CHAPTER 1

Introduction & Review of Literature
Introduction

Nitric oxide (NO) is a highly reactive and diffusible gas molecule that exerts many biological effects which includes smooth muscle relaxation, iron homeostasis, platelet reactivity, neurotransmission and cytotoxic defense mechanism against pathogens. NO is also involved in the pathogenesis of many human pathological conditions, such as inflammatory disease, neurodegenerative disorders and cancer (Mocellin et al., 2007).

Mammalian cells generate NO from the amino acid L-arginine and this conversion is catalysed by nitric oxide synthases (NOS). There are three main isoforms of the NOS enzyme, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Coulter et al., 2008). The half life of NO is of only few seconds and upon contact with haemoglobin it rapidly gets inactivated (Valance, 2003).

NO alone does not interact with proteins or nucleic acids, it can combine with local electron-accepting species to form reactive NO species (RNOS). Further, these RNOS can cause tissue injury and may lead to cell death (Kim et al., 2001). NO can modify proteins through direct chemical reactions, rather than using enzymatic mechanisms. NO has the ability to oxidize nitrate or nitrosylate proteins (Mannick and Schonhoff, 2002). One of the best studied actions of NO is in cardiovascular system where it plays important role in the mechanisms of cardiovascular regulation such as vasodilation, inhibition of platelet adhesion and aggregation.

Many evidences are accumulating which points towards the function of NO as an onco-preventive agent as well as a novel therapeutic to overcome tumor cell resistance (Bonavida et al., 2006). Interestingly, NO can play both pro and antiapoptotic function and these effects are dose dependent and cell type specific. NO induce apoptosis in a variety of cell types by various mechanisms such as p53 accumulation, poly (ADP ribose) polymerase (PARP) activation and Bcl-2 down-regulation (Umansky et al., 2001; Li and Wogan, 2005). A series of new compounds have been also synthesized in which a NO-releasing group is linked to parent molecules. This strategy employed the identification of novel molecules with an improved profile of pharmacological activity either in terms of enhanced therapeutic efficacy or reduced side effects (Keeble and Moore, 2002).

NO is also known to inhibit nuclear transcription factor-κB (NF-κB), which has been implicated in carcinogenesis because of its critical roles in cell survival, cell adhesion, inflammation, differentiation and cell growth. Under normal condition, NF-κB is present in
the cytoplasm as an inactive heterotrimer consisting of p50, p65 and inhibitor of κB (IκBα) subunit. Stimulation of cells lead to phosphorylation of IκBα at Ser-32 and Ser-36, ubiquitination at Lys-21 and Lys-22 and degradation of IκBα by 26S proteasome (Aggarwal, 2000; Mayo and Baldwin, 2000). Several gene products viz. Bcl-xl, Bcl-2, c-FLIP, IAPs, XIAP, Bfl/A1 that negatively regulate apoptosis in tumor cells are controlled by NF-κB activation (Sethi et al., 2008). Constitutive activation of NF-κB has been reported in many human malignancies viz. pancreatic cancer, colon cancer (Wang et al., 1999), breast cancer (Nakshatri et al., 1997), T-cell leukemia (Mori et al., 1999) and lymphoma (Pham et al., 2003). NO can inhibit NF-κB in both constitutively active and inducible NF-κB system, however the mechanism of NO mediated inhibition of NF-κB differs from cell type to cell type. NO can inhibit NF-κB activity by the inhibition of phosphorylation and degradation of IκBα or by inhibiting DNA binding activity of NF-κB (Katsuyama et al., 1998; Matthews et al., 1996; Marshall et al., 2004). It has also been shown that NO inhibits NF-κB in human vascular endothelial cells by induction and nuclear translocation of IκBα (Spiecker et al., 1997).

One of the cancer in which NF-κB is constitutively active is cutaneous T cell lymphoma (CTCL), which is a type of cancer of the immune system resulting from clonal proliferation of neoplastic T lymphocytes. Global or country (India)- specific data are not available, but, according to literature, approximately 1000 new cases of CTCL are definitively diagnosed each year (Panda, 2007). The most frequent forms of CTCL are mycosis fungoides (MF) and its leukemic counter part, the Sézary syndrome (SS). MF proceeds mostly very slowly (up to 5-10 years) but leads ultimately to death, which is often caused in the late phase by rapidly growing and ulcerating tumors and immune disorders. Patients with SS besides having generalized erythroderma also have leukaemic T cells in the blood and their life expectancy is generally shorter (3 years) than that of patients suffering from MF (Zhang et al., 2003). It has been demonstrated that CTCL cell lines, HuT-78, MyLa and SeAx as well as peripheral blood lymphocytes derived from patients with SS express NF-κB constitutively (Sors et al., 2006; Giri and Aggarwal, 1998) and this constitutive NF-κB plays a crucial role in cell survival and apoptosis resistance in CTCL cells (Izban et al., 2000). Recently, it has been shown that arsenic trioxide and proteasome inhibitors viz. N-acetyl-L-leucyl- L-leucyl- L-norleucinal (ALLN), Z-Leu-Leu-Leu-al (MG132), bortezomib, (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile (BAY 11-7082)
and (E)-3-(4-t-butylphenylsulfonyl)-2-propenenitrile (BAY 11-7085) overcome the resistance of CTCL cells to apoptosis by inhibiting NF-κB activity (Tun-Kyi et al., 2008; Sors et al., 2006; Izban et al., 2000). However, the NF-κB inhibiting potential of NO has not been explored in case of CTCL cells.

HuT-78, a CTCL cell line constitutively expresses NF-κB and tumor necrosis factor alpha (TNF-α). TNF-α, a pleotropic cytokine acts as an autocrine growth factor for HuT-78 cells, since proliferation of HuT-78 cells can be inhibited by lowering TNF-α level using anti-TNF antibody (O'Connell et al., 1995; Giri and Aggarwal, 1998). With this knowledge, in the present study we first time attempted to target constitutive NF-κB of CTCL cells by nitric oxide generating compound, sodium nitroprusside. In addition the effect of anticancer agent, pentoxifylline which is known to inhibit NF-κB as well as TNF-α is also being studied on CTCL cells.

Review of Literature

Cancer means 'crab' in Latin, which was coined by Hippocrates to describe a disease in which particular tissues grow and spread unrestrained throughout the life eventually choking off life. The death toll from infectious disease is decreasing and giving way to non communicable diseases. Cancer is the second most common cause of death in the USA, led only by heart disease. In 2000, there were 10 million new cancer cases, 6 million deaths attributed to cancer and 22 million people living with cancer. In 2020 there are predicted to be 15 million new cases and 10 million deaths due to cancer (Parkin, 2001). In 2007, cancer accounted 7.9 million deaths worldwide. About 80% of all cancer deaths in 2007 occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continue rising with an estimated 9 million deaths in 2015 and 11.4 million deaths in 2030 from cancer (http://www.who.int/cancer/en/). Every year about 850,000 new cancer cases are diagnosed in India resulting in about 580,000 cancer related death every year (http://cancerindia.net/cancerstatistics).

1.1. Cancer
In most organs and tissues, a balance is maintained between cell renewal and cell death. The various types of mature cells in the body have a given life span as these cells die, new cells are generated by the proliferation and differentiation of various types of stem cells. Under normal circumstances, the production of new cells is tightly regulated. In contrast, certain cells violate this scheme that no longer respond to normal growth control mechanisms but follow their own internal agenda for reproduction. These cells give rise to clones of cells that can expand to a considerable size producing lump or mass of cells, referred as tumor or neoplasm (Weinberg, 1996).

Cancer in humans doesn’t develop overnight, rather it is a multistep process and these steps reflect genetic alterations that progress the transformation of normal human cells into highly malignant derivatives (Fig. 1.1.). It is now well established that most cancers may indeed be genetically unstable and this instability exists at two distinct levels. In a small subset of tumors, the instability is observed at the nucleotide level and results in base substitutions, deletions or insertions of a few nucleotides, while in most others, the instability is observed at the chromosome level, resulting in losses or gains of whole or large portions of chromosomes (Dahle and Kvam, 2003).

1.1.1. Classification of cancer

In terms of behaviour, tumors can be classified into two categories:
1. **Benign tumor**: These are generally slow-growing expansive masses that do not invade surrounding tissues and pose little threat to life.
2. **Malignant tumor**: These are rapidly growing tumors that invade surrounding tissues, get into circulatory system and set up area of proliferation away from site of their original appearance. In this way a primary tumor at one site can give rise to a secondary tumor at another site, a process known as metastasis. It becomes life threatening as they spread throughout body.

Cancer is generally classified according to the tissue from which the cancerous cells originate (Goldsby et al., 2003).
1. **Carcinoma**: These are most commonly encountered type of cancer involving more than 80% of cancers. It is a cancer of tissues of ectodermal and mesodermal origin, arising from epithelial cells that cover external and internal body surfaces. The majority of cancers of the colon, breast, prostate, and lung are carcinomas.
Fig. 1.1. Overview of transformation of normal cell into cancerous cell
2. **Sarcoma**: It is a cancer of tissues of mesodermal origin involving bone, fat and cartilage. This type of cancer arises less frequently.

3. **Lymphoma and leukemia**: This type of cancer arises from the cells of blood and lymphatic origin. Lymphoma term is used when cancer cells are restricted to solid tumor masses (benign) while leukemia corresponds to cancer cells circulating in blood stream (malignant).

   The vast majority of mutations in cancer is somatic and is found only in an individual’s cancer cells. However, about 1% of all cancers are hereditary. The individual with this type of cancer carry a particular germline mutation in every cell of their body (Fearon, 1997).

**1.1.2. Alterations in a cancer cell**

A cancer cell genotype is manifested by six essential alterations in cell physiology that collectively command malignant growth (Fig. 1.2.). These alterations includes: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. These six capabilities are acquired by most if not all cancers (Hanahan and Weinberg, 2000).

**1.1.3. Agents of cancer**

Agents causing cancer fall into three broad groups: physical, chemical compounds and biological agent (certain viruses). Various physical agents (viz. ultraviolet radiation and ionizing radiation) and chemical agents (viz. DNA-alkylating agents) are mutagenic and carcinogenic. Induction of cancer by these agents involves multiple steps and atleast two distinct phases: initiation and promotion. Initiation involves irreversible changes in the genome but cannot elicit malignant transformation on its own. After initiation, promoters stimulate cell division and lead to malignant transformation. Certain viruses (RNA as well as DNA viruses) known as tumor viruses can cause transformation as a consequence of their ability to integrate their genetic information into the host chromosomal DNA. This transformation by viruses is related to the presence of oncogenes in viral genome (Goldsby et al., 2003).
Fig. 1.2. Acquired capabilities of cancer

Adapted from Hanahan and Weinberg, 2000
1.2. Cutaneous Lymphoma

Cutaneous lymphoma (CL) is a class of non-Hodgkin lymphoma, which is heterogeneous group of neoplasia that is characterized by an accumulation of mononuclear, mostly lymphocytic cells in the skin (Dummer et al., 2007). The overall frequency of CL is approximately 1 per 100,000 inhabitants per year (Willemze et al., 2005). The early stages of CL involve occurrence of persistent symptoms such as pruritus (itching) thus affecting the quality of life whereas in addition to skin problem the advanced stage is accompanied by systemic deviations of the immune response thus resulting in increased risk of infections and secondary malignancies (Dummer et al., 2007).

CL can be subdivided into pseudolymphomas (PSLs), abortive (semi malignant) lymphoma and malignant lymphoma. Cutaneous PSLs is reactive benign lymphoproliferative process, which regresses completely and permanently if causative factor is removed. Abortive lymphoma is confined to skin and does not show systemic spread, however it can not be cured but it never kills the patient. On the other hand malignant lymphoma includes lymphoproliferative diseases with a progressive course. Progression leads to the involvement of extracutaneous compartments and is fatal in most cases (Burg et al., 1997).

Recently both World Health Organization (WHO) and European Organization for Research and Treatment of Cancer (EORTC) have developed a new WHO-EORTC classification which takes into account different clinical behavior of primary cutaneous lymphomas as well as their distinct histological, phenotypical and molecular features. The new consensus WHO-EORTC classification has been validated by data of 1905 patients with primary cutaneous lymphomas derived from Dutch and Austrian registries. According to new WHO-EORTC classification, cutaneous lymphomas are categorized into three groups namely, Cutaneous T-cell and NK-cell lymphomas, Cutaneous B-cell lymphomas and Precursor hematologic neoplasm (Willemze et al., 2005). Among cutaneous lymphoma, T cells types constitute 65% whereas B cells types constitute only 25% (Burg et al., 1997).

1.3. Cutaneous T cell Lymphoma

In 1975, Edelson and Lutzner first coined the term cutaneous T-cell lymphoma (CTCL) (Lutzner et al., 1975). CTCL is a group of lymphoproliferative disorders of skin
resulting from clonal expansion of T cells. Approximately 1,000 to 1,500 new cases are reported each year in the United States (Mann et al., 2007). The most frequent forms of CTCL are Mycosis Fungoides (MF) and its leukemic counterpart, the Sézary syndrome (Zhang et al., 2003).

Mycosis Fungoides was first described in 1806 by French dermatologist Jean-Louis-Marc Alibert. The name *mycosis fungoides* is somewhat misleading— it loosely means "mushroom-like fungal disease". The disease, however, is not a fungal infection but rather a type of non-Hodgkin's lymphoma. It was so named because Alibert described the skin tumors of a severe case as having a mushroom-like appearance. MF is also known as Alibert-Bazin syndrome. MF proceeds slowly over years and is characterized by sequential appearance of patches, developing into plaques and finally into tumors, however in patients in tumor stage MF tumors tend to ulcerate (Fig. 1.3). MF typically affects older adults (median age at diagnosis: 55-60 years; male-to-female ratio: 1.6-2.0:1), but may occur in children and adolescents. Chromosomal loss at 10q and abnormalities in p15, p16 and p53 tumor suppressor genes are commonly found in patients with MF (Willemze et al., 2005).

The prognosis of patients with MF is dependent on stage and in particular the type and extent of skin lesions and the presence of extracutaneous disease. Patients with limited patch/plaque-stage MF have a similar life expectancy to an age, sex, and race matched control population. In recent studies, 10-year disease-specific survivals were 97%-98% for patients with limited patch/plaque disease (covering less than 10% of the skin surface), 83% for patients with generalized patch/plaque disease (covering more than 10% of the skin surface), 42% for patients with tumor stage disease, and about 20% for patients with histologically documented lymph node involvement (Willemze et al., 2005).

Sézary syndrome (SS) is a type of cutaneous lymphoma which was first described by Albert Sézary in 1938. SS is characterized by triad of erythroderma, generalized lymphadenopathy and presence of neoplastic T cells (Sézary cells) in skin, lymph nodes and peripheral blood. In SS, blood shows more than 1000/mm³ sézary cells count. SS occurs exclusively in adults (Willemze et al., 2005). Patients with SS also manifest prominent immunologic defects due to the production of T-helper 2 (Th2) cytokines and by the depressed production of Th1 cytokines (Contassot et al., 2008). The prognosis of SS is worse than in MF, showing approximately a 10% versus 60% to 70%, 5 year survival rate in SS and MF respectively (Willemze et al., 2005). Both MF and SS are characterized by
Fig. 1.3. A, Mycosis fungoides, patch stage: eczematous patchy lesion. 
B, Mycosis fungoides, plaque stage: palpable erythematous plaques. 
C, Mycosis fungoides, tumor stage: exophytic nodular tumors with ulceration.
clonal expansion of cells bearing CD3+, CD4+, CD45RO+ phenotype of memory T lymphocytes (Sors et al., 2006).

The prognosis is generally poor, with median survival between 2 and 4 years. The disease specific 5-year survival of 54 SS patients included in the Dutch and Austrian registries were 24%. Most patients die of opportunistic infections that are due to immunosuppression (Willemze et al., 2005).

1.3.1. Stage classification for patients with Mycosis Fungoides and Sézary syndrome.

Lamberg and Bunn first described the overall staging of MF/SS patients is based on the TNMB (tumor: T1-4, node: N0-3, metastasis: M0-1, blood: B0-2) classification system (Table 1), which takes into account the extent of skin involvement, the presence of lymph node or visceral disease, and the detection of Sézary cells in the peripheral blood (Lamberg and Bunn, 1979). Patients with stage IA disease (T1, N0, M0) have limited patch/plaque disease with less than 10% of body surface area involvement. Patients are usually asymptomatic and may remain at this stage for years. Patients with stage IB (T2, N0, M0) have patches and plaques affecting more than 10% of the body surface area. Stage IIA disease (T1-2, N1, M0) includes stage IA or IB disease plus the presence of lymphadenopathy. Stage IIB (T3, N0/1, M0) is associated with the development of skin tumors, which may arise de novo or in pre-existing patches or plaques with or without associated lymphadenopathy. Patients with erythroderma are classified as having stage III disease (IIIA: T4, N0, M0; IIIB: T4, N1, M0). Sézary syndrome is a distinct form of erythrodermic CTCL, characterized by exfoliative erythroderma, lymphadenopathy, lymphocytosis, intense pruritus and circulating Sézary cells. Enlarged nodes are found in about 47% of all patients and in 83% of erythrodermic patients. Involvement of nodes by tumor cells (N2, N3) without visceral organ involvement is classified as stage IVA (T1-4, N2-3, M0). Patients with extracutaneous manifestations and/or bone marrow involvement are classified as stage IVB (T1-4, N0-3, M1) (Rosen and Querfeld, 2006).

1.3.2. Treatment of CTCL

At present, no curative therapy for CTCL exists. Systemic single-agent or multi-agent chemotherapies are used to treat advanced and aggressive forms of CTCL to palliate
### Clinical staging system for Mycosis Fungoides and Sézary syndrome

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>T1, limited patch or plaque</td>
<td>N0, nodes uninvolved</td>
<td>M0, no visceral involvement</td>
</tr>
<tr>
<td>IB</td>
<td>T2, generalized patch or plaque</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIA</td>
<td>T1-2</td>
<td>N1, nodes enlarged, histologically uninvolved</td>
<td>M0</td>
</tr>
<tr>
<td>IIB</td>
<td>T3, tumors</td>
<td>N0-1</td>
<td>M0</td>
</tr>
<tr>
<td>IIIA</td>
<td>T4, erythroderma</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIIB</td>
<td>T4</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>IVA</td>
<td>T1-4</td>
<td>N3, nodes enlarged, histologically involved</td>
<td>M0</td>
</tr>
<tr>
<td>IVB</td>
<td>T1-4</td>
<td>N0-3</td>
<td>M1, visceral involvement</td>
</tr>
</tbody>
</table>

### B Classification

- **B0**, No circulating Sézary cells
- **B1**, Peripheral blood Sézary cells (PBSC) > 20%, <1000/mm³ by morphologic traits
- **B2**, Sézary syndrome defined as ≥1 of the following:
  - PBSC ≥1000/mm³, CD4/CD8 ratio ≥10,
  - CD4+CD7− cells ≥40% or CD4+CD26− cells ≥30% of lymphocytes

*Adapted from: Tsai et al., 2006*

**Table 1. Clinical staging system for Mycosis Fungoides and Sézary syndrome**
patients. Despite moderate response rates, however, no treatment has been shown to prolong disease-free or overall survival (Querfeld et al., 2006). Presently, only limited compounds are approved by Food and Drug Administration (FDA) for treatment of CTCL cells, which includes; extracorporeal photochemotherapy, Denileukin difftitox, Bexarotene, Vorinostat.

Extracorporeal photochemotherapy (ECP) or photopheresis is a form of chemotherapy which had received FDA approval in 1988 for treatment of SS. ECP involves removal of blood through a vein and isolation of white cells, which includes circulating CTCL cells. The removed cells are mixed with photoactivable drug, 8-methoxypsoralen, which sensitizes the cells to ultraviolet light. Thereafter, the cells are exposed to ultraviolet light to activate the drug and then returned to the patient through a vein. This process needs to be repeated multiple times to gain full effect (Senturk and Sahin, 2003).

Denileukin diftitox, a recombinant fusion protein is designed to direct cytotoxic action of diphtheria toxin to cells expressing interleukin-2 (IL-2) receptor, received accelerated approval in February 1999 for treatment of patients with persistent or recurrent CTCL whose malignant cells express the CD25 component of the IL-2 receptor. Denileukin diftitox binds to T cells receptor of CTCL and thereafter it kills the T lymphocyte (Mann et al., 2007).

Bexarotene, a synthetic rexinoid received approval in December 1999 in both oral capsule and topical gel formulations for the treatment of cutaneous manifestations of CTCL. Clinical trails have shown that bexarotene is safe and effective for the treatment of all stages of CTCL. In addition, topical bexarotene is also effective in clearing CTCL skin lesions (Zhang et al., 2002).

In addition, Vorinostat or suberoylanilide hydroxamic acid (SAHA) has also been approved by FDA in 2006 for treatment of CTCL cells. Vorinostat inhibits class 1 and class 2 histone deacetylase (HDACs). HDAC inhibitors have been shown to induce differentiation, cell-cycle arrest and apoptosis. It also inhibits migration, invasion, and angiogenesis in many cancer cell lines. In addition, these compounds inhibit tumor growth in animal models and show antitumor activity in patients (Martinez-Iglesias et al., 2008).

Studies have indicated that abnormal histone deacetylase activity may lead to aberrant expression of oncogenes and/or tumor suppressor genes, resulting in cancer. In many solid tumors HDACs is over expressed. It has been reported that HDACs activity is required for the transcriptional activity mediated by the signal transducer and transcription

9
activator (STAT) proteins in CTCL. Thus, inhibiting HDAC activity can prevent expression of STAT target genes and restore normal transcription of genes leading to differentiation and apoptosis (Dummer et al., 2007). It has been demonstrated that Vorinostat causes accumulation of hyperacetylated histones, inhibition of proliferation and induction of apoptosis in transformed cells. Vorinostat or suberoylanilide hydroxamic acid (SAHA) was the first HDAC inhibitor approved by the US FDA to enter the clinical oncology market for treating CTCL and is being tested for other malignancies (Martinez-Iglesias et al., 2008).

1.4. Apoptosis

The term apoptosis was first used in a now-classic paper by Kerr, Wyllie and Currie in 1972 to describe a morphologically distinct form of cell death (Kerr et al., 1972). Apoptosis come from the Greek word apo (away from, with the implication of separation) and the root ptosis (to fall). Apoptosis literally means to fall away from, as leaves fall away from a tree. The term ‘programmed cell death’ (PCD) adopted from developmental biology is now used as a synonym for apoptosis to appreciate genetic programs that regulate cell death (Brune et al., 1998). It is well established that animals have a built-in-suicide, or death program, which largely became established through genetic studies in Caenorhabditis elegans that identified genes that seem dedicated to the death program and its control, and then through the findings that some of these genes have mammalian homologs. In C. elegans, 131 of 1090 somatic cells formed during adult development undergo apoptosis. These cells die at particular points during developmental process. The most important clue to the molecular nature of the death program came initially from genetic studies in C. elegans that identified a gene called ced-3 that is required for the 131 programmed cell death that occur during the development of the worm. The gene encodes a cysteine protease that is homologous to interleukin-1β-converting enzyme (ICE), a mammalian cysteine protease that produces the proinflammatory cytokine IL-1β from its precursor protein. At least 11 members of the Ced-3/ICE family of proteases have now been identified in humans and a number of them have been implicated in PCD. All cleave their substrates after specific aspartic acids and are themselves activated by cleavage at specific aspartic acids. They are now therefore referred to as caspases. Moreover, the ced-9 gene, which acts to inhibit PCD in C. elegans, is homologous to the bcl-2 gene, which acts to inhibit PCD in
mammalian cells. The human bcl-2 gene is even able to inhibit PCD in the worm. A number of Ced-9/Bcl-2 family members have been identified in mammals. Some, such as Bcl-2 and Bcl-xl, inhibit PCD, whereas others, such as Bax and Bak, promote PCD (Brune et al., 1998; Jacobson et al., 1997; Hengartner and Horvitz, 1994; Yuan et al., 1993).

Apoptosis also plays an important role in cellular homeostasis in the immune system in the selection of T cell repertoire. An estimated 95-99% of all thymocytes progeny undergoes programmed cell death within in thymus without ever maturing. The high death rate probably results primarily from the elimination of thymocytes that cannot recognize foreign antigenic peptides displayed by self-MHC molecules and of thymocytes that recognize self peptides displayed by self-MHC molecules. Light and electron microscopy have identified the various morphological changes that occur during apoptosis. Apoptosis is associated with blebbing of plasma membrane, cell shrinkage, chromatin condensation, separation of cell fragments into apoptotic bodies, these bodies are subsequently phagocytosed by phagocytic cells, ensuring that their intracellular contents including proteolytic and other lytic enzymes are not released into the surrounding tissue. Macrophages that engulf and digest apoptotic cells are called “tangible body macrophages” (Goldsby et al., 2003).

Apoptosis differs markedly from the alternative cell death known as necrosis, which is considered to be a toxic process and follows an energy-independent mode of cell death. In necrosis, the injured cells swells and bursts, releasing its intracellular contents, which are cytotoxic to other cells into surroundings which leads to inflammatory response. On the other hand there is essentially no inflammatory reaction associated neither with apoptosis nor with the removal of apoptotic cells. Furthermore, necrosis is an uncontrolled and passive process that usually affects large fields of cells whereas apoptosis is controlled and energy dependent process which can affect individual or clusters of cells (Elmore, 2007).

Apoptosis is mediated by two major pathways, the extrinsic or death receptor-mediated pathway and the intrinsic pathway, which is mediated via mitochondria and the endoplasmic reticulum (Fig. 1.4). Although, at least at the beginning, the two pathways are apparently separate from each other, at the end they converge in a single crucial point, i.e. the conversion of procaspase into caspase, a protease whose activation is the biochemical event that has the strongest influence on the structural modifications of the apoptotic cell. The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interaction. It involves the binding of death ligands to cell surface receptor known
Fig. 1.4. **Intrinsic and extrinsic pathways of apoptosis.** Intrinsic pathway is triggered by stress inducing stimuli resulting in perturbation of mitochondria while extrinsic pathway is activated via death receptor resulting in rapid activation of the initiator caspase-8.
as death receptors, these receptors are members of tumor necrosis factor (TNF) receptor gene superfamily. The death domain of receptor plays an important role in transmitting the death signal from the cell surface to the intracellular signaling pathways. The intrinsic signaling pathway gets triggered following cellular stress. Cellular stress may be in the form of withdrawal of growth factors, exposure to radiations, chemicals. In intrinsic pathway, mitochondria release a series of molecules, including cytochrome c. In cytosol, the association of cytochrome c with the adaptor protein Apaf-1 and several procaspase-9 molecules gives rise to the formation of apoptosome, which is responsible for bringing several procaspase-9 molecules into close contact with one another in order to allow their self-processing. Caspase-9 is thus able to recruit and activate caspase-3, which is an effector caspase of both pathways (Elmore, 2007; Russo et al., 2006).

1.4.1. Apoptosis and cancer

In cancer, the balance between proliferation and programmed cell death is disturbed and defects in apoptotic pathways allow cells with genetic abnormalities to survive (Sjostrom and Bergh, 2001). The possibility that apoptosis serves as a barrier to cancer was first raised in 1972, when Kerr, Wyllie and Currie described massive apoptosis in hormone dependent tumors following hormone withdrawal (Kerr et al., 1972; Hanahan and Weinberg, 2000). Tumor cells can suppress apoptosis by a variety of mechanisms. Tumor cells can acquire resistance to apoptosis by the expression of anti apoptotic proteins such as Bcl-2 or by the downregulation or mutation of proapoptotic proteins such as Bax. The expression of both Bcl-2 and Bax is regulated by the p53 tumor suppressor gene. Certain forms of human B cell lymphoma have overexpression of Bcl-2, and this is one of the first and strongest lines of evidence that failure of cell death contributes to cancer (Elmore et al., 2007). It is reported that CTCL cells survive and resist apoptosis due to the constitutive activation of transcription factor, NF-κB (Sors et al., 2006). Most cytotoxic agents as well as radiation, ultimately kill cancer cells by causing irreparable cellular damage that triggers apoptosis. Consequently, the efficacy of cancer treatments depends not only on the cellular damage they cause but also on the cell's ability to respond to the damage by inducing apoptotic machinery (Sjostrom and Bergh, 2001).

1.5. Death receptor and its signaling
Apoptosis can be triggered by a number of factors, including UV or γ-irradiation, chemotherapeutic drugs or signaling by death receptors. Death receptors are cell surface receptors that belong to the TNF receptor gene superfamily. Binding of death ligands to death receptors results in the transduction of either apoptotic or survival signals (Lavrik et al., 2005). So far, eight human death receptors have been characterized, namely: tumor necrosis factor receptor 1 (TNFR1; also known as DR1, CD120a, p55 and p60), CD95 (also known as DR2, APO-1 and Fas), DR3 (also known as APO-3, LARD, TRAMP and WSL1), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-1; also known as DR4 and APO-2), TRAIL-2 (also known as DR5, KILLER and TRICK2), DR6, ectodysplasin A receptor (EDAR) and nerve growth factor receptor (NGFR). All of these death receptors are type I membrane proteins that contains two to four cysteine rich extracellular domains and cytoplasmic region of approximately 80 residues termed as death domain (DD). When death ligands binds to their corresponding death receptors, a number of molecules are recruited to the DD resulting in signaling cascade (Bhardwaj and Aggarwal, 2003; French and Tschopp, 2003). Death ligands also interact with decoy receptors (DcRs), which do not possess DDs, thus this binding does not result in signaling cascade. To date, four decoy receptors have been characterized: TRAILR3 (also known as DcR1), TRAILR3 (also known as DcR2), DcR3 and osteoprotegrin (OPG) (Lavrik et al., 2005).

1.5.1. Signaling by CD95 (Fas)

Among death receptors, signaling triggered by binding of FasL to Fas receptor is the best studied death receptor signaling, to date (Fig. 1.5.) (French and Tschopp, 2003). Binding of FasL to Fas induces trimerization of the Fas receptor, which results in recruitment of DD containing adaptor molecule Fas associated death domain (FADD; composed of an amino terminal death effector domain (DED) and a carboxyl terminal DD). FADD binds to Fas via homophilic DD-DD interaction and recruits the DED-containing procaspases-8 (also called FLICE or MACH) to the receptor via homophilic DED–DED interactions. Procaspase-8 within this newly formed, multi protein complex known as death-inducing signaling complex (DISC), then proteolytically autoactivate itself and initiate apoptosis by subsequent cleavage of downstream effector caspases (French and Tschopp, 2003; Peter and Krammer, 2003; Lavrik et al., 2005).
Fig. 1.5. Models for apoptosis signaling by Death Factors. Upon ligation with FasL, Fas undergoes trimerization and recruits various adaptor molecules resulting in apoptosis. TNF trimerizes TNFR1 upon binding and recruits various adaptor molecules viz. TRADD, RIP, TRAF2 leading to activation of NF-κB. FADD can couple with TNFR1-TRADD complex leading to apoptosis via activation of caspase-8.

Adapted from: Ashkenazi and Dixit. 1998
1.5.2. Signaling by TNFR1

Tumour necrosis factor (TNF) was discovered by Carswell in 1975 (Carswell et al., 1975). It is produced by a wide variety of cell types in response to various inflammatory stimuli. TNF is a pleiotropic cytokine that plays an important role in apoptosis, cell proliferation, immunomodulation, inflammation, viral replication, allergy, arthritis, septic shock, insulin resistance and autoimmune diseases. TNF is a homodimer with trimerizes TNFR1 upon binding. Subsequently an adapter protein, TNFR-associated death domain (TRADD) binds to death receptor via homophilic DD-DD interaction. TRADD recruits TNFR-associated factor-2 (TRAF-2) and receptor interacting protein (RIP). TRAF-2 and RIP activates NF-κB- inducing kinase (NIK), which in turn activates inhibitor of κB (IκB) kinase complex, IKK. IKK via phosphorlyation of IκB leads to activation of NF-κB (Ashkenazi and Dixit, 1998).

In contrast to Fas mediated death signaling, the TNFR1-induced proapoptotic signaling pathway requires the formation of two distinct signaling complexes. The rapidly formed plasma membrane bound complex I is composed of TNFR1, TRADD, RIP, TRAF2 and triggers a NF-κB response, but no apoptosis. A second complex, which includes FADD initiates apoptosis via activation of caspase-8 (Fig. 1.5.) (Micheau and Tschopp, 2003).

1.6. Bcl-2 family of proteins

The hallmark 14;18 chromosome translocation in human follicular B cell lymphoma was found to link the immunoglobulin heavy chain locus to a novel gene denoted bcl-2 (Tsujimoto et al., 1984). The protein Bcl-2 is the founding member of a rapidly expanding family of pro and antiapoptotic molecules. It has become evident that apoptosis is tightly regulated by Bcl-2 family gene products, some of them suppress apoptosis and others potentiate apoptosis (Lincz, 1998).

There are at least 20 Bcl-2-related proteins in mammalian cells (Fig. 1.6.). Bcl-2 family members can be categorized into three subfamilies on the basis of function and structure (Cory et al., 2003):

1. **Bcl-2 subfamily**: It represents a group of antiapoptotic channel forming proteins with four Bcl-2 homology (BH) domains (BH1-4). It includes members like: Bcl-2, Bcl-xl, Mcl-1, A1/Bfl-1.
Fig. 1.6. Three subfamilies of Bcl-2-related proteins. The Bcl-2 subfamily promotes cell survival, whereas the Bax and BH3 subfamilies promote apoptosis.

Adapted from: Cory et al., 2003
2. **Bax subfamily:** It includes a group of proapoptotic channel forming proteins with three BH domains (BH1-3). It includes trio: Bax, Bak and Bok.

3. **BH3 subfamily:** It represents proapoptotic ligands that contains only BH3 domain. It includes members like: Bik, Bad, Bid, Blk, BimL, BNIP3. These disparate ‘BH3 only proteins’ cannot kill in the absence of Bax and Bak. Once activated, most bind to Bcl-2 and other antiapoptotic homologues, neutralizing their prosurvival function, the amphipathic BH3 α-helix docks within the BH1/2/3 hydrophobic groove of the antiapoptotic protein.

The mechanisms of action of Bcl-2 family of proteins include protein-protein interactions and heterodimerization. For e.g. Bcl-xl, a member of Bcl-2 subfamily physically interacts with Apaf-1 to prevent the activation of associated caspases, thus suppress caspase cascade and subsequent apoptosis (Hu et al., 1998). On the other hand, proapoptotic members heterodimerize with antiapoptotic members of Bcl-2 family and promote cell death (Oltvai et al., 1993).

### 1.7. Nuclear factor-κB

Nuclear factor-κB (NF-κB) is a nuclear transcription factor that was first identified in 1986 by Ranjan Sen and David Baltimore (Sen and Baltimore, 1986). It was so named because it was found in the nucleus bound to an enhancer element of the immunoglobulin kappa light chain gene in B cells. It was initially considered to be a B cell specific transcription factor but was later shown to be present in every cell type. It is expressed ubiquitously in the cytoplasm of all cell types from Drosophila to man (Aggarwal, 2004).

The mammalian NF-κB family contains 5 members that belong to two classes: the first class includes c-Rel, RelB, and RelA (p65), proteins that are synthesized as mature products and do not require proteolytic processing. The second group is encoded by the *Nfkbi* and *Nfkb2* genes, whose products are first synthesized as large precursors, p105 and p100, respectively, that require proteolytic processing to produce the mature p50 and p52 NF-κB proteins (Karin and Lin, 2002). These proteins can form homodimers or heterodimers that give diverse combinations of dimeric complexes. These NF-κB family members share a Rel homology domain (RHD), which mediates DNA binding, dimerization and interactions with specific inhibitory factors, the IκBs, which retain NF-κB dimers in the cytoplasm (Luo et al., 2005a). IκB proteins contain a tail of ankyrin repeats that are important in binding to NF-κB family members. Many stimuli activate NF-κB,
mostly through IκB kinase–dependent (IKK-dependent) phosphorylation and subsequent degradation of IκB proteins. Upon receiving a signal, IκBα is phosphorylated at two conserved serine residues (S32 and S36) in its N-terminal regulatory domain. NF-κB remains bound to phosphorylated IκBs and IκBs undergo a second post-translational modification called polyubiquitination. The major ubiquitin acceptor sites in human IκBα are lysines 21 and 22. Protein ubiquitination occurs through E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin protein ligases. After ubiquitination, IκBs are degraded by 26S proteasomes, leading to the release of NF-κB dimers (Sethi et al., 2008). Furthermore, the liberated NF-κB dimers enter the nucleus, where it regulates the expression of almost 400 gene products linked with inflammation, cell survival, proliferation, angiogenesis, invasion and metastasis (Ahn and Aggarwal, 2005). The IKK complex consists of two highly homologous, catalytic kinase subunits, IKKα and IKKβ, and a nonenzymatic regulatory component, IKKγ/NEMO (Luo et al., 2005b).

There are two distinct NF-κB activation pathways described (Fig. 1.7). The first pathway, the canonical or classical pathway, is normally triggered in response to microbial and viral infections or exposure to proinflammatory cytokines that activate the tripartite IKK complex, leading to phosphorylation-induced IκB degradation. This pathway, which mostly targets p50:RelA and p50:c-Rel dimers, depends mainly on IKKβ activity. The other pathway, the alternative pathway, leads to the selective activation of p52:RelB dimers by processing of the NF-κB2/p100 precursor protein, which mostly occurs as a heterodimer with RelB in the cytoplasm. This pathway is triggered by certain members of the TNF cytokine family, through selective activation of IKKα homodimers by the upstream kinase NIK. Both pathways regulate cell survival and death; the classical pathway is responsible for inhibition of programmed cell death (PCD) under most conditions, on the other hand, the alternative pathway is important for survival of premature B cells and development of secondary lymphoid organs (Luo et al., 2005a,b).

1.7.1. NF-κB and cancer

NF-κB has been implicated in carcinogenesis because of its critical roles in cell survival, cell adhesion, inflammation, differentiation and cell growth. Several animal
Fig. 1.7. Schematic representation of the canonical and alternative pathways of NF-κB activation

Adapted from: Dolcet et al., 2005
models have provided evidences for a role of NF-κB in cancer. For example, v-Rel, a viral homologue of c-Rel, was identified as the transforming gene of an avian retrovirus, which is highly oncogenic and causes aggressive lymphomas and leukemias in chickens (Gilmore, 1999). Moreover, some viral oncoproteins may interact with the IKK complex and cause NF-κB activation. The Tax oncoprotein from the human T-cell leukemia virus I (HTLV-I) induces NF-κB activity and activation of NF-κB was shown to be required for transformation of rat fibroblasts by HTLV-I tax protein (Xiao et al. 2001). Besides these, chromosomal rearrangements or deletions affect in g the NF-κB2 locus on chromosome 10q24 which results in the loss of ankyrin repeats of p100 and generation of constitutively active p52 protein have been associated with B cell and T cell lymphomas and multiple myelomas (Neri et al., 1996).

Several genes that play an important role in cell proliferation are regulated by NF-κB. These include growth factors such as TNF-α, IL-1β and IL-6. For instance, it is reported that TNF acts as a growth factor for glioblastoma (Mukhopadhyay et al., 2002) and CTCL (O’Connell et al., 1995). IL-1β acts as a growth factor for acute myelogenous leukemia (Estrov et al., 1998) and IL-6, a growth factor for multiple myeloma (Bharti et al., 2003) and head and neck squamous cell carcinoma (Kato et al., 2000). In addition to growth factors, many cell cycle-regulatory proteins like cyclin D1, which is required for transition of cells from G1 to S phase is also regulated by NF-κB (Mukhopadhyay et al., 2002).

Extensive research has indicated that NF-κB regulates the expression of several genes whose products inhibit apoptosis (Fig. 1.8). These include cellular inhibitors of apoptosis (c-IAPs), X-chromosome-linked inhibitor of apoptosis (XIAP), survivin, TNFR-associated factor 1 (TRAF1) and TRAF2, c-FLIP, Bcl-2, Bel-xi, A1/Bfl-1. c-FLIP inhibits apoptosis by interfering with caspase-8 activation. c-IAP and XIAP directly bind and inhibit effector caspases, acting downstream of initiator caspases (Sethi et al., 2008).

1.7.2. Constitutive activation of NF-κB in cancer

Many reports have suggested the prevalence of constitutive NF-κB in cell lines and this contributes to malignant progression and therapeutic resistance in most of the human cancers. As explained above, the activation of NF-κB occurs as it is transported from the
Fig. 1.8. Overview of role of NF-κB in cancer. NF-κB regulate various gene products that play critical roles in cell survival, proliferation, cell adhesion, metastasis and inflammation.
cytoplasm to the nucleus upon degradation of the inhibitory subunit. In the nucleus, NF-κB binds to specific sites on the DNA and mediates the expression of a number of genes involved in the cellular response to various stresses. Thus, when NF-κB is found to persist in the nucleus, it is referred to as constitutive activation.

Constitutive activation of NF-κB has been reported in many human malignancies viz. pancreatic cancer, colon cancer (Wang et al., 1999), breast cancer (Nakshatri et al., 1997), head and neck cancer (Jackson-Bernitsas et al., 2007), prostate cancer (Suh et al., 2002), T-cell leukemia (Mori et al., 1999) and lymphoma (Pham et al., 2003). Besides cell lines, constitutive activated NF-κB has also been noted in tissue samples derived from patients. For instance, NF-κB has been implicated in an aggressive phenotype of renal cell carcinoma (RCC). Out of 45 cases of RCC, 33% (15 cases) showed greater than 200% higher NF-κB activity than normal renal tissue. In locally advanced cases, 64% showed increased activity. Tissue from metastases showed an even greater increase in NF-κB activity. Serum C reactive protein elevation correlated with the increase in NF-κB activation; therefore, NF-κB may be a cause of the inflammatory paraneoplastic syndrome (Oya et al., 2003). Sors et al. reported that, peripheral blood lymphocytes (PBLs) of all 30 Sézary syndrome patients examined, exhibited constitutively activated NF-κB, while in contrast, no evidence of NF-κB nuclear translocation could be detected in PBLs from 6 healthy donors. The constitutive activated NF-κB in CTCL cells plays a key role in their survival and resistance to apoptosis. The mechanism(s) underlying the constitutive activation of NF-κB in CTCL cells remain(s) unknown and might be due to genetic alterations of NF-κB or IκB genes or to an uncontrolled activation of IKK (Sors et al., 2006).

1.7.3. NF-κB and cancer therapy

Several studies have emphasized the identification of putative NF-κB inhibitors as therapeutic agents for cancer. Since NF-κB activation is the result of a multi-step signaling pathway, these compounds may target different points of the signaling process. A variety of compounds have been screened for their ability to suppress NF-κB. For instance, some anti-inflammatory agents: aspirin and sodium salicylate may inhibit NF-κB by interfering with IKK activity in vitro and in vivo (Yin et al., 1998). Other substances such as curcumin
(derived from turmeric), trans-resveratrol (derived from grapes) or parthenolide (derived from the plant feverfew) are natural compounds that have been demonstrated to inhibit IKK activity (Shishodia et al., 2005; Holmes-McNary and Baldwin, 2000; Saadane et al., 2007). Another way to approach NF-κB inhibition is to target the process of proteasome degradation. Proteasome inhibitors prevent NF-κB activation by blocking the degradation of IκBs, NF-κB1/p105 or NF-κB2/p100. Recently, it has been reported that proteasome inhibitors viz. ALLN, MG132 and bortezomib overcome resistance of CTCL cells to apoptosis by inhibiting NF-κB activity (Sors et al., 2006). Additionally, NF-κB can be inhibited by acetylation inhibitors. Acetylation of RelA is required both for transactivation and prevention of nucleocytoplasmic trafficking of NF-κB. HDAC3 acts directly upon nuclear RelA to enable its association with IκBα and its subsequent export from the nucleus (Chen and Greene, 2003). Recently FDA has approved vorinostat, a HDACs inhibitor for treatment of CTCL (Mann et al., 2007).

1.8. Activator protein-1 transcription factor

Activator protein-1 (AP-1) is one of the first mammalian transcription factors to be identified but its biological relevance and physiological functions are still being elucidated (Shaulian and Karin, 2002). The mammalian AP-1 proteins are homodimers and heterodimers composed of basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), Maf (c-Maf, MafB, MafA, MafG/F/K and Nrl), Jun dimerization partners (JDP1 and JDP2), activating transcription factor (ATF2, LRF1/ATF3 and B-ATF) subfamilies, which recognizes either 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (TRE, 5'-TGAG/CTCA-3') or cAMP response elements (CRE, 5'-TGACGTCA-3'). Besides forming heterodimers with Fos and ATF family members, Jun proteins can also homodimerize among themselves and bind to TRE. Although, it is reported that dimerization of c-Jun with c-JunB attenuates c-Jun's transcriptional activity. Fos proteins cannot homodimerize but could bind to DNA by forming stable heterodimer with Jun proteins. In addition, it is known that heterodimerization of c-Jun with c-Fos enhances c-Jun’s DNA binding activity through formation of more stable dimers. On the other hand, ATF can form both homodimers and
1.8.1. AP-1 and apoptosis

AP-1 activity can be induced by various stimuli including; growth factors, proinflammatory cytokines, neurotransmitters, polypeptide hormones, cell-matrix interactions, bacterial and viral infections and variety of physical and chemical stress. In turn, AP-1 is known to regulate a wide range of cellular processes which include cell differentiation, proliferation, survival and death (Shaulian and Karin, 2002). Furthermore, the outcome of AP-1 activation is highly cell specific. In some cells AP-1 may be involved in the survival of cells, while in others it induces apoptosis (Shaulian and Karin, 2001). Various reports have indicated the proapoptotic function of AP-1. For instance, c-Fos expression is persistently induced and correlated with neuronal cell death in the brains of mice treated with kainic acid, a potent activator of glutamate receptors (Smeyne et al., 1993). In addition, ectopic expression of c-Jun or c-Fos can induce apoptosis in sympathetic neurons, mouse fibroblasts, Syrian hamster embryo cells and a human colorectal carcinoma cell line (Bossy-Wetzel et al., 1997; Ham et al., 1995; Preston et al., 1996). Antisense oligonucleotides directed against c-Fos and c-Jun mRNAs were also found to increase the survival of growth factor deprived lymphoid cells (Colotta et al., 1992). Moreover, it is reported that c-Jun induction precedes the onset of apoptosis in the cells exposed to genotoxic stress such as alkylating agents or short-wavelength UV radiation (Shaulian and Karin, 2002). Paraquat, a herbicide is found to induce apoptosis in pheochromocytoma cells (PC 12) and induced activation of transcription factor AP-1 (Li and Sun, 1999). Similarly, taxotere, a mitotic inhibitor activates transcription factor AP-1 in association with apoptotic cell death in gastric cancer cell lines (Kim et al., 1999). Furthermore, AP-1 activates various genes, such as FasL, Bim or Bcl-3, whose products are either positive or negative regulators of apoptosis. It is actually the balance between the pro-and antiapoptotic target genes that determine whether the outcome will be cell survival or cell death. This balance may be dependent on type and duration of stimulus used to activate AP-1, as well as on the activation of other transcription factors (Shaulian and Karin, 2002).
1.9. Nitric oxide

Nitric oxide (NO) is a hydrophobic molecule and a highly diffusible free radical, generated through oxidation of L-arginine to L-citrulline (Fig. 1.9.) by a family of NO synthase (NOS) isoenzymes. Three different isoforms of the NOS family have been identified: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). The gene symbol nomenclatures are: NOS1 for nNOS, NOS2 for iNOS and NOS3 for eNOS (The nNOS and eNOS isoforms are constitutively expressed in a variety of cell types including the endothelium, platelets and neurons and is transiently activated by an increase in cytosolic calcium which promotes the release of NO over several minutes. In contrast, activation of iNOS is not calcium dependent and after stimulation of immunological or inflammatory reactions produce large amounts of NO that can persist for several days (Li and Wogan, 2005). NO may also be produced nonenzymatically from nitrite at low pH (<pH 5), for example, during ischemia NO diffuses very rapidly both through water and membranes, so NO can easily diffuse from one cell to the next (Brown and Borutaite, 2002). Various evidences indicate that NO is a potent modulator of homeostasis operationally preventing or inducing apoptosis; these effects may be modulated by both direct and indirect interactions that can be dose dependent and cell-type specific (Fig. 1.10.). In some cell types NO can promote apoptosis, whereas in others it inhibits apoptosis (Li and Wogan, 2005).

1.9.1. NO as a proapoptotic inducer

NO can induce apoptosis in a variety of cell types including macrophages, neurons, pancreatic β-cells, thymocytes, chondrocytes, hepatocytes and tumor cells (Chung et al., 2001). NO mediated apoptosis is often accompanied by an accumulation of tumor suppressor gene p53, changes in the expression of proapoptotic and antiapoptotic Bcl-2 family members, cytochrome c release through mitochondrial membrane potential loss, caspase-3 and poly (ADP ribose) polymerase (PARP) activation (Brune et al., 1999).

In the last several years, reports have emerged that demonstrated that NO inhibits NF-κB activation in a variety of cells, including monocytes and endothelial cells. Multiple mechanisms have been described by which NO inhibits NF-κB. For instance, it has also been shown that NO can increase mRNA expression of IκBα in human vascular endothelial
Fig. 1.9. Schematic representation of generation of nitric oxide

Adapted from: http://www.bmb.leeds.ac.uk/illingworth/metabol/amin.htm#nitric

Fig. 1.10. Nitric oxide biological effects significantly differ depending upon its concentrations.

Adapted from: Mocellin et al., 2007
cells and transfection experiments in these cells by chloramphenicol acetyltransferase reporter gene linked to the IκBα promoter have suggested transcriptional induction of IκBα by NO (Peng et al., 1995). It is also reported that NO can inhibit NF-κB in human vascular endothelial cells through stabilization of IκBα by preventing its phosphorylation and degradation (Katsuyama et al., 1998). It has also been shown that NO inhibits NF-κB activation by increasing the expression and nuclear translocation of IκBα (Spiecker et al., 1997). NO can also directly inhibit DNA binding activity of NF-κB by post translational modifications. Emphasis has been focused on S-nitrosylation of Cys-62 of the p50 subunit, which is known to inhibit the ability of NF-κB to bind DNA (Matthews et al., 1996). NO can modify NF-κB through nitration of tyrosine (Tyr) residues of p65. Tyr nitration of p65 induces its dissociation from p50, association with IκBα and subsequent sequestration of p65 in the cytoplasm by IκBα-mediated export (Park et al., 2005).

1.9.2. NO donors and its role in cancer

One of the most widely used therapeutic agents is non-steroidal anti-inflammatory drugs (NSAIDs). More than 50 NSAIDs are known, which possesses therapeutic action like reduction of inflammation and the production of analgesic and antipyretic effects (Chiroli et al., 2003). Unwanted side effects are also associated with NSAIDs, one of the most adverse side effects is the tendency of NSAIDs to induce gastric or intestinal ulceration. Therefore, the problems associated with NSAIDs leads to the requirement for new anti-inflammatory agents which could show good activity in combination with a high degree of safety. One interesting innovative approach relies on the incorporation of a nitric-oxide (NO) releasing moiety into the structure of established NSAIDs. NO-donating non-steroidal anti-inflammatory drugs (NO-NSAIDs) represent a new class of compounds which are synthesized by the ester linkage of an NO-releasing moiety to conventional non-steroidal anti-inflammatory drugs (NSAIDs). NO-NSAIDs are a novel group of drugs showing at least equivalent or enhanced anti-inflammatory, antipyretics and analgesic potential compared to classical NSAIDs. This new class of compound is also related as an antitumoral agent, which combine the cyclooxygenase (COX) inhibiting property of their NSAID moiety with the antitumoral potential of their NO-donating part (Huguenin et al.,
This class includes compounds such as: NO-aspirin, NO-flurbiprofen, NO-naproxen, NO-ibuprofen (Keeble and Moore, 2002).

Aspirin (acetylsalicylic acid, ASA) prevents colon cancer, but its limited efficacy and side effects preclude its application to cancer prevention. NO-donating aspirin (NO-ASA) consisting of traditional ASA that bears a NO-releasing moiety appears to be safer than ASA and is currently undergoing clinical evaluation for the prevention of colon cancer (Rigas and Kashfi, 2004). Compared with ASA, NO-ASA is greater than 1,000 fold more potent in inhibiting the growth of colon and other cancer lines (Kashfi et al., 2002). It is showed that NO-ASA induces apoptosis through a series of steps that begins with the generation of oxidative stress state, which activates the intrinsic apoptosis pathway (Gao et al., 2005). Similarly, NO-sulindac was found to exert more potent inhibitory effect than its NSAIDs counterpart on the growth of human bladder carcinoma cell lines and colon adenocarcinoma cell lines (Lavagna et al., 2001). Thus, it is predicted that use of NO-NSAIDs instead of NSAIDs could be a safer, more effective, new therapeutic strategies and a promising alternative for fight against cancer (Huguenin et al., 2005).

Besides NO-NSAIDs, other members of nitric oxide donating drugs (NODD) includes, NO-releasing compounds (NO donors) like; DETANONOate, sodium nitroprusside (SNP), glyceryl trinitrate (GTN), S-nitroso-N-acetyl-penicillamine (SNAP) and S-nitroso-glutathione (GSNO) (Table 2). To know the effects of NO on cell survival or death without the involvement of NOS, NO donors are valuable tools (Chung et al., 2001). SNP is cytotoxic towards human hematological malignant cells and epithelial cancer cell line (Tsumori et al., 2002; Sumitani et al., 1997). It has been demonstrated that SNP and GSNO induce apoptosis in murine cell line J774 (D'Acquisto et al., 2001). GSNO is also reported to inhibit cell growth in human colon cancer cell lines; HCA7, HT29, HCT116 and murine cell line; RAW 264.7 (Liu et al., 2003; Glockzin et al., 1999). GTN is known to induce apoptosis in human leukemic T cell line, Jurkat (Umansky et al., 2001). Recently, a novel NO-donor, (S,R)-3-phenyl-4,5-dihydro-5-isoxazole acetic acid-nitric oxide (GIT-27NO) is reported to induce p53 mediated apoptosis in human A375 melanoma cells (Mijatovic et al., 2008).

It is reported that besides acting as a cytotoxic agent, NO delivered by NO donors can also modulate the toxicity of other agents. NO can dramatically enhance the cytotoxicity of clinically used alkylating agent such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Laval and Wink, 1994). NO delivered by the NO-donor, 1,1-diethyl-2-hydroxy-2-
<table>
<thead>
<tr>
<th>Drug</th>
<th>Chemical name</th>
<th>Notes</th>
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<tbody>
<tr>
<td>DETA/NO</td>
<td>(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]-diazene-1-ium-1,2-diolate</td>
<td>Long-acting NODD (half-life: 20 hours) that releases NO in the range produced by activated macrophages; no prior biotransformation is needed</td>
</tr>
<tr>
<td>DETA NONOate</td>
<td></td>
<td>No prior biotransformation is needed</td>
</tr>
<tr>
<td>Spermine-NONOate</td>
<td>N-(2-aminoethyl)-N-(2-hydroxy-nitrosohydrazino)-1,2-ethylenediamine</td>
<td>Is metabolized to NO by the pi isomser of glutathione S-transferase, an enzyme expressed at high levels in many tumors</td>
</tr>
<tr>
<td>PABA/NO</td>
<td>O(2)_[2,4-dinitro-5-(N-methyl-N-4-carboxyphenylamino)phenyl]1-N,N-dimethylamino</td>
<td>Anti-hypertensive drug; is a complex of five cyanide anions (CN⁻) and a NO⁺; interaction of SNP with a reducing agent (e.g. thiols) leads to NO and CN⁻ release</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
<td>Antianginal drug; to be pharmacologically active, enzymatic glutathione-dependent biotransformation of nitrate group to NO is needed</td>
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<tr>
<td>GTN</td>
<td>Glycerol trinitrate (nitroglycerin)</td>
<td>S-nitrosothiols donating nitroxy (NO⁻) and nitrosonium (NO⁺)</td>
</tr>
<tr>
<td>SNAP</td>
<td>S-nitroso-N-acetyl-penicillamine</td>
<td></td>
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<tr>
<td>GSNO</td>
<td>S-nitroso-glutathione</td>
<td></td>
</tr>
<tr>
<td>NO-B₃₂</td>
<td>Nitrosoylcobalamin</td>
<td>As B₃₂ receptors are overexpressed by some tumors, NO delivery is tumor-specific</td>
</tr>
<tr>
<td>NO-ASA</td>
<td>NCX-4016: 2-acetoxybenzoate 2-(1-nitroxy-methyl)phenyl ester</td>
<td>Under clinical investigation as NSAID with less gastric toxicity (like prostaglandins produced by COX-1, NO shows mucosa repair-promoting properties); NO-ASA is also clinically investigated as cancer chemopreventive agent</td>
</tr>
<tr>
<td>NO-naproxen</td>
<td>HCT-3012: (S)-6-methoxy-α-methyl-2-naphthalene acetic acid 4-(nitroxy)butyl ester</td>
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<tr>
<td>NO-ibuprofen</td>
<td>NCX-2210: α-methyl-4-(2-methylpropyl)-2-methoxy-4-[1E,3-[4-nitroxy]butoxy]-3-oxo-1-propenylphenyl ester, benzene acetic acid</td>
<td></td>
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<tr>
<td>YC-1</td>
<td>3,5'-hydroxymethyl-2'-furyl-1-benzyl indazole</td>
<td>NO mimetic, activates the soluble guanylyl cyclase (sGC)/protein kinase-G (PKG) pathway; orally available; induces apoptosis and inhibits hypoxia inducible-1 (HIF-1) pathway</td>
</tr>
<tr>
<td>SIN-1</td>
<td>3-morpholinosydnonimine</td>
<td>Active metabolite of prodrug molsidomine (long-acting antianginal drug enzymatically converted into SIN-1 in the liver); SIN-1 decomposes (enzyme-independently) in a two-step reaction into SIN-1C (stable metabolite) and NO; SIN-1 also releases superoxide, but in excess of superoxide dismutase is converted into a pure NODD</td>
</tr>
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Table 2. Nitric oxide donating drugs (NODD) tested in cancer models.
nitroso-hydrazine (DEA/NO) resulted in enhancement of melphalan-mediated toxicity in Chinese hamster V79 lung fibroblasts and human breast cancer (MCF-7) cells by 3.6- and 4.3-fold, respectively (Cook et al., 1997). It is evidenced that pretreatment of human breast cancer cells and Chinese hamster V79 lung fibroblasts with a bolus of NO for 30 min enhanced the cytotoxicity of doxorubicin and cisplatin respectively (Evig et al., 2004; Wink et al., 1997). It is also documented that NO-donor, S-nitrosocaptopril (CapNO) could enhance taxol induced cytotoxicity in carcinoma cells by increasing influx of taxol into intracellular compartments (Jia et al., 2003).

1.10. Pentoxifylline

Pentoxifylline (PTX), a methylxanthine derivative (1-(5-oxohexyl)-3, 7-dimethylxanthine) (Fig. 1.11.) belongs to the non-selective phosphodiesterase inhibitor. It is widely used as a haemorrheological agent that augments erythrocyte flexibility, decreases blood viscosity resulting in increased microcirculatory blood flow, reduces platelet aggregation. PTX alters membrane fluidity and causes cell deformability in cancer cells which may pass the microcirculation easily and are thus less likely to attach and form metastasis (Dua and Gude, 2006).

PTX is of high therapeutic value. PTX is effective in decreasing proteinuria in diabetic patients with advanced renal failure (Navarro et al., 1999). In addition, PTX provides renoprotection, the possible mechanism underlying this may be linked to the effect of PTX against glomerular mesangial cell proliferation by blocking membrane translocation of Akt and causing arrest of cells in G\textsubscript{1} phase by down regulation of cyclin D1 expression. Indeed, PTX can inhibit mesangial cell proliferation both in vitro and in vivo (Lin et al., 2003). Besides this, the effects of PTX on inflammation and organ dysfunction have been investigated clinically. PTX can improve survival in patients with severe alcoholic hepatitis and this benefit of PTX appears to be related to a significant decrease in the risk of developing hepatorenal syndrome (Akriviadis et al., 2000). Moreover, increased plasma TNF-\textalpha concentration correlates with mortality in sepsis. PTX is shown to have therapeutic value in the treatment of premature infants with sepsis as PTX reduces elevated plasma TNF level in these patients (Lauterbach and Zembala, 1996). Furthermore, PTX can be used as an adjuvant therapy in patients with septic syndrome because of PTX capability to reduce respiratory burst activity in polymorphonuclear granulocyte of patients (Oismuller et
Adapted from: http://en.wikipedia.org/wiki/Pentoxifylline

Fig. 1.11. Structure of pentoxifylline
Cardiopulmonary bypass produces an inflammatory response due to the interaction of blood with a foreign body surface. The lungs are most affected by this inflammatory response. This damage in lungs is minimized by PTX as it can minimize leukocyte sequestration in the lung (Turkoz et al., 1996).

Besides these properties, PTX has also been found to exert a wide range of immunomodulatory properties. It decreases the secretion of proinflammatory cytokines such as TNF-α and IL-6 (Michetti et al., 2003). It is known to reduce Th1 lymphocyte pools thus influencing Th1/Th2 balance (Laurat et al., 2001). PTX modulates iNOS mediated NO production in rodent macrophages and astrocytes (Trajkovic et al., 1997). It is also reported that PTX potentiates NO production and inhibit [3H] thymidine uptake in a synergistic fashion in IFN-γ treated murine fibrosarcoma cell line, L929 (Stosic-Grujicic et al., 1998). It has been demonstrated that PTX downregulates expression of the intercellular adhesion molecule-1 (ICAM-1) in human monocytes both in vitro and in vivo (Neuner et al., 1997).

Moreover, it has been documented that PTX enhances antitumor activity of many chemotherapeutic agents. For instance, PTX in vitro and in vivo sensitizes leukemic cells to adriamycin induced apoptosis (Lerma-Diaz et al., 2006). PTX has been considered as a modulating agent for use with fludarabine to potentiate fludarabine cytotoxicity and circumvent its drug resistance (Alas et al., 2000). PTX enhances antitumor effect of cisplatin and etopside on human lung adenocarcinoma cell lines (Ohsaki et al., 1996). In addition, PTX sensitizes human myelomonocytic leukemic cells to antineoplastic action of phytochemicals like perillyl alcohol (Gomez-Contreras et al., 2006). PTX augments the effect of alkylating agents on FSaIIC fibrosarcoma and mammary adenocarcinoma both in vitro as well as in vivo (Teicher et al., 1991). Besides enhancing antitumor activity of some drugs, PTX can sensitize tumor cells to radiotherapy. PTX can abrogate S and G2 checkpoints which provide an effective strategy for enhancing the cytotoxic and radiosensitizing effects of (E)-2'-deoxy-2'-(fluoromethylene) cytidine (FMdC), a novel inhibitor of ribonucleotide-diphosphate reductase, on human colon-cancer cell line, WiDr (Li et al., 1999). PTX causes radiosensitization in HepG2 cells, which is possibly associated with the abrogation of G2 arrest following radiation exposure (Wu et al., 2004).

Furthermore, PTX itself is associated with anticancer property. It has been demonstrated that PTX inhibits murine B16F10 melanoma solid tumor growth and metastasis to lung, which is mediated via its inhibitory action on cell adhesion, matrix
metalloproteinases (MMP-9 and MMP-2) secretion and tumor angiogenesis (Dua and Gude, 2006). Further, suramin, a polysulphonated napthyl urea when combined with PTX synergizes the antitumor and antimetastatic activity of PTX in B16F10 melanoma (Dua et al., 2007).

1.11. Objectives of the study

CTCL is a cancer which belongs to a class of non-Hodgkin lymphoma and is second most common extranodal lymphoma, characterized by clonal proliferation of neoplastic T lymphocytes. The two frequent forms of this disease are MF and its leukemic counterpart, SS with MF being the most common type of CTCL. It has been found that CTCL constitutively express cell survival gene, Bcl-xl, which is an antiapoptotic member of Bcl-2 family. Many treatments are available for CTCL, but currently, no cure for either form of CTCL has been found (Tun-Kyi et al., 2008).

NF-κB is an important transcription factor involved in immune and inflammatory cellular responses affecting cell growth and survival (Ashikawa et al., 2002). NF-κB regulates expression of a large number of genes that are critical for regulation of programmed cell death or apoptosis (Sethi et al., 2008; Shishodia and Aggarwal, 2002). It is demonstrated that NF-κB is constitutively expressed in CTCL cells and make these cells resistance to apoptosis. The inhibition of NF-κB pathway leads to major alterations of CTCL viability and proliferative capacities (Sors et al., 2006).

Moreover, TNF-α, acts as an autocrine growth factor for CTCL cell line, HuT-78, since proliferation of HuT-78 cells can be inhibited by lowering TNF-α level using anti-TNF antibody (O'Connell et al., 1995; Giri and Aggarwal, 1998). Besides showing resistance to TNF-α induced apoptosis, CTCL cells are also resistant to TRAIL mediated killing (Braun et al., 2007).

Nitric oxide (NO) is a diffusible gas molecule which can induce apoptosis in a variety of cell types (Li and Wogan, 2005). Indeed, it has been found that NO can inhibit NF-κB in both constitutive and inducible system, however the mechanism of NO mediated inhibition of NF-κB differs from cell type to cell type (Marshall et al., 2004; D'Acquisto et al., 2001; Katsuyama et al., 1998). Besides acting as a proapoptotic agent, NO can also sensitize tumor cells to Fas (Garban and Bonavida, 1999), TNF-α (Garban and Bonavida,
2001) and TRAIL (Huerta-Yepez et al., 2004) mediated apoptosis. Furthermore, NO can also enhance cytotoxicity of anticancer agents viz. cisplatin (Wink et al., 1997), taxol (Jia et al., 2003), doxorubicin (Evig et al., 2004) in carcinoma cells.

Pentoxifylline (PTX) is a xanthine derived antioxidant known to lower TNF-α in many cells (Lima et al., 2005). It is also reported that PTX inhibits not only inducible NF-κB but also its constitutive form (Bellas et al., 1995; Wang et al., 1997; Jimenez et al., 2001). Moreover, PTX has been shown to enhance antitumor activity of some drugs and can also sensitize tumor cells to radiotherapy (Rauko et al., 1998; Alas et al., 2000; Lerma-Diaz et al., 2006; Waldeck et al., 2007).

With this background knowledge, the present study was undertaken on HuT-78 cells, a T-cell line derived from a Sézary lymphoma with following laid objectives:

A. To study the cytotoxic potential of nitric oxide generating compound, sodium nitroprusside (SNP) on T lymphoma cell line

- Apoptotic effect of SNP on HuT-78 cells.
- Role of SNP on constitutive NF-κB in HuT-78 cells.
- Effect of SNP on the expression of pro and anti-apoptotic proteins.
- Effect of SNP on TNF-α superfamily members sensitivity in CTCL cells.

B. To study the cytotoxic potential of anticancer agent, pentoxifylline (PTX) either alone or with SNP on T lymphoma cell line

- Apoptotic effect of PTX on HuT-78 cells.
- Role of PTX on constitutive NF-κB in HuT-78 cells.
- Effect of PTX on the expression of pro and anti-apoptotic proteins.
- Effect of PTX on TNF-α superfamily members sensitivity in CTCL cells.
- To study the combined effect of SNP and PTX.