, S., Wang, J., Meyeroff, L., Parsons, R., Sun, L., Lutterbaugh,J., Fan, R., Zborowska, E., Kinzler, K., Vogelstein, B. 1995. Inactivation of the type II TGF-b receptor in colon cancer cells with microsatellite instability. Science 268:1336-1338.

Marsh, S., McLeod, H.L. 2001. Thymidylate synthase pharmacogenomics in colorectal cancer. Clin Colorectal Cancer 1:175-178.

Martinou, J. C. & Green, D. R. 2001. Breaking the mitochondrial barrier. Nature Rev Mol Cell Biol 2:63-67.

McConkey, D.J.Y., Lin, Y., Nutt, L.K., Ozel, H.Z., Newman, R.A. 2000. Cardiac glycosides stimulate Ca2+ increases and apoptosis in androgen-independent, metastatic human prostate adenocarcinoma cells. Cancer Res 60:3807-12.

McDonnell, T. J. 1989. BCL-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell 57:79-88.

Medema, J. P. 2001. Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. Proc Natl Acad Sci USA 98:11515-11520.

Medema, R.H., and Bos, J.L. 1993. The role of p21-ras in receptor perspectyrosine kinase signaling. Crit. Rev Oncog 4:615-661.

Meijer, C., Mulder, N.H., Timmer-Bosscha, H., Sluiter, W.J., Meersma, G.J, de Vries, E.G. 1992. Relationship of cellular glutathione to the cytotoxicity and resistance of seven platinum compounds. Cancer Res 52: 6885-6889.

Meijerink, J. P. 1998. Hematopoietic malignancies demonstrate loss-of-function mutations of BAX. Blood 91: 2991-2997.

Mercurio, F., Manning, A.M. 1999. Multiple signals converging on NF-κB. Curr Opin Cell Biol 11:226-32.

Message, S. D., and Johnston, S. L. 2004. Host defense function of the airway epithelium in health and disease: clinical background J Leukoc Biol 75:5-17.

Micheau, O., Hammann, A., Solary, E., Dimanche-Boitrel, M.T. 1999a. STAT-1-independent upregulation of FADD and procaspase-3 and -8 in cancer cells treated with cytotoxic drugs. Biochem Biophys Res Commun 256(3):603-7.

Micheau, O., Solary, E., Hammann, A., Dimanche-Boitrel, M.T. 1999b. Fas ligand-independent, FADD-mediated activation of the Fas death pathway by anticancer drugs.J Biol Chem 274(12):7987-92.

Middleton, G., Nunez, G., Davies, A.M. 1996. Bax promotes neuronal survival and antagonises the survival effects of neurotrophic factors. Development 122:695-701.

Midis, G. P., Shen, Y. & Owen-Schaub, L. B. 1996. Elevated soluble Fas (sFas) levels in nonhematopoietic human malignancy. Cancer Res 56:3870-3874.

Migliazza, A. 1994. Heterogeneous chromosomal aberrations generate 3' truncations of the NFKB2/Lyt-10 gene in lymphoid malignancies. Blood 84:3850-3860.

Millauer, B., Shawver, L.K., Plate, K.H., Risau, W., and Ullrich, A. 1994. Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. Nature 367-576-579.

Miyashita, T. & Reed, J. C. 1992. BCL-2 gene transfer increases relative resistance of S49.1 and WEHI7.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. Cancer Res. 52:5407-5411.

Modrak, D.E., Rodriguez, M.D., Goldenberg, D.M., Lew, W., Blumenthal, R.D. 2002. Sphingomyelin enhances chemotherapy efficacy and increases apoptosis in human colonic xenografts. Int J Oncol 20:379-84.

Molenaar, J. J. 1998. Microsatellite instability and frameshift mutations in BAX and transforming growth factor-Î² RII genes are very uncommon in acute lymphoblastic leukemia in vivo but not in cell lines. Blood 92: 230-233.

Moller, P. 1994. Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. Int J Cancer 57:371-377.

Moses, H.L., Yang, E.Y., and Pietenpol, J.A. 1990. TGF-b stimulation and inhibition of cell proliferation: new mechanistic insights. Cell 63:245-247.

Mueller, C. M. & Scott, D. W. 2000. Distinct molecular mechanisms of Fas resistance in murine B lymphoma cells. J Immunol 165:1854-1862.

Mukhopadhyay, A., Manna, S.K., Aggarwal, B.B. 2000. Pervanadate-induced nuclear factor-kappaB activation requires tyrosine phosphorylation and degradation of IkappaBalpha.Comparison with tumor necrosis factor-alpha. J Biol Chem 275:8549-8555

Muller, M., Strand, S., Hug, H., Heinemann, E.M., Walczak, H., Hofmann, W.J., Stremmel, W., Krammer, P.H., Galle, P.R. 1997. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. J Clin Invest 99(3):403-13.

Muller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber, M., Friedman, S.L., Galle, P.R., Stremmel, W., Oren, M., Krammer, P.H. 1998. p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. J Exp Med 188(11):2033-45.

Murdoch, C. 2000. CXCR4 : chemokine receptor extraordinaire. Immunol Rev 177:175-184.

Murphy, P.M. 1994. The molecular biology of leukocyte chemoattractant receptors. Annu Rev Immunol 12:593-633.

Murphy, P.M., and Tiffany, H.L. 1991. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. Science 253:1280-1283.

Nagane, M., Levitzki, A., Gazit, A., Cavenee, W.K., Huang, H.J. 1998. Drug resistance of human glioblastoma cells conferred by a tumorspecific mutant epidermal growth factor receptor through modulation of Bcl-XL and caspase-3-like proteases. Proc Natl Acad Sci U S A 95:5724-5729.

Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, J. R. J. & Sledge, J. G. W. 1997. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Mol Cell Biol 17:3629-3639.

Natarajan, K., Singh, S., Burke, T.R., Grunberger, D. Jr, Aggarwal. B.B. 1996. Caffeic acid phenethyl ester (CAPE) is a potent and specific inhibitor of activation of nuclear transcription factor NF-κB. Proc Natl Acad Sci USA 93:9090-5.

Neri, A. 1991. B-cell lymphoma-associated chromosomal translocation involves candidate oncogene Lyt-10, homologous to NF-κB p50. Cell 67:1075-1087.

Neri, A. 1995. Molecular analysis of cutaneous B- and T-cell lymphomas. Blood 86:3160-3172.

Ng, S.S, Tsao, M.S., Nicklee, T., Hedley, D.W. 2001. Wortmannin inhibits pkb/akt phosphorylation and promotes gemcitabine antitumor activity in orthotopic human pancreatic cancer xenografts in immunodeficient mice. Clin Cancer Res 7:3269-3275.

Nguyen, D.M., Chen. G.A., Reddy, R., Tsai, W., Schrump, W.D., Cole, G .Jr. 2004. Potentiation of paclitaxel cytotoxicity in lung and esophageal cancer cells by pharmacologic inhibition of the phosphoinositide 3-kinase/protein kinase B (Akt)-mediated signaling pathway. J Thorac Cardiovasc Surg 127:365-375.

Nowell, P.C. 1976. The clonal evolution of tumor cell populations. Science 194:23-28.

Olayioye, M.A., Neve, R.M., Lane, H.A., Hynes, N.E. 2000. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 19:3159-3167.

Pahl, H.L. 1999. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 18:6853-6866.

Pai, S. I. 1998. Rare loss-of-function mutation of a death receptor gene in head and neck cancer. Cancer Res 58:3513-3518.

Pathak, S., Multani, A.S., Narayan. S., Kumar, V., Newman, R.A. 2000. Anvirzel, an extract of Nerium oleander, induces cell death in human but not murine cancer cells. Anticancer Drugs 11:455-63.

Paulson, T.G., Wright, F.A., Parker, B.A., Russack, V., Wahl, G.M. 1996. Microsatellite instability correlates with reduced survival and poor disease prognosis in breast cancer. Cancer Res 56:4021-4026.

Pegram, M.D., Finn, R.S., Arzoo, K., Beryt, M., Pietras, R.J., Slamon, D.J. 1997. The effect of HER-2/neu overexpression on chemotherapeutic drug sensitivity in human breast and ovarian cancer cells. Oncogene 15:537-547.

Perkins, N. D. 2000. The Rel/NF-κB family: friend and foe. Trends Biochem Sci 25:434-440.

Peters, G.J., Kohne, C.H. Fluoropyrimidines as antifolate drugs. In Antifolate Drugs in Cancer Therapy, Jackman AL (ed). Humana Press: Totowa, NJ, 1999.

Pianetti, S., Arsura, M., Romieu-Mourez, R., Coffey, R.J., Sonenshein, G.E. 2001. Her-2/neu overexpression induces NfkappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN. Oncogene 20:1287-1299.

Pillinger, M. H., and Abramson, S. B. 1995. The neutrophil in rheumatoid arthritis Rheum Dis Clin North Am 21:691-714.

Pitti, R. M. 1998. Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. Nature 396:699-703.

Pluen, A. 2001. Role of tumor-host interactions in interstitial diffusion of macromolecules: cranial vs. subcutaneous tumors. Proc Natl Acad Sci USA 98:4628-4633.

Purcell, N.H., Yu, C., He, D., Xiang, J., Paran, N., DiDonato, J.A., Yamaoka, S., Shaul, Y., Lin, A. 2001. Activation of NF-kappaB by hepatitis B virus X protein through an IkappaB kinaseindependent mechanism. Am J Physiol Gastrointest Liver Physiol 280:G669-G677.

Rampino, N. 1997. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science 275:967-969.

Rathmell, J. C. & Thompson, C. B. 1999. The central effectors of cell death in the immune system. Annu Rev Immunol 17:781-828.

Ray, E., and Samanta, A. K. 1997. Receptor-mediated endocytosis of IL-8: a fluorescent microscopic evidence and implication of the process in ligand-induced biological response in human neutrophils Cytokine 9:587-596.

Reardon, J.T., Vaisman, A., Chaney, S.G., Sancar, A. 1999. Efficient nucleotide excision repair of cisplatin, oxaliplatin, and bisaceto-ammine-dichloro-cyclohexylamine-platinum(IV) (JM216) platinum intrastrand DNA diadducts. Cancer Res 59:3968-3971.

Reed, J.C. 2000. Mechanisms of apoptosis. Am J Path 157:1415-1430

Reed, J. C. 2001. The Survivin saga goes in vivo. J Clin Invest 108:965-969.

Reed, J. C., Cuddy, M., Slabiak, T., Croce, C. M. & Nowell, P. C. 1988. Oncogenic potential of BCL-2 demonstrated by gene transfer. Nature 336:259-261.

Renan, M.J. 1993. How many mutations are required for tumorigenesis? Implications from human cancer data. Mol. Carcinogenesis 7:139-146.

Ricci, A., Felici, L., Mariotta, S., Mannino, F., Schmid, G., Terzano, C., Cardillo, G., Amenta, F., and Bronzetti, E. 2004. Neurotrophin and neurotrophin receptor expression in the human lung. Am J Respir Cell Mol Biol 30:12-20.

Roth, W. 2001. Soluble decoy receptor 3 is expressed by malignant gliomas and suppresses CD95 ligand-induced apoptosis and chemotaxis. Cancer Res 61:2759-2765.

Salvesen, G.S. and Dixit, V.M. 1999. Caspase activation: the induced-proximity model. Proc Natl Acad Sci USA 96:10964-10967.

Salvesen, G.S., Dixit, V.M. 1997. Caspases: intracellular signaling by proteolysis. Cell 91:443-446

Samanta, A. K., Oppenheim, J. J., and Matsushima, K. 1990. Interleukin 8 (monocyte-derived neutrophil chemotactic factor) dynamically regulates its own receptor expression on human neutrophils. J Biol Chem 265:183-189.

Sarkar, A, Sreenivasan, Y., Ramesh, G. T., and Manna S. K. 2004. beta-D-Glucoside suppresses tumor necrosis factor-induced activation of nuclear transcription factor kappaB but potentiates apoptosis. J Biol Chem 279:33768-33781.

Sato, H., Seiki, M. 1993. Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. Oncogene 8:395-405.

Savill, J., Fadok, V. 2000. Corpse clearance defines the meaning of cell death. Nature 407:784-788.

Scaffidi, C. 1998. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 17:1675-1687.

Schmitt, C. A., Rosenthal, C. T. & Lowe, S. W. 2000. Genetic analysis of chemoresistance in primary murine lymphomas. Nature Med 6:1029-1035.

Schwartz, P.M., Moir, R.D., Hyde, C.M., Turek, P.J., Handschumacher, R.E. 1985. Role of uridine phosphorylase in the anabolism of 5-fluorouracil. Biochem Pharmacol 34: 3585-3589.

Senftleben, U., Karin, M. 2002. The IKK/NF-kappaB pathway. Crit Care Med 30:S18-S26.

Sen, R., and Baltimore, D. 1986. Inducibility of kappa immunoglobulin enhancer-binding protein NF-kappa B by a posttranslational mechanism Cell 47:921-928.

Shay, J.W., and Bacchetti, S. 1997. A survey of telomerase activity in human cancer. Eur J Cancer 33:787-791.

Shin, M. S. 2001. Mutations of tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2) genes in metastatic breast cancers. Cancer Res 61:4942-4946.

Shi, Q., Abbruzzese, J. L., Huang, S., Fidler, I. J., Xiong, Q., and Xie, K. 1999. Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic. Clin. Cancer Res 5:3711-3721.

Shiratori, O. 1967. Growth inhibitory effect of cardiac glycosides and aglycones on neoplastic cells: in vitro and in vivo studies. Gann 58(6):521-8.

Singh, R. K., and Varney, M. L. 2000. IL-8 expression in malignant melanoma: implications in growth and metastasis Histol Histopathol 15:843-849.

Singh, S., Aggarwal, B.B. 1995. Activation of transcription factor NF- κB is suppressed by curcumin (Diferulolylmethane). J Biol Chem 270:24995-5000.

Singh, S, Darnay, B.G., Aggarwal, B.B. 1996. Site-specific tyrosine phosphorylation of IkappaBalpha negatively regulates its inducible phosphorylation and degradation. J Biol Chem 271:31049-31054.

Sizemore, N., Leung, S., Stark, G.R. 1999. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-κB p65/RelA subunit. Mol Cell Biol 19:4798-805.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177-182.

Smith, J.A., Madden, T., Vijjeswarapu, M., Newman, R.A. 2001. Inhibition of export of fibroblast growth factor-2 (FGF-2) from the prostate cancer cell lines PC3 and DU145 by Anvirzel and its cardiac glycoside component, oleandrin. Biochem Pharmacol 62:469-72.

Smith, L.M., Wise, S.C., Hendricks, D.T., Sabichi, A.L., Bos, T., Reddy, P., Brown, P.H., Birrer, M.J. 1999. c-Jun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype. Oncogene 18:6063-70.

Soengas, M. S. 2001. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature 409:207-211.

Song, H. Y., Rothe, M. & Goeddel, D. V. 1996. The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF-κB activation. Proc Natl Acad Sci USA 93:6721-6725.

Song, Q., Kuang, Y., Dixit, V.M., Vincenz, C. 1999. Boo, a novel negative regulator of cell death, interacts with Apaf-1. EMBO J 18:167-178.

Sovak, M. A. 1997. Aberrant NF-κB/Rel expression and the pathogenesis of breast cancer. J Clin Invest 100:2952-2960.

Sparmann, A., Bar-Sagi, D. 2004. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. Cancer Cell. 6(5):447-458.

Sporn, M.B. 1996. The war on cancer. Lancet 347:1377-1381.

Sreenivasan, Y., Sarkar, A., and Manna, S. K. 2003. Oleandrin suppresses activation of nuclear transcription factor-kappa B and activator protein-1 and potentiates apoptosis induced by ceramide. Biochem Pharmacol 66:2223-2239.

Stenkvist, B. 1999. Is digitalis a therapy for breast carcinoma? Oncol Rep 6(3):493-6.

Strand, S. 1996. Lymphocyte apoptosis induced by CD95(APO-1/Fas) ligand-expressing tumor cells: a mechanism of immune evasion? Nature Med 2:1361-1366.

Strasser, A., Harris, A.W., Bath, M.L., and Cory, S. 1990. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature 348: 331-333.

Strum, J.C., Small, G.W., Pauig, S.B., Daniel, L.W. 1994. 1-b-D-arabinofuranosylcytosine stimulates ceramide and diglyceride formation in HL-60 cells. J Biol Chem 269:15493-15500.

Surh, Y.J., Chun, K.S., Cha, H.H., Han, S.S., Keum, Y.S., Park, K.K., Lee, S.S. 2001. Molecular mechanisms underlying chemo-preventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. Mutat Res 480-481:243-268.

Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S.,Jacks, T., Van Dyke, T. 1994. p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell 78:703-711.

Takebe, N., Zhao, S.C., Ural, A.U., Johnson, M.R., Banerjee, D., Diasio, R.B . 2001. Retroviral transduction of human dihydropyrimidine dehydrogenase cDNA confers resistance to 5-fluorouracil in murine hematopoietic progenitor cells and human CD34+ enriched peripheral blood progenitor cells. Cancer Gene Ther 8:966-973.

Tam, W. F., Wang, W., Sen, R. 2001. Cell-specific association and shuttling of IκBα provides a mechanism for nuclear NF-κB in B lymphocytes. Mol Cell Biol 21:4837-4846.

Teitz, T. 2000. Caspase-8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nature Med 6:529-535.

Thomas, H., Coley, H.M. 2003. Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting pglycoprotein. Cancer Control 10:159-165.

Thornberry, N., Lazebnik, Y. 1998. Caspases: enemies within. Science 281:1312-1316.

Tsujimoto, Y., Cossman, J., Jaffe, E., Croce, C. M. 1985. Involvement of the BCL-2 gene in human follicular lymphoma. Science 228:1440-1443.

Ugurel, S., Rappl, G., Tilgen, W. & Reinhold, U. 2001. Increased soluble CD95 (sFas/CD95) serum level correlates with poor prognosis in melanoma patients. Clin Cancer Res 7:1282-1286.

Varner, J.A., Cheresh, D.A. 1996. Integrins and cancer. Curr Opin Cell Biol 8:724-730.

Vaux, D. L., Cory, S. & Adams, J. M. 1988. BCL-2 gene promotes haemopoietic cell survival and cooperates with c-MYC to immortalize pre-B cells. Nature 335:440-442.

Vogt, P. K. 2001. Jun, the oncoprotein. Oncogene 20:2365-2377.

Volkmann, M. 2001. Loss of CD95 expression is linked to most but not all p53 mutants in European hepatocellular carcinoma. J Mol Med 79:594-600.

VonKnethen, A., Callsen, D., Brune, B. 1997. Superoxide attenuates macrophage apoptosis by NF-κB and AP-1 activation that promotes cyclooxygenase-2 expression. J Immunol 163:2858-66.

Waddick, K.G., Uckun, F.M. 1999. Innovative treatment programs against cancer. II. Nuclear factor-kappa B (NF-κB) as a molecular target. Biochem Pharmacol 57:9-17.

Walczak, H., Krammer, P.H. 2000. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res 256(1):58-66.

Wang, C.Y., Guttridge, D.C., Mayo, M.W., Baldwin, A.S. Jr. 1999. NF- κB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Mol Cell Biol 19:5923-9.

Wang, Y., Zhao, R., Goldman, I.D. 2003. Decreased expression of the reduced folate carrier and folylpolyglutamate synthetase is the basis for acquired resistance to the pemetrexed antifolate (LY231514) in an L1210 murine leukemia cell line. Biochem Pharmacol 65:1163-1170.

Wattenberg, L. W. 1990. In Antimutagenesis and Anticarcinogenesis, Mechanism II. Kuroda Y, Shankel DM, Waters MD, eds. Plenum Publishing Corp. New York.

Wattenberg, L.W. Inhibition of carcinogenesis by naturally occurring and synthetic compounds. In Antimutagenesis and Anticarcinogenesis, Mechanism II. Kuroda Y, Shankel DM, Waters MD, eds. Plenum Publishing Corp. New York, 1990.

Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. Cell 81:323-330.

Weller, M., Malipiero, U., Aguzzi, A., Reed, J. C. & Fontana, A. 1995. Protooncogene bcl-2 gene transfer abrogates Fas/APO-1 antibody-mediated apoptosis of human malignant glioma cells and confers resistance to chemotherapeutic drugs and therapeutic irradiation. J Clin Invest 95:2633-2643.

Wilkinson, K.D. 2003. Signal transduction: aspirin, ubiquitin and cancer. Nature 424: 738-739.

Wright, W.E., Pereira-Smith, O.M., and Shay, J.W. 1989. Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. Mol Cell Biol 9:3088-3092.

Wunsch, H. 1998. COX provides missing link in mechanism of aspirin in colon cancer. Lancet 351:1864.

Wyllie, A.H., Kerr, J.F., and Currie, A.R. 1980. Cell death: the significance of apoptosis. Int Rev Cytol 68:251-306.

Xiao, G. 2001. Retroviral oncoprotein Tax induces processing of NF-κB2/p100 in T cells: evidence for the involvement of IKKα. Oncogene 20:6805-6815.

Xu, Y., Villalona-Calero, M.A. 2002. Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. Ann Oncol 13:1841-1851.

Yamada, K.M., Araki, M. 2001. Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. J Cell Sci 114:2375-2382.

Yang, D., Chertov, O., and Oppenheim, J. J. 2001. Participation of mammalian defensins and cathelicidins in anti-microbial immunity: receptors and activities of human defensins and cathelicidin (LL-37). J Leukoc Biol 69:691-696.

Yarden, Y., and Ullrich, A. 1988. EGF and erbB2 receptor overexpression in human tumors. Growth factor recepor tyrosine kinases. Annu Rev Biochem 57:443-478.

Yatsunami, J., Tsuruta, N., Ogata, K., Wakamatsu, K., Takayama, K., Kawasaki, M., Nakanishi, Y., Hara, N., Hayashi, S.I. 1997. Interleukin-8 participates in angiogenesis in non-small cell, but not small cell carcinoma of the lung. J Leukoc Biol 69:661-668.

Yuan, A, Chen, J.J.,Yao, P.L., and Yang, P.C. 2005. The role of interleukin-8 in cancer cells and microenvironment interaction. Front Biosci 10:853-865.

Yuan, Z.M., Huang, Y., Whang, Y., Sawyers, C., Weichselbaum, R., Kharbanda, S., Kufe, D. 1996. Role for c-Abl tyrosine kinase in growth arrest response to DNA damage. Nature 382:272-274.

Yu, K. Y. 1999. A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. J Biol Chem 274:13733-13736.

Zamzami, N. & Kroemer, G. 2001. The mitochondrion in apoptosis: How Pandora box opens. Nature Rev Mol Cell Biol 2:67-71.

Zandi, E., Rothwarf, D.M., Delhase, M, Hayakawa, M., Karin, M. 1997. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell 91:243-252.

Zhang, J., Alter, N., Reed, J.C., Borner, C., Obeid, L.M., Hannun, Y.A. 1996. Bcl-2 interrupts the ceramide-mediated pathway of cell death. Proc Natl Acad Sci USA 93:5325-5332.